TESTING MATERNAL CONTRIBUTIONS IN TRIPLOID EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*): A FIELD EVALUATION IN COASTAL ALABAMA

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of
the requirements for the Degree of
Master of Science

Auburn, Alabama
August 7, 2021

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ABSTRACT

With the establishment of off-bottom aquaculture in the northern Gulf of Mexico, triploid oysters have become the mainstay of production due to their well-documented advantages; however, there have been observations in recent years that triploid oysters can suffer from sudden high mortality. This study aimed to investigate the effects of broodstock origin, size, and ploidy on performance of eastern oysters (*Crassostrea virginica*) in coastal Alabama. Six groups of oysters were spawned from wild broodstock collected from three areas with different salinity regime histories to produce half-sibling diploids and triploids: Calcasieu Lake, Sister Lake, and Vermillion Bay in Louisiana. To determine the effect of broodstock, cohorts were deployed at three different sites in Alabama with expected differences in salinity regimes: Grand Bay, Dauphin Island, and Mobile Bay. The effect of size on diploid and triploid performance was determined by a simultaneous field experiment at Grand Bay, AL. Growth and survival were determined by monthly data collection. Both broodstock and ploidy affected growth at all three sites; at Mobile Bay and Dauphin Island broodstock and ploidy had an interactive effect, while broodstock was the only significant effect on daily growth at the Grand Bay site. However, ploidy was the only factor that had a significant effect on mortality (with triploids suffering higher mortality of varying magnitude across sites). Growth rates of Sister Lake oysters were significantly affected by size, with small size oysters growing significantly faster than both medium and large sized oyster, however size and ploidy had an interactive effect on growth rate with triploid Calcasieu Lake oysters growing significantly faster than all other treatments. Triploids experienced higher cumulative mortality than diploids in both Sister Lake and

Calcasieu Lake oysters. From April to May, size and ploidy had a significant effect on interval mortality in Sister Lake oysters, with triploid large sized oysters experiencing significantly higher interval mortality than all other experimental treatments. However, triploid Calcasieu Lake oysters had significantly higher interval mortality than diploids from April to May. Evaluating and understanding the effect of these factors on triploid performance could help alleviate industry concerns and equip commercial hatcheries with information to improve seed stocks for farmers in less-than-ideal grow-out environments.

ACKNOWLEDGMENTS

Throughout the writing of this thesis, I have received a great deal of support and assistance. I would first like to extend my sincerest appreciation to my advisor and mentor, Bill Walton, who took me on as one of his students and transformed me from a technician to a scientist. The experience and knowledge you have provided over the years has been invaluable to my research and experience here at Auburn. A special thank you to Jerome La Peyre, James Stoeckel, and Brian Callam for their guidance and input making the completion of this thesis possible. I also would like to extend my deepest gratitude to Scott Rikard whose knowledge, support, and advice has been vital to my success over the last 3 years. Thank you to Glen Chaplin for your continuous encouragement throughout my time here and your dedication to my monthly sampling. A special thanks to the extraordinary people at the Auburn Shellfish Lab, Sarah Spellman, Caitlin Henning, John Lewis, James Kelly, and Becky Wasden. For allowing me to use space on their farm for my field experiments, thank you to Dottie Lawley and Tyler Meyers. I would also like to thank the staff at the Louisiana State University Animal and Food Science Lab for assisting with sample processing. Lastly, a special thanks to my roommates and grandparents, Pal and Mickey Eastburn, for their support and encouragement throughout the last three years.

I dedicate this thesis to my twin sister, my confidant, Anna Alyce Eastburn, for always encouraging me to lead my own path. To my mother, Laurie Eastburn, for her unwavering support and confidence in me. Lastly, my father, William Eastburn, for instilling a love and respect for our coastal ecosystems, and for the unconditional encouragement through all my endeavors.

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List of Abbreviations

CB	Cytochalasin B
6-DMAP	6-dimethylaminopurine
MSX	Multinucleated Sphere Unknown
nGOM	northern Gulf of Mexico
SL	Sister Lake
CL	Calcasieu Lake
VB	Vermillion Bay
MOB	Mobile Bay
DI	Dauphin Island
GBOP	Grand Bay Oyster Park
BSOP	Bayou Sullivan Oyster Park
AUSL	Auburn University Shellfish Laboratory
2N	Diploid
3N	Triploid
4N	Tetraploid

CHAPTER ONE: A BRIEF REVIEW OF TRIPLOID PRODUCTION IN OYSTER AQUACULTURE

Polyploidy in Oyster Aquaculture

Polyploidy refers to an organism possessing two or more complete sets of chromosomes and is known to provide advantages at the phenotypic and molecular levels (Comai, 2005; Guo and Allen, 1994; Allen et al., 1993; Allen 1983). Wilkins (1981) suggested this genetic manipulation could prove useful in the culture of fish and shellfish. For commercial aquaculture, grass carp (*Ctenopharyngodon Idella*) hybrids (crossed with bighead carp, *Hypophthalmichthys nobilis*) were produced to manage aquatic vegetation, and they were deemed effective due to their sterility that prevents unwanted reproduction. (Sutton et al. 1981). In addition to sterility, another possible advantage of inducing polyploidy in animals is increased meat quality. Breese and Malouf (1977) suggested that preventing decreased meat quality during reproduction and spawning months could potentially increase production by 35%. This led to the idea of culturing nonreproductive oysters that could lead to increased meat quality (Stanley et al., 1981). Stanley et al. (1981) did find success inducing triploidy in eastern oysters (*Crassostrea virginica)*; however, they observed these advantages of polyploidy to be dependent on the time of induction. Using cytochalasin B (CB), triploidy (3n) was induced by blocking meiosis I, and these oysters showed clear growth advantages over diploid oysters induced by blocking meiosis II (Stanley et al., 1984). However, there were concerns about the effectiveness of this method to induce triploidy. Bushek and Allen (1992) investigated the effectiveness of using "stripped" gametes to reduce the variation in ploidy. By carefully controlling the factors that induce spawning, they found that stripping gametes did yield a higher percentage of triploidy, but variation was still observed though the causes were not explicitly stated (Bushek and Allen 1992). Concerns about the toxicity of CB and low induction success rates were addressed by investigating the reproductive characteristics of tetraploidy in Pacific oysters (*C. gigas*) for application in triploid

production (Guo et al. 1996). Tetraploid *C. gigas* was successfully produced from inhibiting polar body 1 in fertilized triploid eggs (Guo and Allen 1994). While most triploids are sterile, some can produce eggs that can be used for tetraploid induction (Guo and Allen, 1994). Guo and Allen (1996) successfully crossed tetraploid males and diploid females to produce triploid *C. gigas.* This method was believed to be the best solution to ploidy variation in triploids and alleviated the concerns of CB toxicity (Guo et al. 1996). Recently, however, Peachey and Allen (2016) published a new method using the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) for tetraploid induction in *C. virginica*, instead of CB. Regardless of their induction mechanism, CB and 6-DMAP had comparable results in both *C. gigas* and *C. virginica* for triploid numbers and survival rates (Peachey and Allen 2016; Desrosiers et al., 1993; Gérard et al., 1999). Because 6-DMAP is hazard free, it is safer for regular hatchery use compared to CB (Peachey and Allen, 2016; Sigma-Aldrich, 2015a). The protocol for using 6-DMAP for tetraploid induction was also found to be simpler than CB because of the different induction mechanism and is recommended as the best method for chemical induction of tetraploidy for *C. virginica* and potentially other *Crassostrea* species (Peachey and Allen, 2016). These advancements in polyploidy induction techniques and technologies have increased the accessibility of triploids for the commercial oyster aquaculture industry.

Overview of Commercial Use of Triploid Oysters

Wadsworth et al. (2019) reported that Auburn University Shellfish Lab (Dauphin Island, AL) produced roughly 99 million triploid C. virginica eyed larvae (pediveliger) in 2017, accounting for approximately 85% of their commercial seed sales that year, while only 6 million diploid seed were ordered the same year (Scott Rikard, per. comm). Since 2017, Cherrystone Aquafarms (Cape Charles, VA) has produced an approximate 400 million seed (Tim Rapine, per.

comm), and Hooper Island Oyster Company (Cambridge, MD) has reportedly produced approximately 54.4 million triploid seed since 2017 (Stephanie Wiegand and Natalie Ruark, per. comm). The commercial industry uses triploids in such large numbers because of their increased summer meat quality, ability to grow and reach market size faster than diploids, and possible resistance to spawning season mortality. Depending on the year and the location, the spawning season for *C. virginica* can span from April to October on the Gulf Coast because of the sub-tropical climate (La Peyre et al., 2013; Supan & Wilson, 2001; Deksheniek, 1993; Hayes & Menzel, 1981; Ingle, 1951). This extended spawning period affords individual oysters the opportunity to spawn several times throughout the season but can also be associated with a prolonged decrease in meat quality of the reproductive diploid oysters. Allen and Dowling (1986) found in *C. gigas* that overall triploids had used less of their glycogen reserves when compared to diploids during the spawning months, suggesting that the reduced gonad development allowed triploids to use their glycogen reserves for somatic tissue growth instead. This reduced usage allows triploids to retain their meat quality during the spawning season, thus becoming more marketable (Nell 2002; Allen and Dowling, 1986). Additionally, it was hypothesized that reduction in gonad development might allow the triploids to resist summer mortality that was associated with the stress caused by the physiological and metabolic demands of spawning (Allen et al., 1989). Summer mortalities of natural diploids broadly are a long-recognized issue in oyster aquaculture with decades of reported summer mortality events from Japan, France, and the west coast of the United States (Burge et al., 2007; Soletchnik et al., 2007; Gagnaire et al., 2006; Cheney et al., 2000; Koganezawa, 1975). While exact causes for these reports were inconclusive there was speculation that oyster size, rising temperatures, decreasing salinity, or a combination, and Koganezawa (1975) concluded the mortality was a response to

physiological disorders that were induced by these environmental changes (Cheney et al., 2000, Gagnaire et al., 2006, Soletchnik et al., 2007). This conclusion was based on earlier studies that linked summer mortalities to accelerated reproductive maturation during the highest seasonal temperatures (Imai et al., 1965; Tamate et al., 1965).

For decades, the investigation into 'summer mortality' in oysters has been inconclusive about specific causes, with a more recent focus on the effect of an association with ploidy; however, there are several generalizations that have been found. 'Summer mortality' does not appear to typically have a single cause but appears to result possibly from a combination of different factors such as salinity, temperature, pathogen presence, and/or farm handling techniques. In 2012, farmers in the Chesapeake Bay began reporting unusual mortalities of *C. virginica* from May to June without any pathological explanation such as MSX or Dermo disease, and by 2014, some farmers reported up to 85% crop loss and these events were associated with triploid oysters and described as "triploid mortality" (Guevelou et al., 2019). Similar mortality events have been reported in adult *C. gigas* and there were no pathogen or environmental condition linked to these events (Cotter et al., 2010; Glude, 1975; Koganezawa, 1975; Maurer and Comps, 1986; Samain and McCombie, 2008; Wendling and Wegner, 2013). In estuaries along the Atlantic and Gulf Coast, temperature and salinity have substantial seasonal fluctuations, which can be impose physiological stress upon estuarine organisms. Eastern oysters (*C. virginica*) are known to have a broad tolerance to these changes (Casas et al., 2018; Rybovich et al., 2016; Shumway, 1996; Galstoff, 1964; Butler, 1954), but do experience mortalities under extreme combinations. Rybovich et al. (2016) found in both lab and field studies that a combination of high temperature (30°C) and low salinity (<5 ppt) negatively impacted seed (<25mm), spat (25-75mm), and market size oysters (>75mm). Market size oysters

had the highest sensitivity to the effects of low salinity and high temperatures, together and separately, while spat had highest sensitivities when salinity was at 1 ppt and the interaction of the effects was only observed when salinity was at 5 ppt (Rybovich et al., 2016). Casas et al. (2018b) emphasized the importance of temperature in relation to clearance rates. Despite oyster tolerance to high temperatures, gaping increased with high oxygen consumption rates (Casas et al., 2018b). Optimal clearance rates are speculated to be between 15-25 ppt, so low salinity environments may be a limiting factor for oyster oxygen consumption (Casas et al., 2018b; Galtsoff 1964; Loosanoff, 1953). Casas et al. (2018a) found that oysters' clearance rates were much lower in summer months at low salinity (< 10ppt) than winter months in low salinity. Casas et al. (2018a) suggested that the water chemistry in low salinity environments may affect physiological processes like cellular metabolism that are used to resist the stress of these environments.

Selective breeding in Oyster Aquaculture

In response to concerns about mortality events, including 'summer mortality', selective breeding has been promoted as a potential means of reducing these losses. Wild oyster stocks have natural variation that can be exploited in a hatchery setting to develop strains of oysters to help maintain the genetic variation and health of oyster populations. Individuals may be selected based on their desired traits such as faster growth, disease resistance, and meat quality (Gjedrem, 1982). In the Chesapeake Bay, MSX (*H. nelson*) ravaged the wild oyster populations in the late 1950's, however in following years surviving oysters' infection intensities began to decrease, suggesting the oysters had naturally built resistance (Allen et al., 1993). Haskin and Ford (1979) found that resistance to MSX infections was a heritable trait and selecting for it was successful. Broodstock was chosen from oysters that experienced intense epizootic events and were bred for

multiple generations to successfully produce disease resistance lines (Haskin and Ford, 1979; Ford and Haskin, 1987; Burreson, 1991). Unfortunately, the heritability of dermo disease (*P. marinus*) resistance is less clear and due to environmental conditions dermo has gradually surpassed MSX as the leading pathogen in the Chesapeake Bay (Frank- Lawale et al. 2014). Burreson (1991) found that when MSX resistant lines were exposed to dermo disease, they were more susceptible to infection, and infection intensities were higher than lines that were not selected, meaning selecting for MSX resistance does not give an individual dual resistance, despite the coexistence of each pathogen in the water column. Ragone Calvo et al. (2003) successfully produced dual resistant lines for MSX and dermo disease by breeding four generations from the York River, VA where they experienced exposure to both, MSX, and Dermo. Frank-Lawale et al. (2014) found that performance of selectively bred lines was dependent on site, line, and site by line interaction (environment x genotype). The genotype x environment interaction can be a limiting factor for breeding programs because oysters from the same line may not have the same advantages under different environmental conditions (Frank-Lawale et al., 2014).

As triploidy becomes more common in commercial culture practices it is important to understand their relationship to pathogenic diseases. According to Allen et al. (1993) when exposed to dermo for one season, the difference between triploid and diploidy mortality was only about 10%; however, by the second season triploids suffered significantly higher mortality and were not recommended for disease resistant testing (Allen et al., 1993). While triploids did grow faster than their diploid counterparts, they were just as susceptible to *P. marinus* infections. Despite the lack of susceptibility differences, triploids reached market size before the second year and could be harvested prior to mortalities from *P. marinus* infection intensities (Baker and

Mann 1991). Through selective breeding hatcheries can help maintain genetic variation and health of oyster populations and potentially address the questions of 'summer mortality' and ploidy. Casas et al., (2017) reported that an F4 dermo selected line (OBOY) had lower cumulative and interval mortality (%) than F0 unselected lines, as well has consistently higher shell height (mm). The multi-generational selection allowed the line to outperform other lines at the Alabama grow-out sites, including lines produced from wild Alabama stocks (Casas et al., 2017). Genetic variation between Gulf of Mexico and Atlantic populations has also been recorded, with evidence supporting the idea that subpopulation created from genetic variability may allow populations to adapt to fluctuating estuarine conditions (Leonhardt et al. 2017; Burford et al. 2014; Varney et al. 2009; Murray and Hare, 2006; Rose et al. 2006; Reeb and Avise 1990). Low salinity events have been shown to be an effective disease management technique, consequently, if the low salinity is prolonged more than 3 weeks it can potentially cause stress on the oysters that they are not capable of withstanding (McCarty et al., 2020; La Peyre et al., 2013). McCarty et al. (2020) found that surviving acute low salinity is a heritable trait in oysters and could be an appropriate trait for selective breeding programs. With some limitations, this study demonstrated successful selection for survival in low salinity (2.5ppt) in higher temperatures (27℃) (McCarty et al., 2020). Frequent freshwater events from excess rain in the spring and river run-off have created low salinity environments in estuaries along the Gulf coast and having the ability to selectively breed oysters to survive these events could be beneficial for off-bottom oyster farmers that experience these conditions throughout the growing season.

In addition to environmental factors and pathogens, oyster growers, especially those using the more intensive off-bottom culture methods, may impose a variety of stressors on the oysters through culture practices. Tumbling and desiccating oysters has become regular practice for farmers along the Gulf Coast, so they can control biofouling, shape, and size of the cultured oysters. Tumbling allows farmers to sort and grade their crop by size and encourages better shell shape by breaking off new growth (Ring 2012). Exposing oysters to ambient air temperatures through desiccation has been proven to reduce infestation of other marine organisms, like barnacles, that can grow on the shells of the oysters and the grow-out gear (Mallet et al., 2009; Callow and Callow, 2002). Farmers that desiccate typically do so for about 18-24hr once a week (Bodenstein et al., 2021). However, there have been concerns that these management practices combined with stressful environmental conditions could lead to increased oyster mortality. Bodenstein et al. (2021) investigated the effects of these stressors on both diploid and triploid oysters during summer months at three different sites on the Gulf Coast. Despite both diploid and triploids being subjected to the same stressors, triploids had higher cumulative mortality (Bodenstein et al., 2021). Regardless of tumbling regimes, triploids had significantly higher mortality at 48 hours of desiccation, while diploid mortality was elevated only when they were tumbled and desiccated for 48 hours (Bodenstein et al., 2021). Triploids did have a higher mortality than diploids, but when compared to controls both ploidies had higher mortality when exposed to any stress treatment (desiccation or tumbling). Additionally, higher mortality was observed when water temperatures were higher, so farmers should be conscientious when managing their oysters during summer months (Bodenstein et al., 2021). Furthermore, exposing triploids to less stress during this time could potentially lower their susceptibility to ‘summer mortality’ (Bodenstein et al., 2021).

In addition to environmental conditions and farm management techniques, effect of oyster pathogens has been also associated with mortality. On the Atlantic coast there are two major diseases that cause mortality in eastern oysters (*C. virginica*), MSX and dermo. MSX (Multinucleated Sphere Unknown) and dermo are caused by two different protists *Haplosporidium nelsoni* and *Perkinsus marinus* (Burreson and Stokes 2000). These parasitic protistan pathogen can be lethal to *C. virginica* through reduced growth and reproduction. While there has been no detection of MSX in the Gulf of Mexico, *P. marinus* is found in wild oyster populations across the Atlantic and Gulf coast (Ulrich et al., 2017; VIMS Fact Sheet). First described by Mackin et al. (1950), *P. marinus* transmission is direct from infected dying oysters to healthy ones that become new hosts, and all stages (meront, prezoosporangia, and biflagellate zoospores) are thought to be infective or become infectious once the oyster dies and the deteriorated tissue enters the water column (Chu 1996). Hosts typically become infected through the digestive tract and the proliferation of the parasite rapidly increases in high temperatures and salinities (Andrews 1996). Diploid and triploid oysters of the same species (*C. gigas* or *C. virginica*) did not differ in prevalence or intensity of Dermo; however, *C. virginica* diploids had higher mortality than conspecific triploid (Meyers et al., 1991). Additionally, several studies cite the detrimental effects of *P. marinus* infections on oyster growth and reproduction. Andrews and Ray (1988) observed that transplanting oysters to lower salinity areas (and harvesting oysters prior to large *P. marinus* infestations) could encourage disease control (Andrews and Ray 1988). Paytner and Burreson (1991) observed a reduction in shell length (mm), occasionally up to 60%. When evaluating *P. marinus* effect on condition index, observed differences tended to be more of a response to sampling time (season/month), however, the data proves that infection can reduce condition index (Paytner and Burreson, 1991). La Peyre et al. (2009) examined how

freshet events (pulsed freshwater event) affect intensity of *P. marinus* infections. When infected oysters were exposed to low salinities (<5 ppt) for a short period of time (~3 weeks) there was a reduction in infection intensity (La Peyre et al. 2009).

Overview of Research Goals

Within the last several years, farmers in Virginia and the northern Gulf of Mexico have raised concerns about the survival of triploid *C. virginica* and their viability as a crop for off-bottom oyster aquaculture in the northern Gulf of Mexico. While several studies have documented a trend of triploids potentially experiencing higher levels of mortality (Wadsworth et al., 2019), these studies have not had consistent, predictable results about the causes of this triploid mortality. To achieve our research objectives, first generation triploid lines were produced from three different stocks of wild Louisiana oysters: Sister Lake, Calcasieu Lake, and Vermillion Bay. These sites were chosen for broodstock collection because of their historical differences in temperature, salinity, and dermo prevalence and their potential selection for tolerance to different salinity regimes. Three additional lines of half-sibling diploids were produced from the same wild broodstock for experimental controls. Experimental replicates were deployed at 2 commercial farms and one research farm in coastal Alabama: Mobile Bay, Dauphin Island, and Grand Bay. Through monthly monitoring growth and survival were recorded as well as condition index and Dermo disease status quarterly. Based on previous work by Wadsworth et al. (2019), in concert with industry feedback, a second hypothesis of oyster size affecting mortality in triploids, specifically larger oysters dying more quickly than smaller sized oysters. A second study was deployed at one site in Alabama (Grand Bay) to determine the effect of oyster size on grow out performance. This was achieved by deploying diploid and triploid progenies from the above mentioned wild broodstock (Calcasieu Lake and Sister Lake) that were

mechanically graded into three different size classes (as a proxy for growth). Growth and survival were monitored monthly, and condition index and dermo disease status were determined after final collection. Additionally, temperature and salinity conditions were monitored daily at each study site. Understanding the additive effects of broodstock origin, size, and ploidy selection on triploid performance under different environmental conditions is important for the success of off-bottom oyster aquaculture on the Gulf Coast.

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CHAPTER TWO: CHAPTER TWO: DECREASING MORTALITIES OF TRIPLOID EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) IN THE ESTUARIES OF THE NORTHERN GULF OF MEXICO: FIELD EVALUATION IN COASTAL ALABAMA

Introduction

The Eastern oyster, *Crassostrea virginica,* is highly valued for its ecological, economic, and social role along the estuaries of the northern Gulf of Mexico (nGoM). Historically, commercial production relied heavily on harvest of market-sized oysters from public grounds, wild spat collection, or on-bottom culture of seed oysters, all of which depended on natural recruitment. With the establishment of off-bottom oyster farming in the nGOM and its use of hatchery- produced seed, oyster farmers in the region have widely adopted triploid oysters as the mainstay of their production, (Wadsworth et. al., 2019a) due to their faster growth and better summer meat condition across a range of different off-bottom gear (Wadsworth et al., 2019b; Walton et al. 2013; Maxwell & Supan 2010).

In 2016, however, oyster farmers in Alabama and Louisiana voiced concerns about elevated mortality in their crops that they associated with triploids, echoing similar concerns from Virginia growers (Matt et al. 2020; Guévélou et al 2017). Similarly, elevated mortalities were observed in some lines of triploid Pacific oysters, *Crassostrea gigas*, in France (Houssin et al. 2019), though another study found that triploids survived better than diploids (Gagnaire et al. 2006). These abnormal mortality events seem to overlap with the broader issue of 'summer mortalities' regardless of ploidy. Summer mortalities broadly are a long-recognized issue in oyster aquaculture with decades of reported summer mortality events from Japan, France, and the west coast of the United States (Burge et al., 2007; Soletchnik et al., 2007; Gagnaire et al., 2006; Cheney et al., 2000; Koganezawa, 1975). Early studies have shown that seasonal temperatures rising as oysters reproductively mature and may inhibit their ability to withstand environmental changes (Soletchnik et al., 2007; Gagnaire et al., 2006; Cheney et al., 2000; Imai et al.,1965; Tamate et al., 1965). but clear and consistent triggers have not been identified and the additional effects of triploidy require further investigation.

Allen and Dowling (1986) found during months that diploids were spawning, triploid *C. gigas* tended to use less of their glycogen reserves and continued to grow. Due to reduced gamete maturation, triploids utilize less of their glycogen reserves so it was hypothesized that this would provide a tolerance to stressful environmental conditions that are observed during summer spawning months (Guo and Allen,

1996; Allen and Dowling, 1986). In France, Dégremont (2006), found that juvenile oysters' (*C. gigas*) tolerance to summer mortality has a high genetic correlation that suggested selective breeding could be advantageous to improve production. Suggesting that selecting wild broodstock from local environments could potentially contribute to a higher survival in offspring. Callam et al. (2016) explored a link between genetic contribution from diploid broodstock (when crossed with tetraploids) to triploid offspring performance in differing salinity regimes. While triploids did not outperform diploids in terms of growth in low salinity environments, they maintained higher condition index throughout the summer (Callam et al. 2016); they suggested that farmers in lower salinity regions, such as the Maryland portion of the Chesapeake Bay (<15 ppt), might use triploids for the condition index benefit despite no growth advantage.

In addition to ploidy, managing the presence of *Perkinsus marinus* parasites is pertinent to the success of the oyster populations in the nGOM. La Peyre et al. (2003) used laboratory-controlled freshet events to simulate the possible changes in the Louisiana estuaries and recorded the effects on *P. marinus* infection intensities. Oysters with moderate to heavy *P. marinus* infections survived spring and winter freshets, but not summer. High initial infection rates coupled with high summer temperature, overwhelmed oysters exposed to significant amounts of freshwater, ultimately leading to high mortality (La Peyre et al. 2003). Casas et al (2017) found that during mid to late summer when water temperature was the warmest and *P. marinus* infection intensity was found the be the highest, cumulative mortality was significant among the different stocks. Harvesting prior to these stressful months is recommend but not always possible as the environmental conditions might have not been favorable for oysters to reach market size that early. Selecting wild broodstock from different salinity regimes and *P. marinus* exposures could potentially create different stocks of oysters that would have growth enhancement and disease resistance during grow out seasons (Casas et al. 2017).

In response to concerns about triploid mortality and 'summer mortalities' within the nGOM, Wadsworth et al (2019a) quantitatively compared triploid and diploid oysters in a field study at four sites in Alabama; while triploids outgrew diploids during the main grow-out season, triploid mortality was

significantly greater than diploid mortalities at each of the four sites (of differing salinities), though the magnitude and timing of mortality varied greatly. No clear cause was found for the mortalities across all four sites, though salinity was implicated as a cause in at least one site (Wadsworth et al. 2019a). Bodenstein et al. (2021) found that farm handling techniques such as desiccation and tumbling can affect ploidy differently. Triploid mortality significantly increased when desiccation was 48 hours regardless of whether they were tumbled or not, while diploid mortality only significantly increased when tumbled and desiccated for 48 hours (Bodenstein et al. 2021). Matt et al. (2020) observed a triploid mortality event at one of their four experimental sites, however there were no differences between oyster lines despite their different genetic lineages suggesting that triploid mortality in the Chesapeake Bay may involve more than broodstock source and/or specific genetic crosses (Matt et al. 2020). Similarly, Guévélou et al. (2019) documented the first triploid mortality in the Chesapeake Bay that was not connected to major parasitic diseases (e.g., MSX, Dermo). In this study, they suggested that some oysters may be more susceptible than others, however the mortality coincided with peak gametogenesis stages (Guévélou et al. 2019)

Given this prior work, in this study we conducted two related tests of causes of triploid mortality. First, we tested the effect of broodstock collected from different salinity regimes to produce half-sibling triploids and diploids to evaluate the impact of maternal diploid genetic contributions to oyster survival at three sites with differing salinity regimes. Second, we tested the effect of oyster size within a cohort (as a proxy for growth) on oyster survival between triploids and diploids within a line because previous work in addition to industry feedback suggest disproportionate mortality associated with larger oysters. Oyster performance was assessed through survival, growth, condition index, and *Perkinsus marinus* infection intensity. The intention of this work was to determine if different strains of triploids improve performance, improve culture practices, and increase quality of triploid production.

Materials and Methods

Oysters

Broodstock (*C. virginica)* were collected from three estuaries in Louisiana with differing salinity regimes (Figure 1): Calcasieu Lake (29.860348° N, -93.309144° W, February 5th, 2019), Sister Lake (29.129673° N, -90.945780° W, January 28th, 2019), and Vermillion Bay (29.717930° N, -91.955926° W, January 15th, and February 6th, 2019). Historically, Calcasieu Lake estuaries experience relatively intermediate (10-20ppt) to high (>20) salinities annually (20.4 ± 2.2), Sister Lake experiences low (<10) to intermediate salinities (10.4 ± 2.5), and Vermillion Bay experiences highly variable annual salinities (1.2-22.9) (Leonhardt et al., 2017; Lowe et al., 2017).

Broodstock oysters were maintained on longlines in the field in Grand Isle, LA until they were sent to AUSL on July 17th, 2019. Prior to shipping, the oysters were scrubbed clean and placed on a dry burlap overlaying a bed of ice packs in a cooler. The tetraploid broodstock (4MC18) was produced at the Auburn University Shellfish Laboratory (AUSL, Dauphin Island, AL) and is a Gulf of Mexico lineage from the original tetraploid line (4nGNL) created by the Louisiana State University Sea Grant Hatchery on Grand Isle, LA. 4MGNL15 (tetraploid generic line) were mated to produce 4MC18 on July 31, 2018; 21 females and 16 males. All tetraploid sperm was verified by flow cytometry prior to fertilization. Thermal heat shock was used to induce spawning. All spawning, larval rearing, and nursery care took place at AUSL prior to field deployment.

Broodstock was spawned in individual containers to contain released gametes and induced through “thermal shock”. Cold and warm water flowed through the containers to trigger spawning and mimic natural spawning season conditions. For both diploid and triploids, diploid

eggs are used, however, diploid sperm is used to fertilize eggs to produce diploids, while tetraploid sperm was stripped from male individuals and used to fertilize diploid eggs to produce triploids (Table 1). Due to space constraints, spawns of each broodstock were done on different dates. On July 18th, 2019, Calcasieu Lake broodstock were spawned; 14 females and 7 males were spawned for diploid F1 stock (2CL) and 14 females and 10 tetraploid males were spawned for F1 triploid stock (3CL). On July 23rd, 2019, Sister Lake broodstock were spawned; 51 females and 33 males were used to spawn F1 diploid stock (2SL), and 51 females and 8 tetraploid males were used to create F1 triploid stock (3SL). On July 25th, 2019, Vermillion Bay broodstock were spawned; 40 females and 46 males were used to create F1 diploid stock (2VB), and 46 females and 8 tetraploid males were used to create triploid F1 stock (3VB).

Broodstock (2n)	2N	4MC18
SL	2SL	3SL
CL	2CL	3CL
VB	2VB	3VB

Table 1: Spawning design for *C. virginica* produced from crossing diploid oysters (2N) from Sister Lake (SL), Calcasieu Lake (CL), and Vermillion Bay (VB) in a 3 x 1 matrix to produce three diploid lines (2SL, 2CL, & 2VB) as well as same diploid female oysters to tetraploid male oysters (4N) 3 x 1 matrix to produce three triploid lines (3SL, 3CL, & 3VB).

Figure 1: A map of Louisiana estuaries. Broodstock collection sites indicated by stars: Calcasieu Lake, Vermillion Bay, and Sister Lake.

Ploidy Verification:

Ploidy for each mated treatment was verified by flow cytometry performed at AUSL. For any verification, twenty-five oysters were tested per treatment. If ploidy was not 100% in the first 25 individuals 25 more oysters were then tested for ploidy. Specifically, when testing triploids, if the second group of 25 individuals were not 100% triploid they were labeled as a mixture and not pure triploids. Each individual spat was placed in a centrifuge tube in a ~1ml solution of DAPI/DMSO. Using a sterilized syringe, the solution was filtered through a 20µm screen into a 3.5mL Röhren tube with ~1ml of DI water and then inserted into the flow cytometer. On August 12th, 2019 (~2.5 wks. after spawning), the spat of the CL and SL lines were tested and verified to be 100% of the putative ploidy, respectively. In that same verification, discrepancies were observed in the original verification of 3VB; therefore, a second

test was run on additional 25 individual seed of each VB line. Out of 50 seed of 2VB, 49 oysters were found to be diploid (98%). For 3VB, 45 out of 50 individuals were triploid (95%). A second test was run on 3VB gill samples on February 12, 2020, and 44 out of the 50 samples were found to be triploid (88%). This is attributed to contamination during fertilization by regular diploid sperm. Most likely, deactivation of sperm was not complete (where deactivated sperm are used to help induce spawning in thermal shock spawning at AUSL). During nursery grow-out 3VB, seed was tested based on their "set" (metamorphosis) date to see if there was a higher or lower triploid percentage. Oysters set after August 7th were $\leq$ 88% triploid and were discarded. In February 2020, 3VB gill samples were verified again and were 88% triploid. By industry standard triploids must be 94% to be sold as triploid oysters, so despite Vermillion Bay triploids being 88% we deemed this a high enough percentage to be able to observe triploid effect on our response variables.

Nursery Grow Out

In response to the threat of Hurricane Dorian, seed from all six treatments were split into three groups, with two groups deployed on August 23rd, 2019, at two research farms (Grand Bay Oyster Park, 30.375436° N, -88.315730° W, and Bayou Sullivan Oyster Park, 30.364474° N, -88.215714° W) and a third group held in a nursery upweller at AUSL (30.247232° N, -88.078430 W°). By September 11, 2019, seed held at AUSL were divided between the two research farms and deployed. Seed remained at these two sites until the start of the experimental test of the effect of broodstock origin in November 2019. For the experimental test of the effect of oyster size, the seed from the two research farms were combined, sorted by size, and deployed in March 2020.

Site Descriptions

The test of the effect of broodstock origin study was conducted at three different sites along the Alabama coast of the northern Gulf of Mexico (nGoM, Figure 2), intended to represent three different salinity regimes approximating the salinity regimes of the areas where the broodstock were harvested (relatively low, relatively high and variable). The first site in Alabama was on the western shore of Mobile Bay (MOB) in cooperation with Bama Bay Oyster Farm on Mon Louis Island, Alabama (30.441373° N, -88.104191° W) and was intended to be the relatively low salinity site. The second site was on the Mississippi Sound shore of Dauphin Island (DI), Alabama in cooperation with Massacre Island Oyster Company (30.253738° N, -88.169057° W) and was intended to be the relatively high salinity site. The third site was Grand Bay Oyster Park (GBOP) in Grand Bay Alabama, an AUSL research site (30.375436° N, -88.315730° W) and was intended to be the variable salinity site based on prior years. The oysters for the size effect study were only deployed at GBOP.

Environmental conditions were monitored with a combination of continuous in-water data loggers (salinity, temperature) and handheld devices when visiting sites monthly.at all three Alabama sites (salinity and temperature). An Onset U24-002-C HOBO Saltwater Conductivity Data Logger was deployed on the most “offshore” cage (see experimental design below). Every 2-3 weeks this logger was switched with a new one to keep biofouling from accumulating on the logger. When a logger was switched out, real time measurements were taken onsite with YSI 6600 V2 Multi-Parameter Sondes (Yellow Spring Instruments, Yellow Spring, Ohio) to use for calibration points.

Experimental Design

For the effect of broodstock origin study, 6 floating aluminum framed cages (built by Apalachicola Oyster Company) were deployed in October 2019 at each site (Figure 2). Floating cages were chosen for the experiment because it is a common farming gear type and could be used across all three sites. Each cage held six 9-mm mesh Vexar divided bags (90.5cm x 46.99cm x 6.35cm*)* in a randomized complete block design with each of the three broodstock origins for each ploidy (6 treatments: 2SL, 3SL, 2CL, 3CL, 2VB and 3VB) assigned to one randomly assigned bag (with a divider in the bag). All oysters were approximately 5 months old, half-sibling diploid and triploids within a broodstock origin treatment. Each bag was initially stocked with 100 oysters total (50 on each side of the divider). There were thirty-six bags at each site for a grand total of one hundred and eight bags. There were no imposed stressors (i.e., desiccation) throughout the study which concluded in June at MOB and September at DI and GBOP.

On June 23rd, 2020, sampling at the MOB site was concluded due to a high mortality event that began in April 2020. On April 3rd, the cooperating farmer notified AUSL of a mortality event experienced by nearby farmers and themselves. In response, mortality was assessed immediately and again two weeks later during the normal sampling period. This mortality, coupled with some oysters lost to Tropical Storm Cristobal in early June 2020, meant that sampling could not continue past the June sample. At the June sampling, the remaining oysters were sent to LSU for assessment of condition index, histology, and pathology. In addition, at the other sites, tropical storms and bag failures led to the loss of several replicates.

For the test of the effect of oyster size impact study, oysters were deployed in March 2020 at GBOP, using the respective half-sibling diploid and triploid progeny oysters from CL and SL broodstock. We chose to deploy this at one site due to space constraints at the

commercial farm sites with the assumption that environmental conditions would be ideal for grow-out performance (i.e.: salinity higher than Mobile Bay).For this, seed oysters of each line were graded separately on a mechanical tube sorter (Chesapeake Bay Oyster Co. Quicktube sorter ¼", ½") to sort into three size classes of small, medium, and large that were identical for each broodstock line (Table 2). Due to gear limitations, the oysters were deployed in a total of six floating cages (Oyster Gro™), each stocked with six divided bags with each bag half randomly assigned a different treatment (allowing 12 treatments per cage). Each cage contained one replicate of each of the 12 treatments (2 ploidies x 2 broodstock, and 3 sizes) for a randomized complete block design.

Broodstock	Ploidy	Size	Shell Height (mm)
Sister Lake	Diploid	*SMALL*	35.9 ± 0.50
		MEDIUM	51.9 ± 0.49
		LARGE	59.4 ± 0.56
	Triploid	*SMALL*	41.8 ± 0.51
		MEDIUM	56.2 ± 0.48
		LARGE	65.9 ± 0.74
Calcasieu Lake	Diploid	*SMALL*	41.9 ± 0.41
		MEDIUM	54.7 ± 0.52
		LARGE	64.0 ± 0.61
	Triploid	*SMALL*	38.8 ± 0.51
		MEDIUM	55.8 ± 0.56
		LARGE	66.9 ± 0.71

Table 2. Average (mean ± SE) initial shell height (March 2020) for Sister Lake and Calcasieu Lake diploids and triploids for the size effect study.

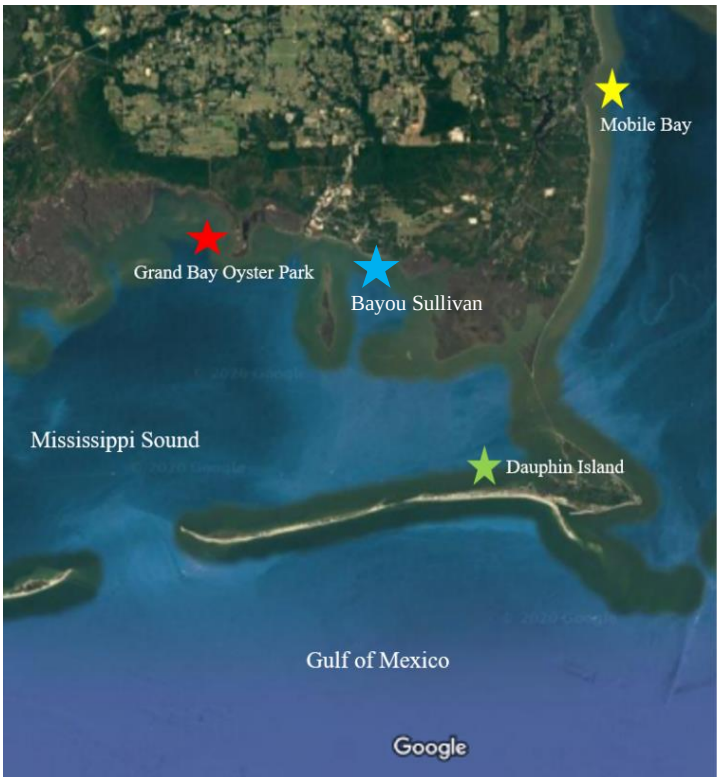

Figure 2: A map of Mobile Bay and the Mississippi Sound study sites: Mobile Bay (MOB), Dauphin Island (DI) and Grand Bay Oyster Park (GBOP). GBOP was also the nursery site and study site for the study of the effect of size.

Data Collection

The effect of broodstock origin study oysters were sampled monthly from January to September 2020 (30 haphazardly collected oysters per replicate). For the effect of size category study, oysters were sampled monthly from April to September. Shell height (Figure 3) was measured to the nearest 0.1mm using Mitutoyo IP67 ABS Coolant Proof calipers using the protocol of Galstoff (1964), and oysters were returned to the appropriate bag. Shell height measurements were used to calculate average growth rate (Equation 5) for periods between each sampling. Prior to deployment, initial shell height was measured for 100 oysters for each treatment for both studies, and the appropriate mean was used as the initial size at deployment. Live and dead oysters were counted monthly, and dead oysters were discarded but not replaced.

Mortality calculations were made following the protocol of Ragone Calvo et al. (2003) including interval mortality, adjusted interval mortality, cumulative mortality, and growth rate (Equations 1-4), accounting for oysters collected and removed for sampling.

For the effect of broodstock origin study, in March, May and September, 30 additional oysters per treatment (2-3 per replicate) were haphazardly collected and shipped overnight to LSU Animal and Food Science Laboratory in Baton Rouge, LA to determine whole wet weight, wet shell weight, dry tissue weight, condition index, *Perkinsus marinus* (dermo) infection intensity, and histology. Similarly, for the effect of size category study, this sampling occurred in November 2020. In each of these samples, 20 oysters were used for condition index and dermo processing and 10 for histology. Condition index of the oysters was determined by the procedures described by La Peyre et al. (2009). The condition index was calculated by dividing the dry weight of tissue by the whole oyster wet weight minus its wet shell weight and multiplying by 100, a variation of Hopkins formula as recommended by Abbe & Albright (2003). Because of COVID-19 restrictions on personnel, in May only 10 oysters per treatment were sent for histopathology processing.

Perkins marinus infection intensity is defined by the parasite per gram of oyster wet tissue and determined by using the whole-oyster procedure described by Fisher and Oliver (1996) and modified by La Peyre et al. (2003).

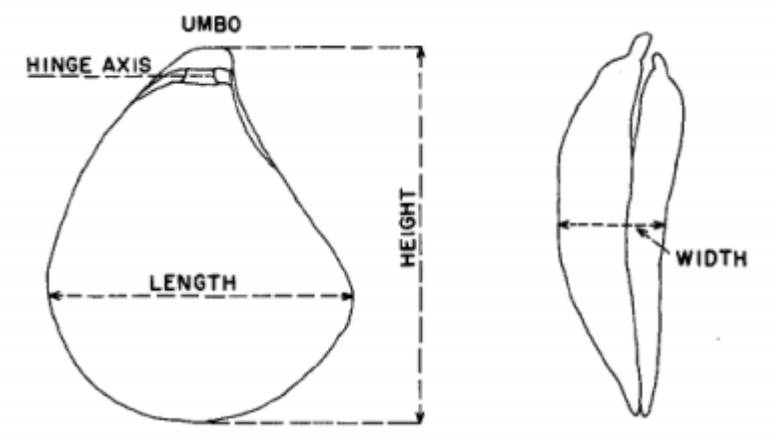

Figure 3: Shell metrics used to determine shell height (Galtsoff 1964)

Equation 1

Interval Mortality= No. dead oysters in current Interval ÷ total No. of oysters

Equation 2

Adjusted interval mortality= Interval Mortality x (1- CM of Preceding interval) x Current Interval's CM

Equation 3

Cumulative Mortality= Adjusted Interval mortality + Previous months cumulative mortality

Equation 4

Growth rate = (Average Shell Height at Final Collection - Average Shell Height at Deployment) ÷No. of Days between Deployment and Final Collection

Data Analysis

Data analysis was completed using R© and RStudio© statistical program, using packages *nlme, lme4, dplyr, emmeans* (R Development Core Team 2020). Data collected from January-September 2020 for the broodstock origin study were analyzed for each site by broodstock (2 df), ploidy (1 df), and the interaction between ploidy and broodstock (2 df) for the following response variables: growth (Equation 5) percent cumulative mortality, percent interval mortality, condition index, and *P. marinus* infection intensity (body burden). Data from the effect of size study were analyzed for each broodstock line by size (2 df), ploidy (1 df), and the interaction between size and ploidy (2 df) for the following response variables: growth, percent cumulative mortality, percent interval mortality, condition index, and *P. marinus* infection intensity. Analysis of variance tests (ANOVA) were used to determine statistical significance between, respectively, 1) broodstock, ploidy, and the broodstock x ploidy interaction, and 2) size category, and the size category x ploidy interaction followed by a Tukey's Multiple comparison test when significant differences were found ($p<0.05$). The statistical model included cage as a random block effect. Condition index and Dermo infection intensity data were non-normal and were log transformed to satisfy homogeneity variance and achieve normality. Interval salinity and temperature data were analyzed using Kruskal-Wallis one factor analysis of variance (ANOVA) test followed by Dunn's multiple comparison test when significance ($p<0.05$) was found.

Results

Environmental Conditions

Average daily water temperature and salinity across the study period were significantly different among the three Alabama sites ($p<0.01$, Table 3). MOB and DI had significantly lower mean water temperature (°C± SD) than Grand Bay ($p=0.04$ and $p<0.01$, respectively). MOB had the lowest mean salinity of 5.62 ± 5.30 and was significantly lower than both DI and Grand Bay salinity ($p<0.01$), and GBOP had significantly lower salinity than DI ($p=0.02$). From March to April 2020, GBOP had its lowest average salinity of 8.34 ± 0.60 ppt (Figure 5). DI had its lowest average salinity from May to June at 11.6 ± 1.03 ppt (Figure 5). From January to June (at which point data were no longer collected due to extreme levels of oyster mortality), MOB did not have an average salinity over 5 ppt, with March and April being the lowest at 1.4 ± 0.21 ppt (Figure 5).

Site	Temperature (°C ± STD)	Daily Min (°C)	Daily Max (°C)	Salinity (ppt ± Std)	Daily Min (ppt)	Daily Max (ppt)
MOB^a	21.3 ± 5.75	8.43	33.2	5.62 ± 5.30	0.31	21.6
DI^c	21.3 ± 5.60	11.7	30.8	20.8 ± 5.77	3.40	32.6
$GBOP^b$	22.5 ± 6.56	10.9	32.6	19.2 ± 5.42	4.38	28.8

Table 3: Average (mean ± SD) temperature and salinity at three study sites in Alabama with daily minima and maxima. Grand Bay and Dauphin Island data were collected from October 2019-September 2020. *Mobile Bay data were collected from October 2019-June 2020.

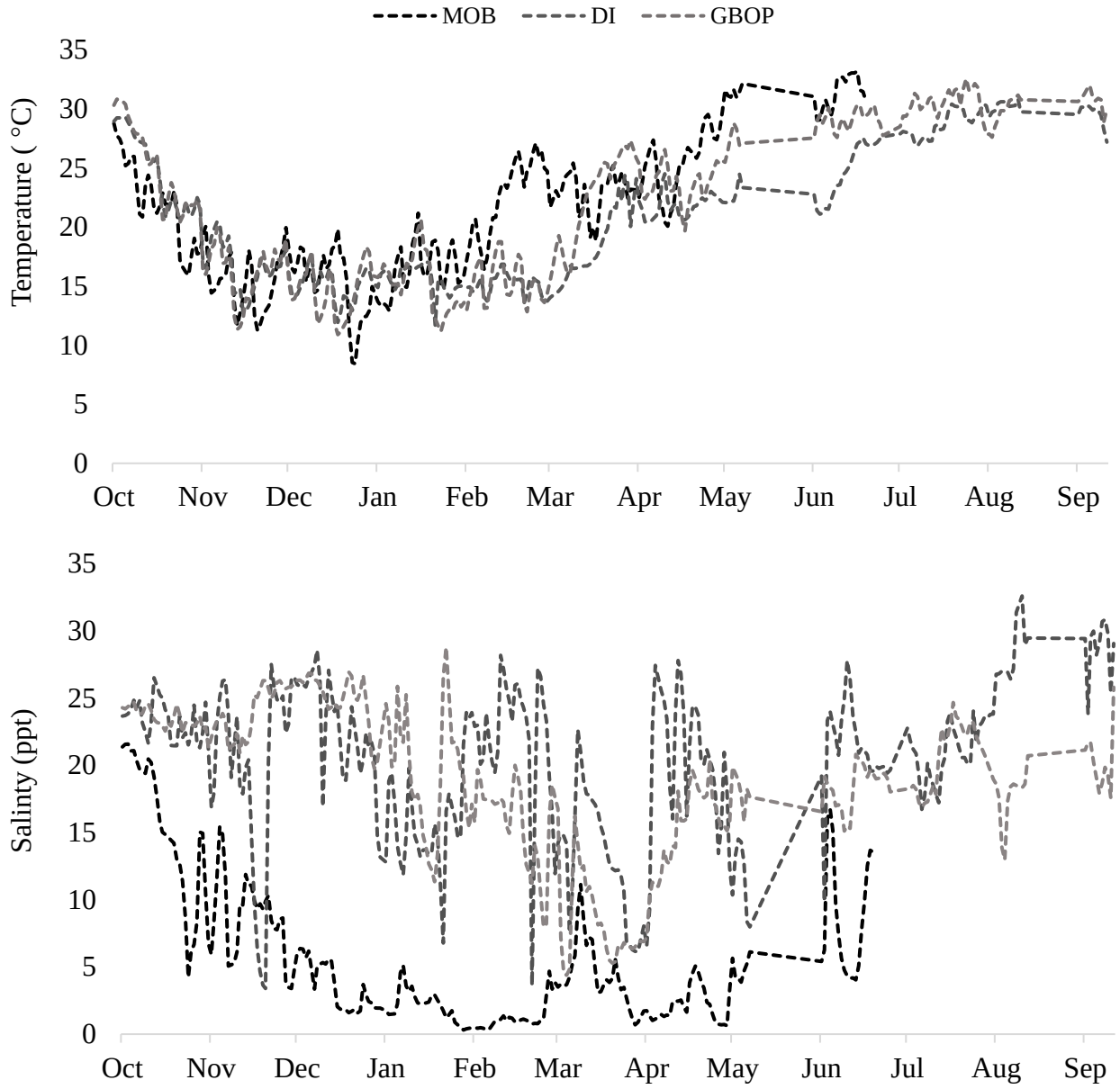

Figure 4: Daily water temperature and salinity at all three study sites from October 2019 to September 2020. *Mobile Bay data collected from October 2019 to June 2020. Logged at each site with an Onset U24-002-C HOBO Saltwater Conductivity Data Logger.

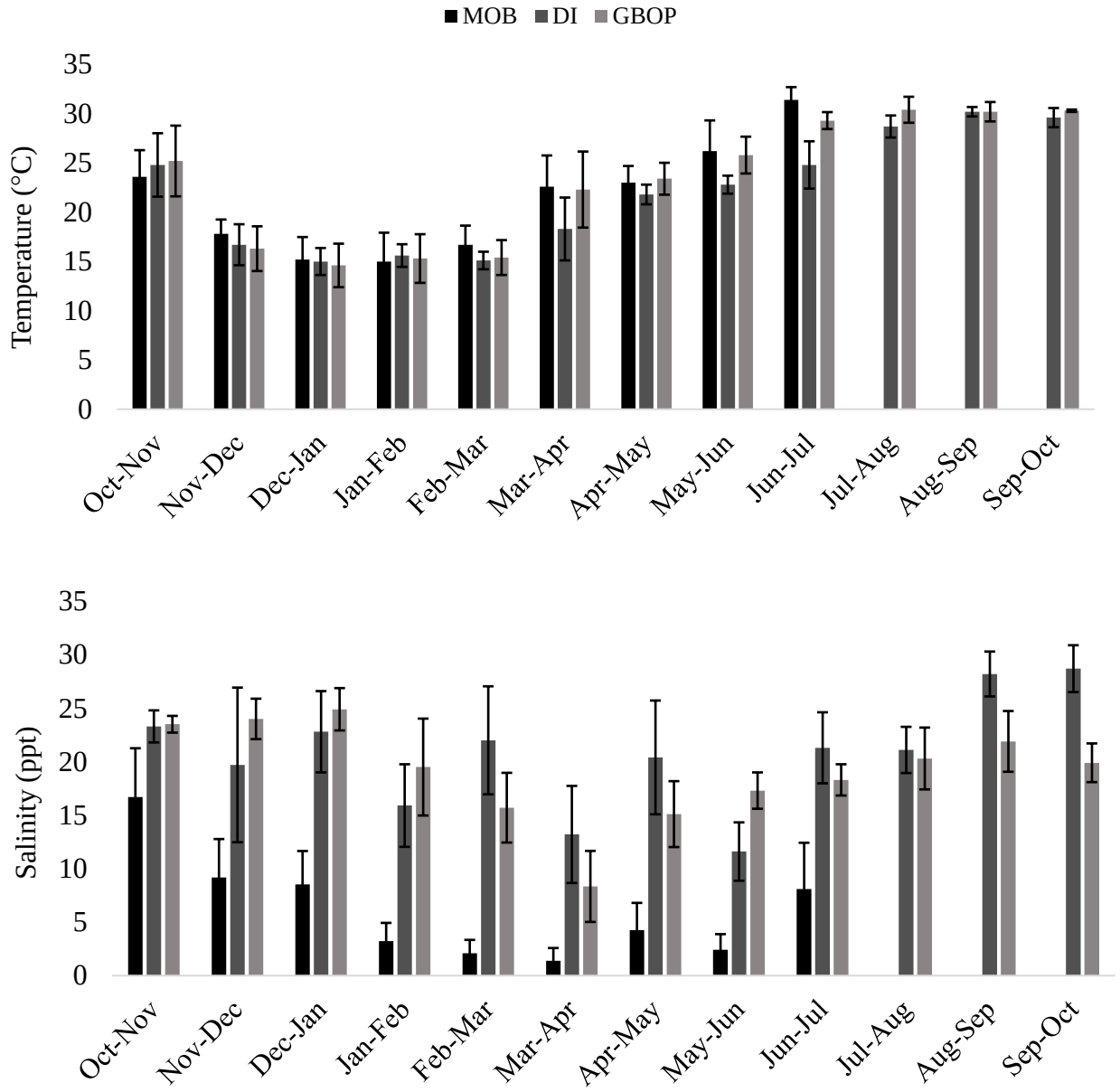

Figure 5: Mean (± SE) interval water temperature and salinity at all three sites from October 2019 to September 2020. *Mobile Bay data collected from October 2019 to June 2020. Error bars represent standard deviation.

Effect of Broodstock Origin and Ploidy on Cumulative Mortality

Cumulative mortality was only affected by ploidy as all three sites regardless of maternal broodstock origin, with triploids experiencing significantly greater mortality than diploids (Table 4, Figure 6). Cumulative mortality was by far the highest at MOB; MOB triploids experienced 95.8% (± 0.019) average cumulative mortality, while diploids experienced an average of 78.1% (± 0.034) cumulative mortality ($p<0.01$). At DI (where mortality was the lowest), triploids experienced 12.1% (± 0.02) cumulative mortality while diploids experienced 3.9% (± 0.0077, $p<0.01$). At GBOP, triploids experienced 17.5% (± 0.022) mortality while diploids experienced 5.1% (± 0.0083, $p<0.01$).

Factor	Site	df	F	p
Broodstock Origin	MOB	2	1.80	0.18
Ploidy		1	16.27	**<0.01**
Broodstock Origin x Ploidy		2	1.72	0.12
Broodstock Origin	DI	2	1.99	0.12
Ploidy		1	32.82	**<0.01**
Broodstock Origin x Ploidy		2	0.43	0.67
Broodstock Origin	GBOP	2	0.085	0.92
Ploidy		1	28.80	**<0.01**
Broodstock Origin x Ploidy		2	0.076	0.93

Table 4: Analysis of variance (ANOVA) of cumulative mortality (%) of broodstock origin, ploidy, broodstock origin x ploidy for all three sites: Mobile Bay (MOB), Dauphin Island (DI), & Grand Bay (GBOP).

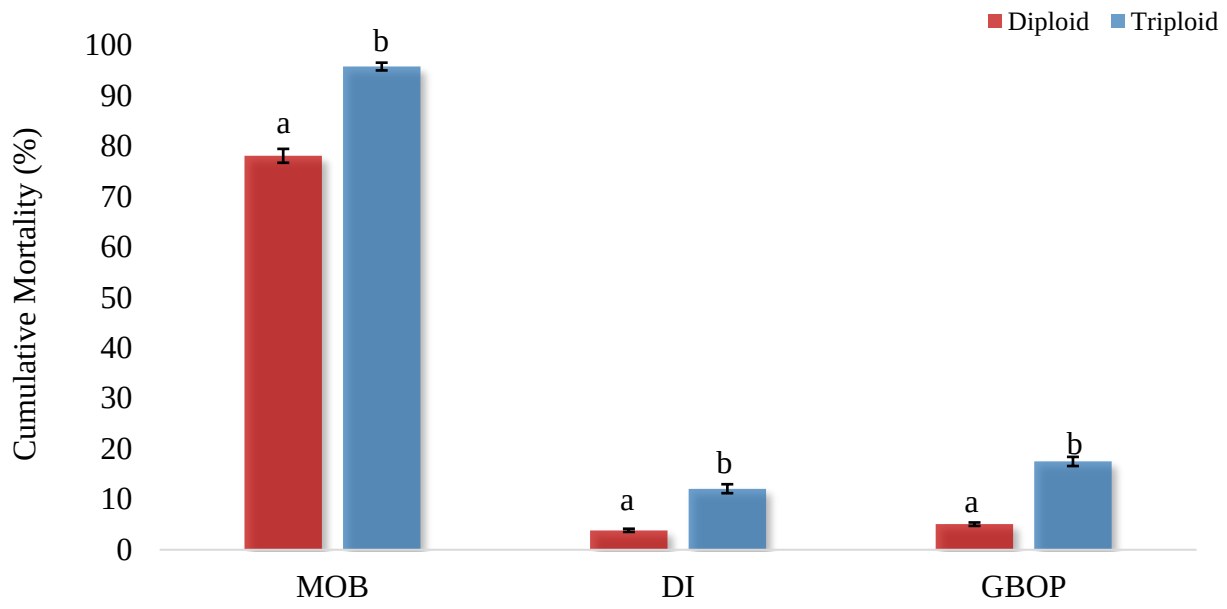

Figure 6: Mean (± SE) cumulative mortality of diploid and triploid oysters at the three study sites; Mobile Bay (MOB), Dauphin Island (DI), and Grand Bay (GBOP). Superscripts denote statistical differences between ploidy at each site ($p \leq 0.05$).

Effect of Broodstock Origin and Ploidy on Interval Mortality

Qualitatively, cumulative mortality appeared to be driven primarily by an elevation in interval mortality from March to April at all three sites, with a secondary pulse of mortality from August to September at DI and GBOP (Figures 7-9).

At MOB, from March to April there was significant effect of broodstock origin ($p=0.01$) and ploidy on interval mortality ($p<0.01$) but no interaction ($p=0.07$) For ploidy, diploids experienced significantly lower mortality than triploids ($p<0.01$). For broodstock origin, interval mortality was significantly lower for CL oysters compared to SL oysters ($p=0.01$), with VB not differing from either.

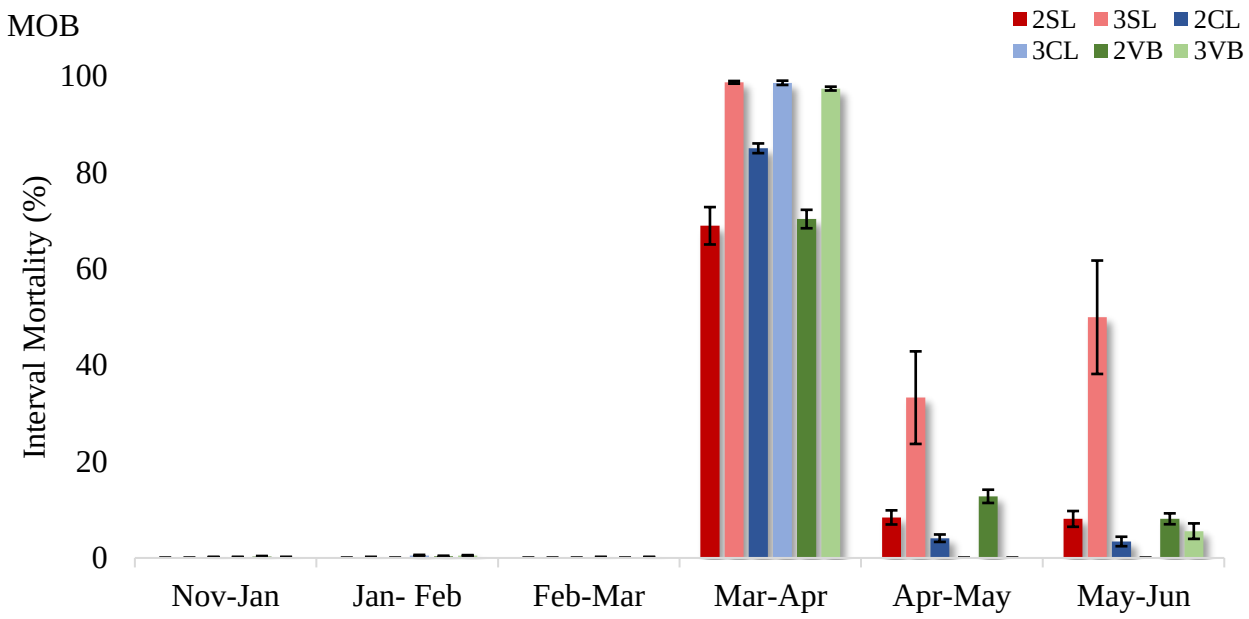

Figure 7: Mean (± SE) interval mortality (%) at Mobile Bay (MOB) from November 2019 to June 2020. Error bars represent standard error.

At DI from March to April (Figure 8) there was significant effect of broodstock origin on interval mortality($p=0.02$) and ploidy ($p<0.01$), and there was no broodstock x ploidy interaction ($p=0.10$). Among broodstock origin, mortality in VB was significantly lower than either SL or CL ($p\leq0.02$). Additionally, mortality was significantly higher in triploids relative to diploids ($p<0.01$). Within the August to September interval at DI (Figure 8), mortality was only significantly affected by ploidy, with mortality again higher in triploid oysters ($p<0.01$) with no effect of or interaction with broodstock origin ($p\geq0.53$).

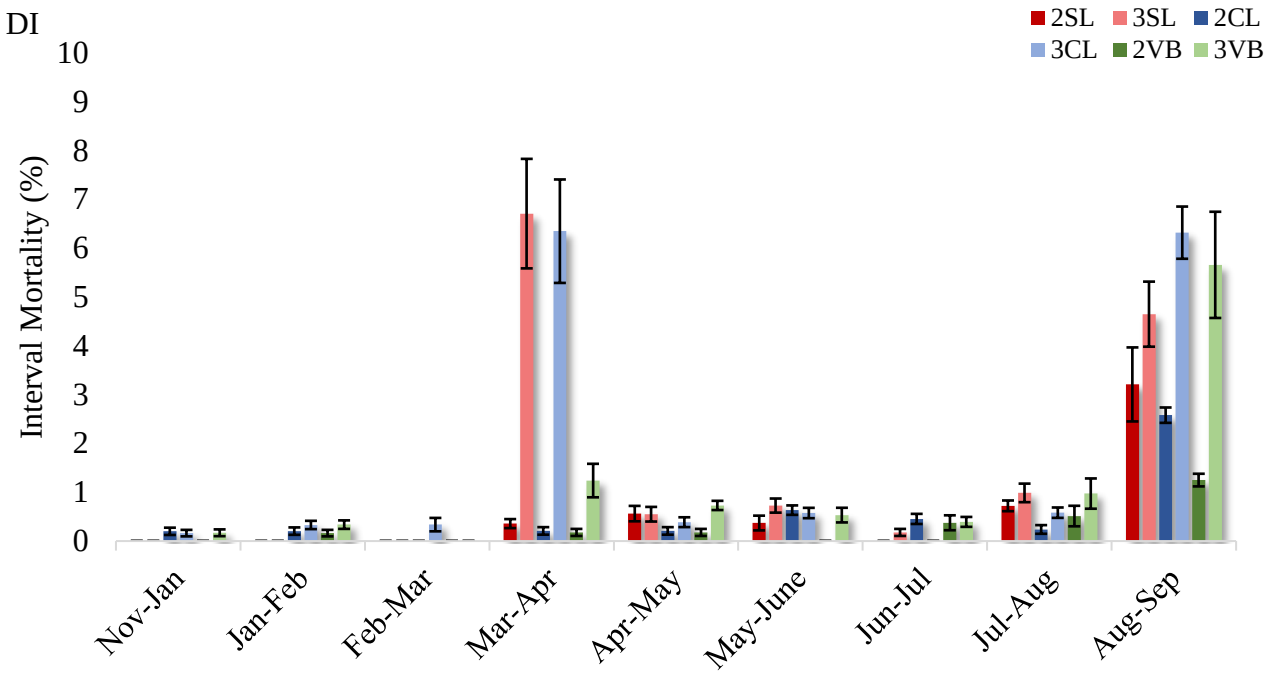

Figure 8: Mean (± SE) interval mortality (%) at Dauphin Island (DI) for all six experimental treatments: diploid (2) and triploid (3) Sister Lake (SL), Calcasieu Lake (CL), and Vermillion Bay (VB). Error bars represent standard error. Note that the Y-axis scale is different from MOB figure to show mortality differences.

Interval mortality at GBOP from March to April (Figure 9) was also significantly affected by ploidy ($p<0.0001$) but not by broodstock origin nor an interaction with broodstock origin ($p\geq 0.96$) with, triploid oysters suffering higher interval mortality than diploids ($p\leq 0.01$).

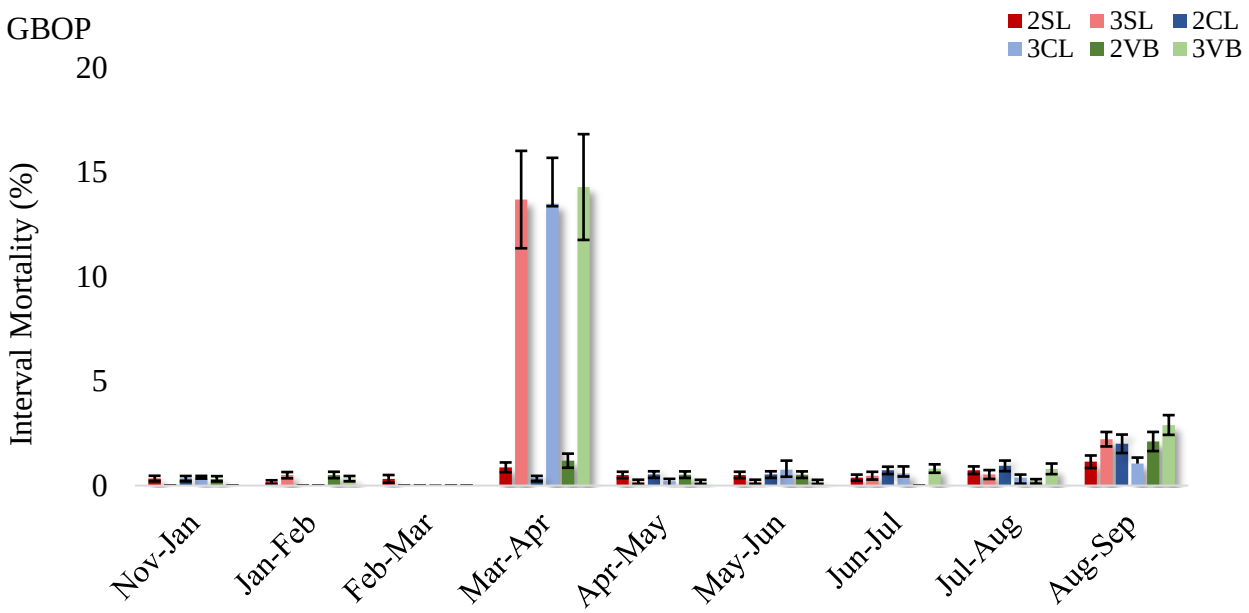

Figure 9: Mean (± SE) interval mortality (%) at Grand Bay (GBOP) for all six experimental treatments: diploid (2) and triploid (3) Sister Lake (SL), Calcasieu Lake (CL), and Vermillion Bay (VB). Error bars represent standard error. Note that the Y-axis scale is different from MOB and DI figure to show mortality differences.

Effect of Broodstock Origin and Ploidy on Growth Rate

Within each site, there were several differences in growth rates (Tables 5). At MOB (Figure 10), there was a broodstock origin x ploidy interaction (p=0.03, Table 6). Diploid VB grew significantly slower than diploid CL, and diploid and triploid SL (p=0.01, Table 6), with no other significant differences among the treatments. In addition (Figure 11), while interval growth rates were relatively low throughout the study, interval growth dropped even further in spring 2020 (concurrent with the lowest salinities) and mortality may have affected the overall growth results (larger oysters dying more quickly).

Treatment	Site	Initial Shell Height (mm ± SE)	Final Shell Height (mm ± SE)	Days Deployed	Average Daily Growth Rate (mm/day ± SE)
2SL	MOB	33.2 ± 0.53	32.8 ± 0.49	182	-0.0011 ± 0.0027
3SL		39.2 ± 0.62	42.4 ± 0.77		0.018 ± 0.0042
2CL		36.1 ± 0.67	35.1 ± 0.72		-0.0054 ± 0.004
3CL		47.6 ± 0.86	43.6 ± 0.55		-0.035 ± 0.015
2VB		36.4 ± 0.60	32.6 ± 0.49		-0.021 ± 0.0027
3VB		44.1 ± 0.74	41.2 ± 1.76		-0.016 ± 0.0097
2SL	DI	33.2 ± 0.53	64.9 ± 0.86	304	0.10 ± 0.0028
3SL		39.2 ± 0.62	78.1 ± 0.80		0.13 ± 0.0026
2CL		36.1 ± 0.67	71.2 ± 0.77		0.12 ± 0.0026
3CL		47.6 ± 0.86	81.7 ± 0.79		0.11 ± 0.0026
2VB		36.4 ± 0.60	70.4 ± 0.72		0.11 ± 0.0024
3VB		44.1 ± 0.74	83.3 ± 0.79		0.13 ± 0.0026
2SL	GBOP	33.2 ± 0.53	70.5 ± 0.71	304	0.12 ± 0.0023
3SL		39.2 ± 0.62	76.3 ± 0.68		0.12 ± 0.0022
2CL		36.1 ± 0.67	70.1 ± 0.53		0.11 ± 0.0017
3CL		47.6 ± 0.86	81.7 ± 0.64		0.11 ± 0.0021
2VB		36.4 ± 0.60	69.8 ±0.56		0.11 ± 0.0018
3VB		44.1 ± 0.74	79.8 ± 0.68		0.12 ± 0.0022

Table 5: Mean (± SE) initial and final shell height of each treatment, days deployed and calculated average daily growth rate (mm/day) over the days deployed at all three experimental sites: Mobile Bay (MOB), Dauphin Island (DI), & Grand Bay (GBOP).

Factor	Site	df	F	P
Broodstock Origin	MOB	2	29.84	**<0.01**
Ploidy		1	1.14	0.30
Broodstock Origin x Ploidy		2	3.72	**0.03**
Broodstock Origin	DI	2	2.86	**0.02**
Ploidy		1	35.98	**<0.01**
Broodstock Origin x Ploidy		2	13.41	**<0.01**
Broodstock Origin	GBOP	2	8.64	**<0.01**
Ploidy		1	1.10	0.30
Broodstock Origin x Ploidy		2	1.58	0.20

Table 6: Analysis of variance (ANOVA) of daily growth rate (mm/day) for broodstock origin, ploidy, and broodstock origin x ploidy for all three sites: Mobile Bay (MOB), Dauphin Island (DI), & Grand Bay (GBOP).

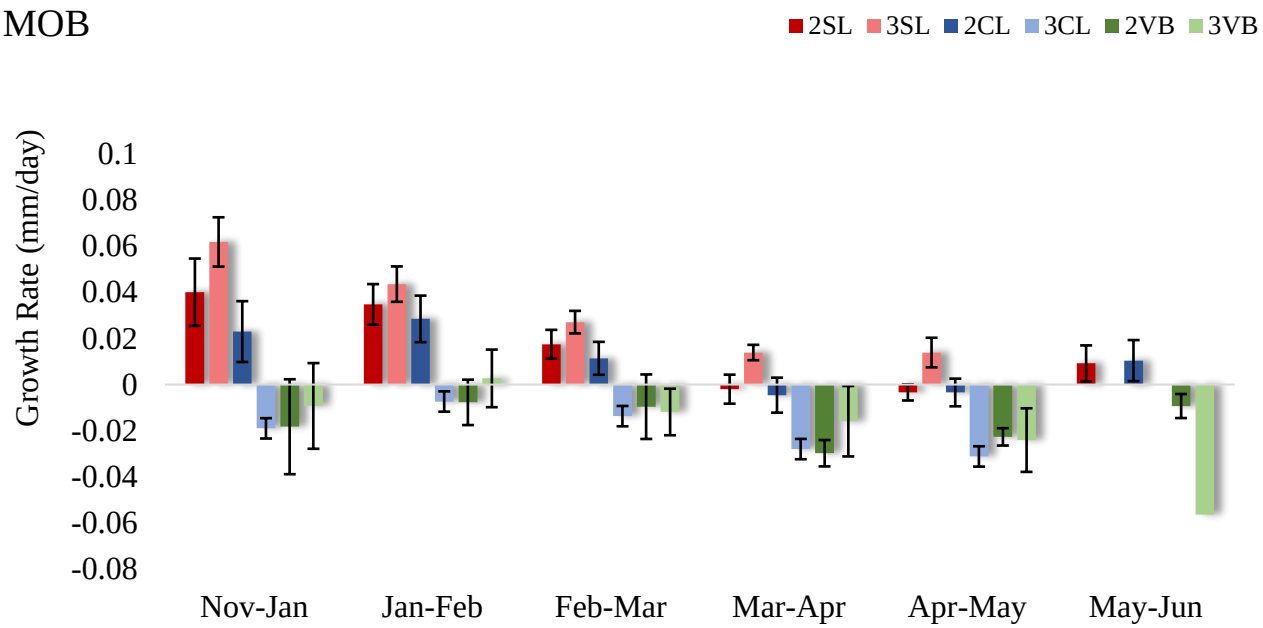

Figure 10: Mean (± SE) daily growth rates (mm/day) from deployment to final collection date (November 2019- June 2020) of each experimental treatment at Mobile Bay (MOB). Superscripts denote statistical difference ($p \leq 0.05$). Error bars represent standard error.

At DI (Figure 11), there was also a broodstock origin x ploidy interaction ($p<0.01$, Table 6). At this site, triploid VB and SL grew faster than all other treatments ($p\leq0.04$). All other treatments were not significantly different ($p\geq0.10$).

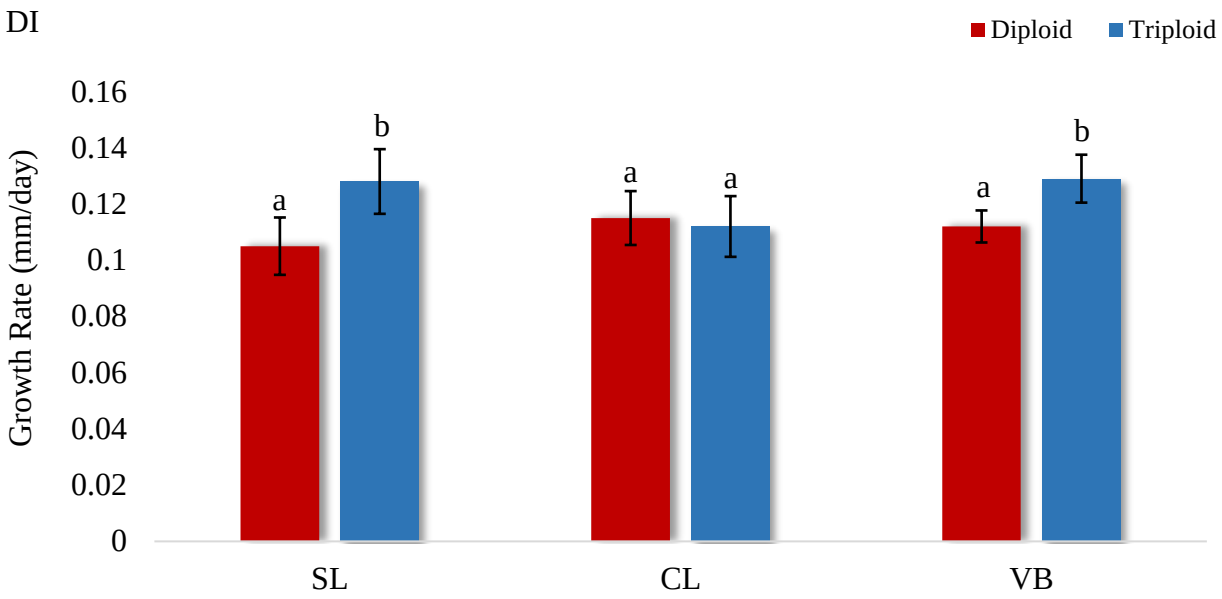

Figure 11: Mean (± SE) daily growth rates (mm/day) from deployment to final collection (November 2019 - September 2020) of each experimental treatment at Dauphin Island (DI). Superscripts denote statistical difference ($p\leq0.05$).

At GBOP, daily growth rates were only significantly affected by broodstock origin ($p<0.01$, Figure 12) and not by ploidy ($p=0.30$) nor interaction between broodstock x ploidy ($p=0.20$, Table 6). Regardless of ploidy, SL oysters grew significantly faster than CL & VB oysters ($p<0.01$), which did not differ from each other ($p=0.90$).

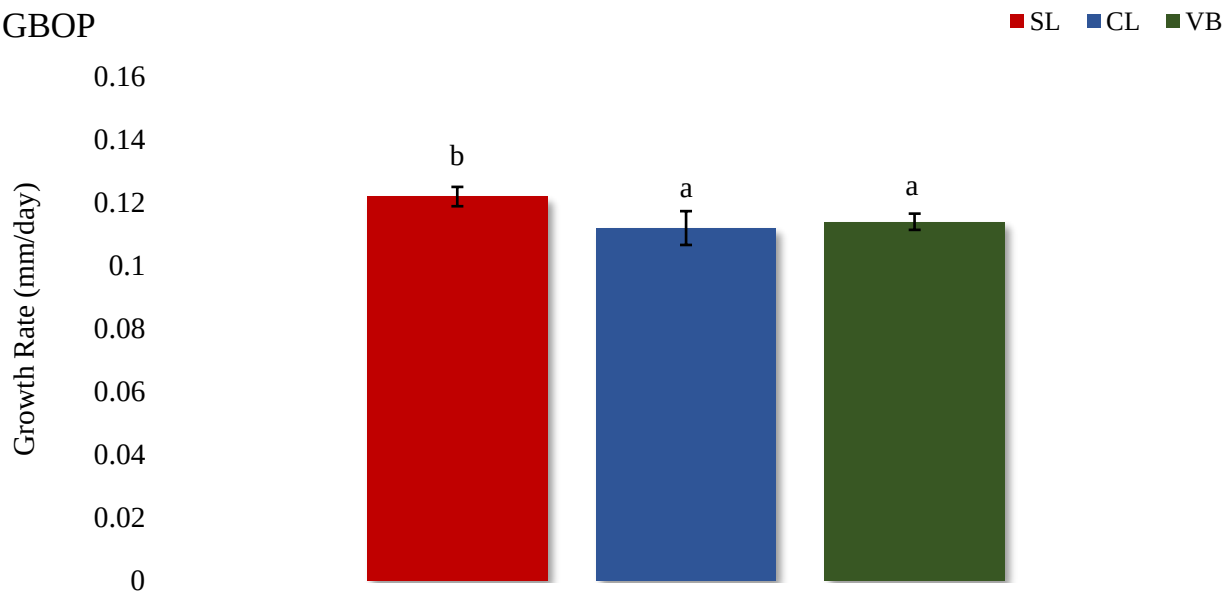

Figure 12: Mean (± SE) daily growth rates (mm/day) at Grand Bay (GBOP) of each experimental treatment from deployment to final collection (November 2019- September 2020). Superscripts denote statistical differences ($p<0.05$). Error bars represent standard error.

Effect of Broodstock Origin and Ploidy on Condition Index

At MOB, for condition index [(dry meat weight)/ (whole wet weight-dry shell weight) x 100] in March (Figure 13), there was only a significant effect of broodstock origin ($p=0.01$) but not ploidy ($p=0.29$) nor an interaction between broodstock and ploidy ($p=0.38$). Among broodstock origin, SL had significantly higher condition index than CL ($p=0.01$), while oysters from VB did not differ from either ($p\geq0.06$).

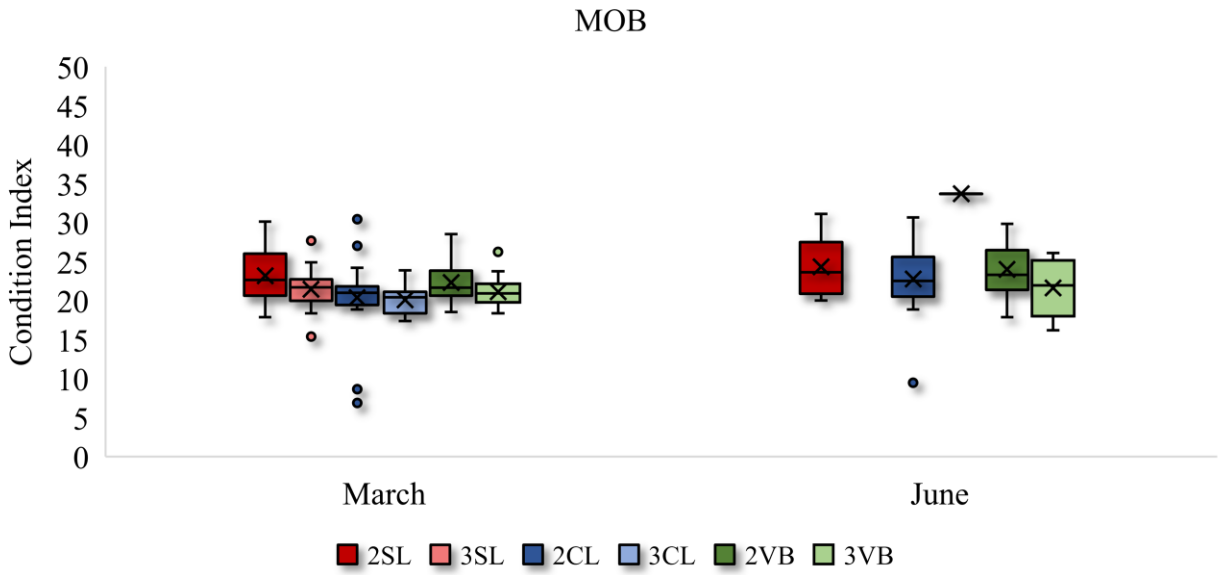

Figure 13: Condition index [(dry meat weight)/ (whole wet weight-dry shell weight) x 100] at Mobile Bay (MOB) in March and June. Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

For condition index at DI in March and June (Figure 14), there were no significant effects of broodstock origin or ploidy ($p \geq 0.13$), and there was not an interaction between broodstock origin x ploidy ($p \geq 0.66$). However, in September there was a significant effect of broodstock origin ($p=0.01$) and ploidy ($p<0.01$), but there was no interaction between broodstock origin x ploidy ($p=0.38$). Triploids had significantly higher condition index than diploids ($p<0.01$). Among broodstock, SL had significantly higher condition index than CL and VB ($p \leq 0.04$), and there were no differences between CL and VB ($p=0.85$).

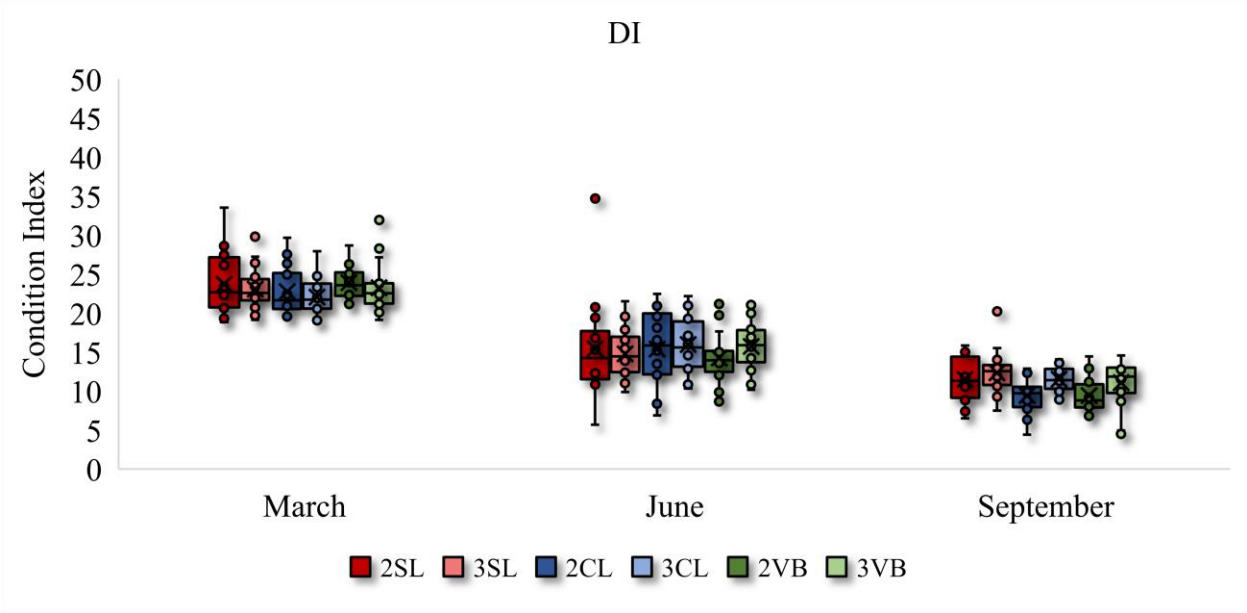

Figure 14: Condition index [(dry meat weight)/ (whole wet weight-dry shell weight) x 100] at Dauphin Island (DI) in March, June, and September 2020. Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

For condition index at GBOP in March, there was no significant effect of broodstock origin or ploidy ($p \geq 0.10$), and no interaction between broodstock origin x ploidy ($p=0.90$). In June, there was no significant effect of broodstock origin ($p=0.42$), but there was a significant effect of ploidy ($p=0.01$, Figure 15) with no interaction between broodstock origin x ploidy ($p=0.83$). In June, the condition index of triploids was higher than diploids. In September, there was a significant effect of broodstock origin ($p=0.04$) and ploidy ($p<0.01$), but there was not an interaction between broodstock x ploidy ($p=0.42$). In September, triploids again had significantly higher condition index than diploids ($p<0.01$). In terms of the effect of broodstock origin, SL had significantly higher condition index than CL ($p=0.03$), while VB did not differ from either ($p \geq 0.35$).

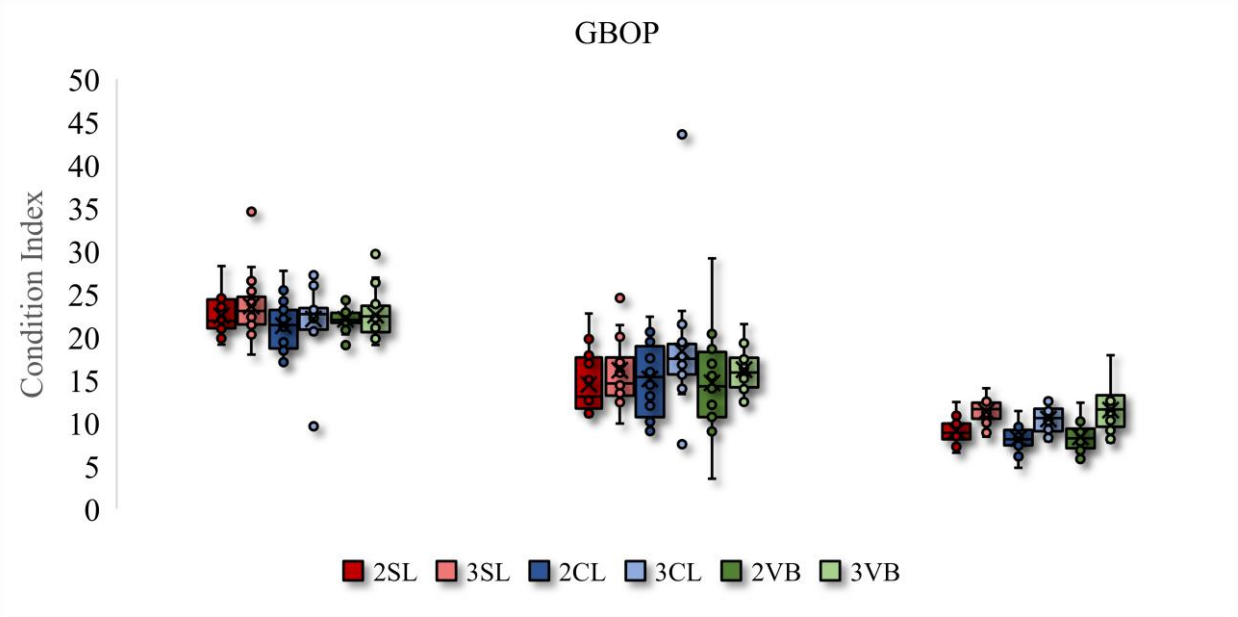

Figure 15: Condition index [(dry meat weight)/(whole wet weight-dry shell weight) x 100] at Grand Bay (GBOP) for March, June, and September 2020. Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

Effect of Broodstock Origin and Ploidy on Perkinsus marinus Infection Intensities

Perkinsus marinus infection intensity increased over time at all three sites (Figures 16-18). At MOB in March, there was not a significant effect of broodstock origin (p=0.2) or ploidy (p=0.7) on P. marinus infection intensity, and there was no significant interaction between broodstock origin x ploidy (p=0.3).

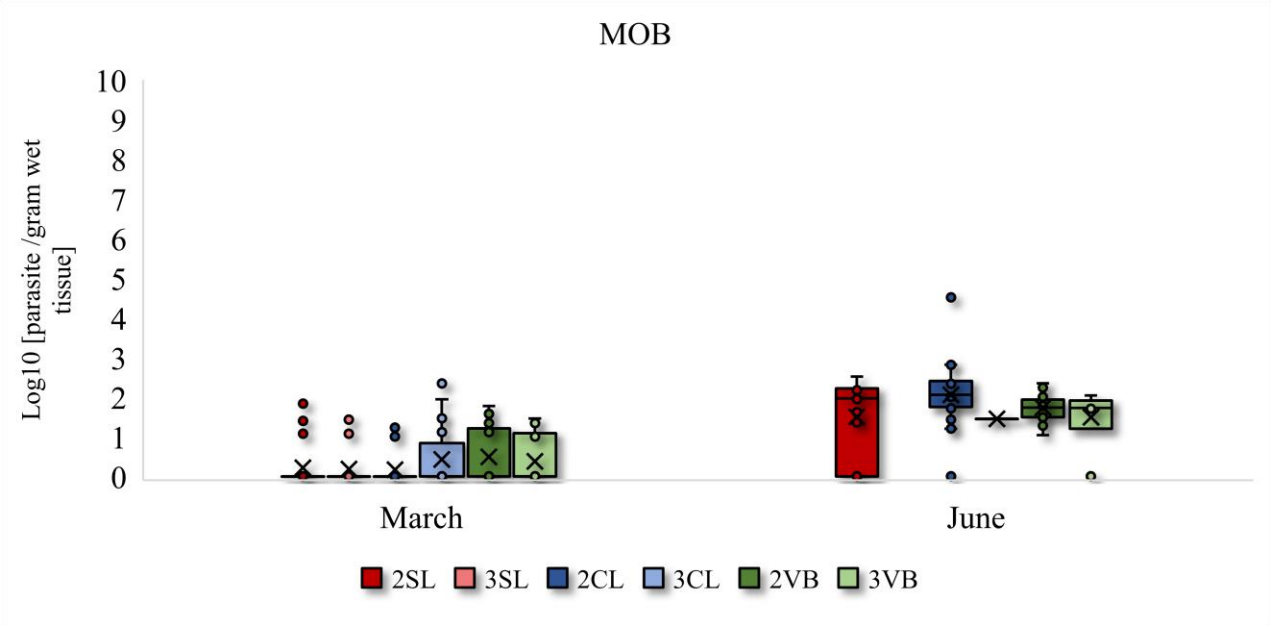

Figure 16: *Perkinsus marinus* infection intensity [$\mathrm{Log}_{10}(\ \mathrm{g}^{-1}\mathrm{wet\ tissue})$] at Mobile Bay (MOB) for March and June 2020. Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

For *P. marinus* infection intensity at DI in March, June and September, there was not a significant effect of broodstock origin ($p>0.2$) or ploidy ($p>0.6$) on infection intensity, and there was no significant interaction between broodstock origin x ploidy ($p>0.69$). There was a substantial increase, however, from June to September in all treatments (Figure 17).

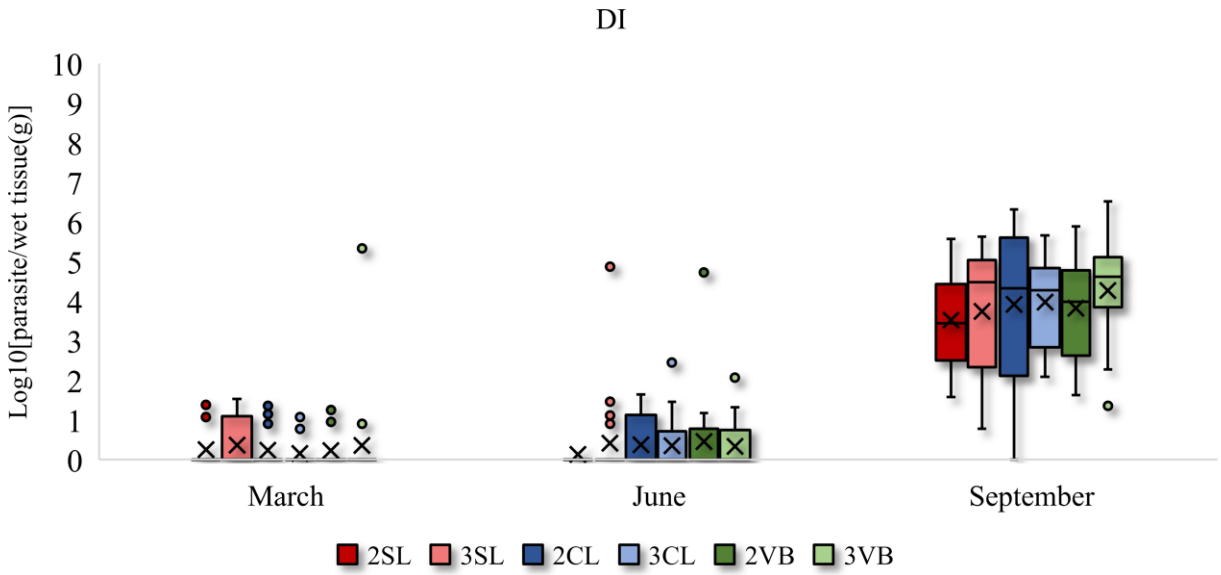

Figure 17: *Perkinsus marinus* infection intensity [$\mathrm{Log}_{10}(\mathrm{g}^{-1}\text{wet tissue})$] at Dauphin Island (DI) for March, June, & September 2020. Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

For *P. marinus* infection intensity at GBOP in March, there was no significant effect of broodstock origin (p=0.17) or ploidy (p=0.12) on *P. marinus* infection intensity, and there was no interaction between broodstock x ploidy (p=0.67, Figure 18). In June, however, there was a significant effect of broodstock origin on infection intensity (p=0.02), with no significant effect of ploidy (p=0.15) and no interaction between broodstock origin x ploidy (p=0.63). For the broodstock origin effect, SL oysters had significantly lower infection intensities than CL and VB ($p \leq 0.05$). In September, there was a significant interaction between broodstock origin x ploidy (p=0.04). At this sampling time, triploid CL oysters had significantly lower infection intensities than triploid VB (p=0.04) with no other significant differences among the treatments.

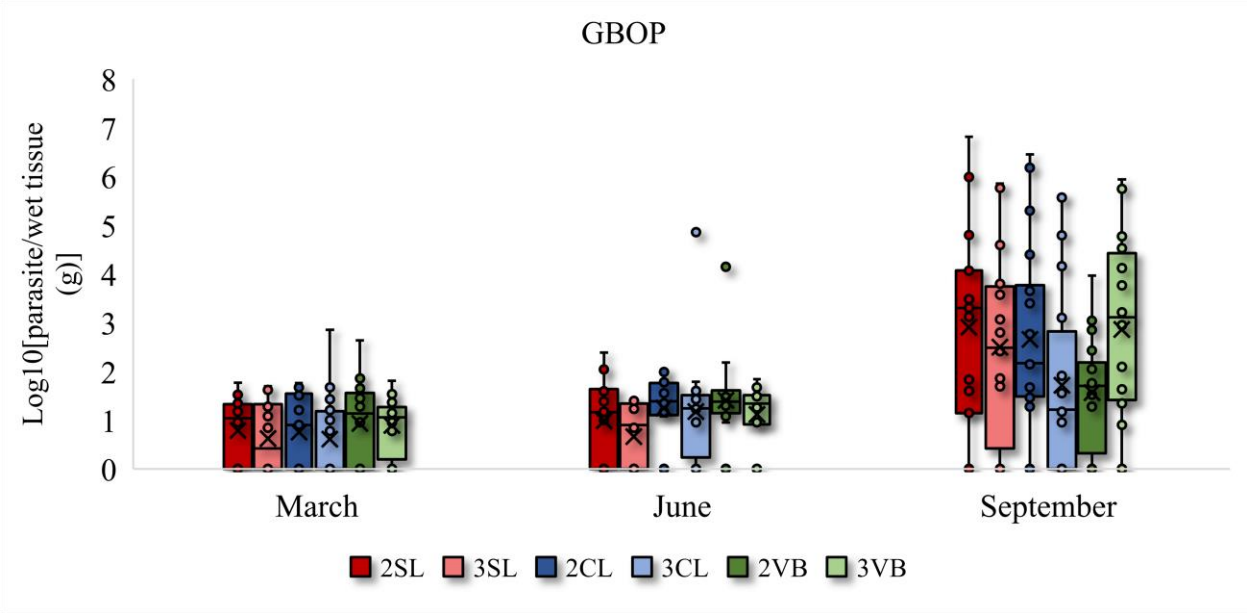

Figure 18: *Perkinsus marinus* infection intensity [$Log_{10}(g^{-1}$wet tissue)] at Grand Bay (GBOP) for March, June, & September 2020. Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

Effect of Ploidy and Oyster Size on Cumulative Mortality

Cumulative mortality for SL oysters was only significantly affected by size and ploidy ($p=0.01$), but there was no interaction ($p=0.17$). Triploids experienced significantly higher cumulative mortality than diploids ($p<0.01$). Large sized SL oysters experienced higher mortality than both medium and small ($p<0.0001$), which also differed from each other ($p<0.001$, Figure 19). Cumulative mortality for CL oysters was affected only by ploidy ($p<0.01$), and not by size category nor any interaction ($p\geq0.51$). Triploids had an average cumulative mortality of 14.3% ± 0.007, which was significantly higher than diploids (7.93% ± 0.0046, $p<0.001$)

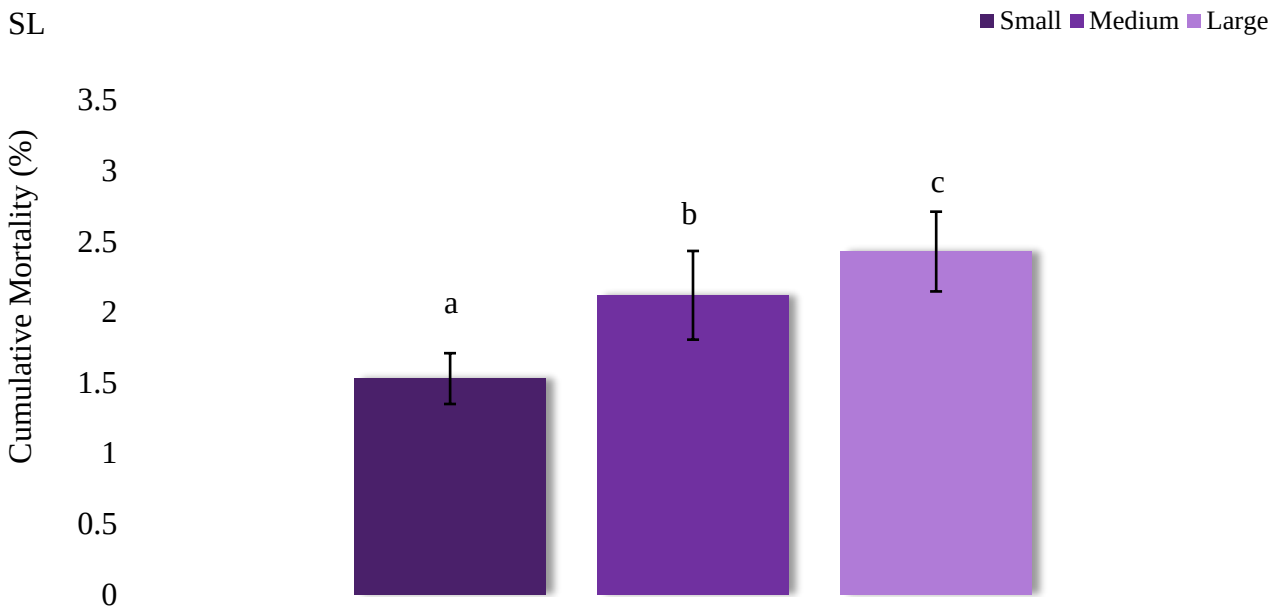

Figure 19: Mean (± SE) cumulative mortality for Small, Medium, and Large sized Sister Lake (SL) Oysters in September 2020. Superscripts denote statistical significance ($p<0.05$). Error bars represent standard error.

Factor	Broodstock	df	F	p
Size	SL	2	2.77	0.08
Ploidy		1	7.21	**0.01**
Size x Ploidy		2	0.18	0.83
Size	CL	2	0.19	0.83
Ploidy		1	15.86	**<0.01**
Size x Ploidy		2	0.69	0.51

Table 7: Analysis of variance (ANOVA) table for cumulative mortality for size category, ploidy, & size category x ploidy interaction for Sister Lake (SL) and Calcasieu (CL) oysters.

Effect of Ploidy and Oyster Size on Interval Mortality

From April to May, there was an observed spiked in interval mortality for both SL and CL oysters at Grand Bay. Size and ploidy had interactive effect on interval mortality in SL oysters ($p<0.01$, Figure 20). Diploid large sized oysters experienced the highest mortality during this interval, while diploid small sized oyster had the lowest mortality ($p<0.001$). There was no

size category effect on interval mortality in CL oysters (p=0.32), however, triploids experienced significantly higher interval mortality than diploids (Figure 21, p=0.01).

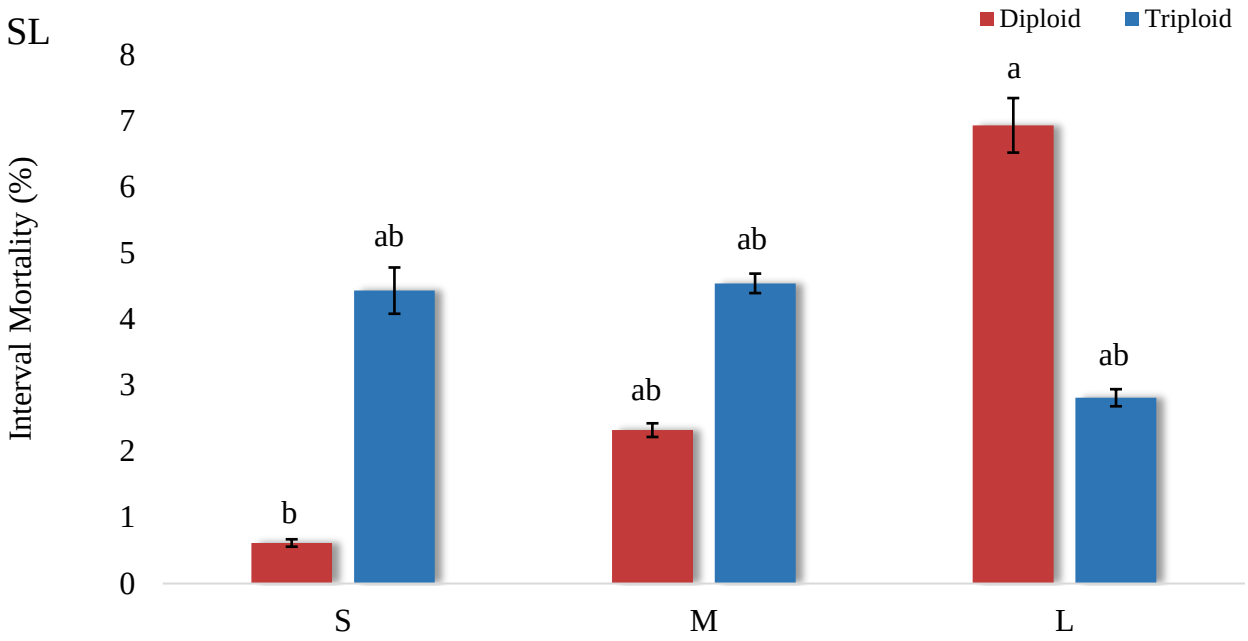

Figure 20: Mean (± SE) interval mortality for diploid and triploid Sister Lake (SL) oysters during April to May. Superscripts denote statistical significance (p<0.05). Error bars represent standard error.

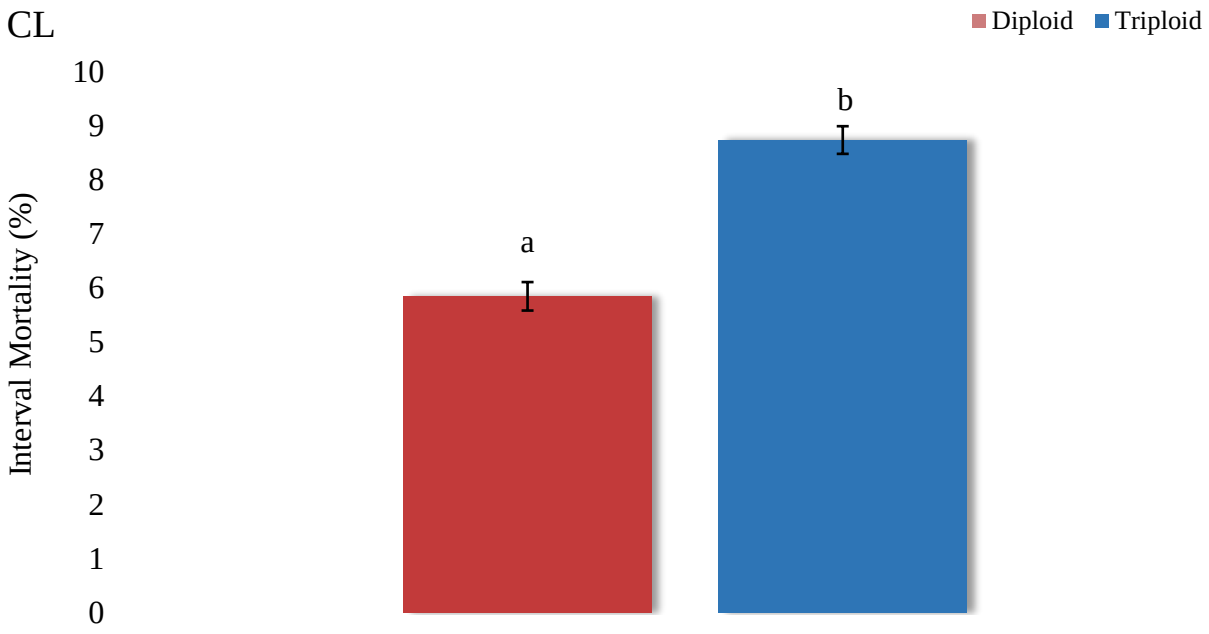

Figure 21: Mean (±SE) interval mortality (%) for diploid and triploid Calcasieu Lake (CL) oysters from April to May. Superscripts denote statistical significance($p<0.05$). Error bars represent standard error.

Effect of Ploidy and Oyster Size on Growth Rates

Amongst SL oysters, growth rates (mm/day, Table 9) were significantly different among size category ($p<0.001$, Figure 22) with small oysters growing significantly faster than medium oysters ($p<0.01$) which, in turn, grew significantly faster than large oysters ($p<0.01$, Table 8).

Factor	Broodstock	df	F	P
Size	SL	2	74.26	< **0.01**
Ploidy		1	4.10	0.07
Size x Ploidy		2	1.49	0.33
Size	CL	2	63.63	< **0.01**
Ploidy		1	69.20	< **0.01**
Size x Ploidy		2	11.74	< **0.01**

Table 8: Analysis of variance table (ANOVA) for daily growth rates (mm/day) for size category, ploidy, & size category x ploidy for both broodstock origins: Sister Lake (SL) and Calcasieu (CL).

Treatment	Initial Average Shell Height	Final Average Shell Height (mm)	Average Daily Growth Rate
2SL- Small	35.9 ± .50	65.8 ± 1.25	0.20 ± 0.0052
2SL- Medium	51.9 ± 0.49	74.5 ± 1.46	0.15 ± 0.0095
2SL- Large	59.4 ± 0.56	78.8 ± 1.33	0.13 ± 0.0087
3SL- Small	41.8 ± 0.51	71.4 ± 2.0	0.19 ± 0.013
3SL- Medium	56.2 ± 0.48	81.1 ± 0.92	0.16 ± 0.0059
3SL- Large	65.9 ± 0.74	87.7 ± 1.29	0.14 ± 0.0085
2CL- Small	41.9 ± 0.41	67.5 ± 0.94	0.17 ± 0.0044
2CL- Medium	54.7 ± 0.52	76.8 ± 2.03	0.14 ± 0.0058
2CL- Large	64 ± 0.61	81.2 ± 1.71	0.11 ± 0.0066
3CL- Small	38.8 ± 0.51	75.1 ± 2.69	0.24 ± 0.0076
3CL- Medium	55.8 ± 0.56	80.2 ± 0.95	0.16 ± 0.0053
3CL-Large	66.9 ± 0.71	90.4 ± 1.85	0.15 ± 0.0065

Table 9: Mean (± SE) shell height (mm) in September 2020 for Sister Lake (SL) and Calcasieu (CL) experimental treatments.

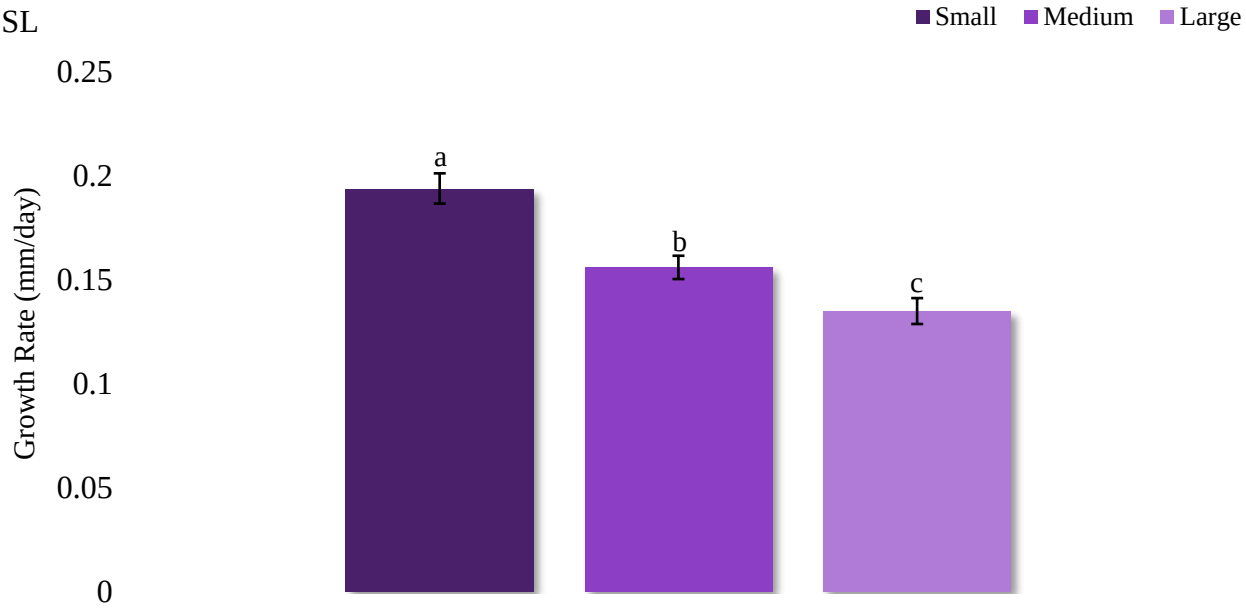

Figure 22: Mean (± SE) daily growth rates (mm/day) for the three different sizes of Sister Lake (SL) oysters. Superscripts denote statistical differences ($p \leq 0.05$). Error bars represent standard error.

Amongst CL oyster's, growth rates (mm/day, Table 9) were affected by the interaction between size category and ploidy (p=<0.01, Figure 23). Large diploids had the significantly slowest growth rates (p<0.01), which was exceeded significantly by the growth rates of small diploids, medium diploids, medium triploids, and large triploids (p≤0.007), which, in turn, was significantly less than the growth rates of small triploids (p<0.01, Table 8).

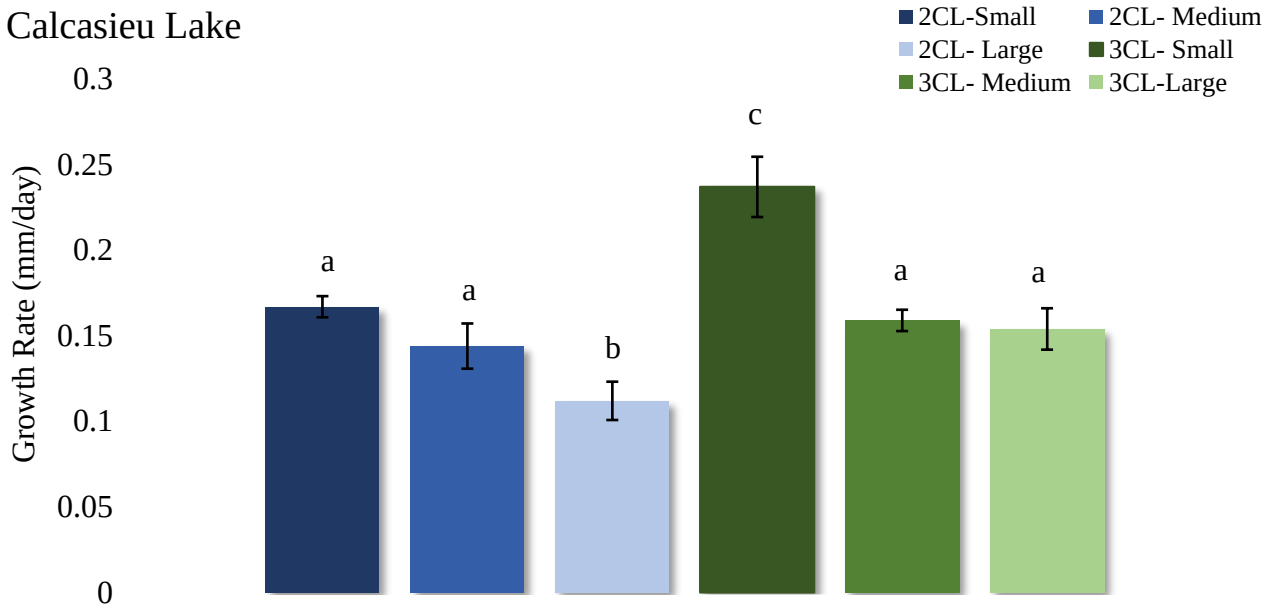

Figure 23: Mean (± SE) daily growth rate (mm/day) for the three different sizes of Calcasieu Lake (CL) oysters. Superscripts denote statistical differences (p ≤0.05).

Effect of Ploidy and Oyster Size on Condition Index

Condition index of SL oysters in November 2020 (Figure 24) was significantly different across oyster size categories (p<0.01) and ploidy (p<0.01) with no interaction between these factors (p=0.12). For the effect of size categories, the condition index of small oysters was significantly greater than the other size categories (p≤0.01), and the condition index of medium oysters was significantly greater than the large oysters (p<0.01). Additionally, the condition index of triploids (1.06 ± 0.01) was greater than diploids (0.99 ± 0.13, p<0.01).

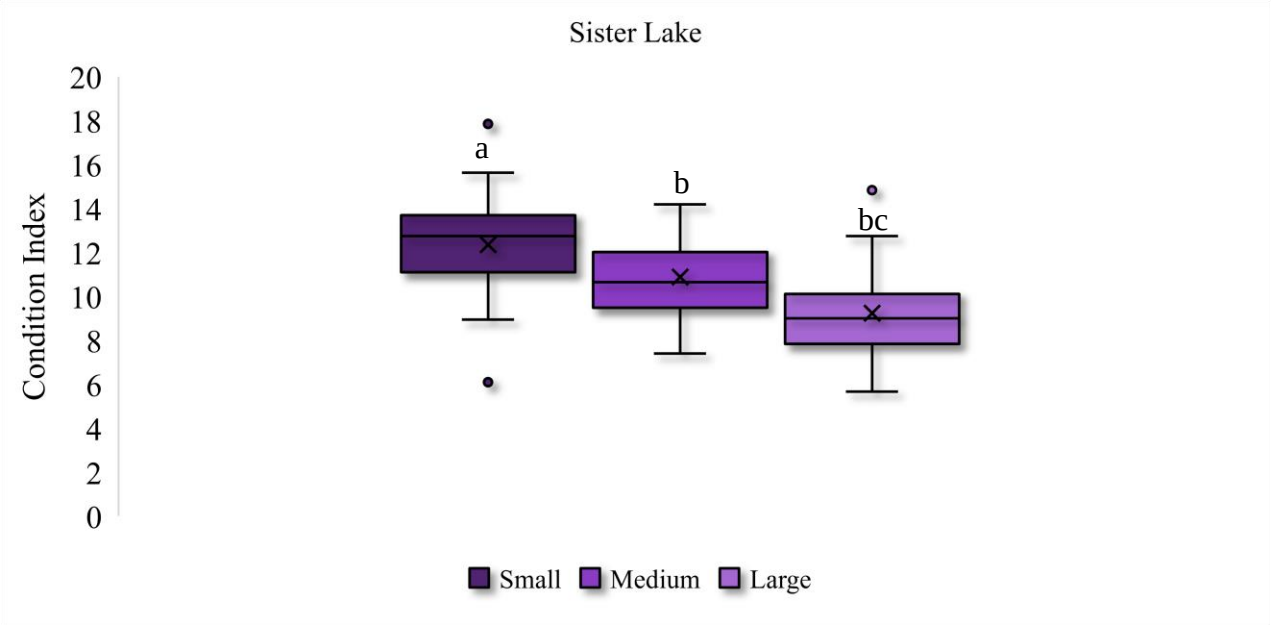

Figure 24: Condition index for Sister Lake (SL) oysters (Small, Medium, & Large sizes). Superscripts denote statistical differences ($p \leq 0.05$). Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

Condition index of CL oysters in November 2020 (Figure 25) was significantly different across oyster size categories ($p<0.01$) and ploidy ($p<0.01$) with no interaction between these factors ($p=0.84$). Triploids had significantly higher condition index than diploids ($p<0.01$). Small sized oysters had significantly higher condition index than both medium and large oysters ($p<0.01$), with no difference between medium and large size oysters ($p=0.95$).

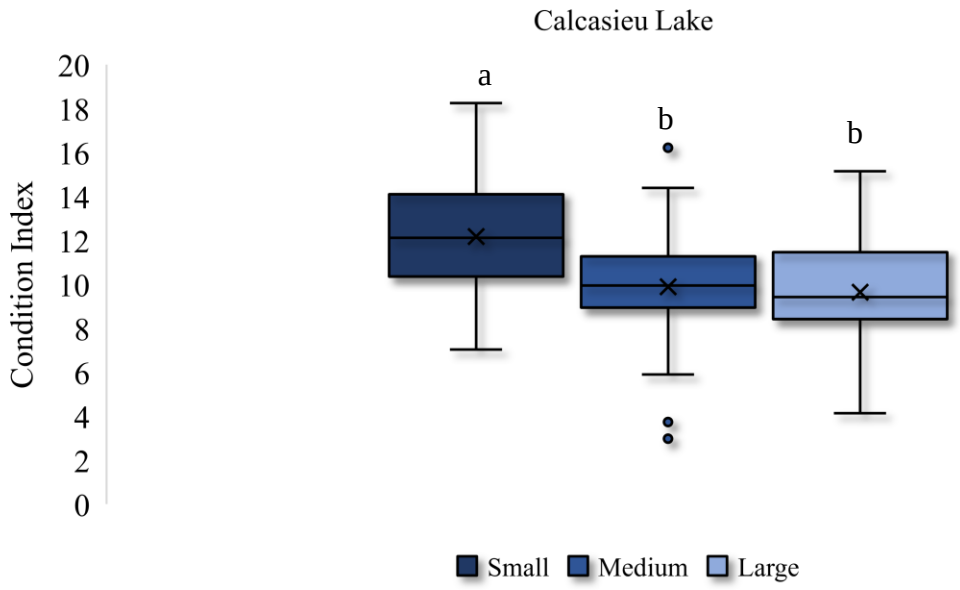

Figure 25: Condition index for Calcasieu Lake (CL) oysters (Small, Medium, & Large sizes). Superscripts denote statistical significance ($p \leq 0.05$ Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

Effect of Ploidy and Oyster Size on Perkinsus marinus Infection Intensity

Perkinsus marinus infections in SL oysters (Figure 26) were significantly affected by the size category x ploidy interaction ($p=0.05$). Triploid small oysters had significantly higher infection than diploid large oysters ($p=0.04$). There were no other significant differences among treatments ($p \geq 0.1$)

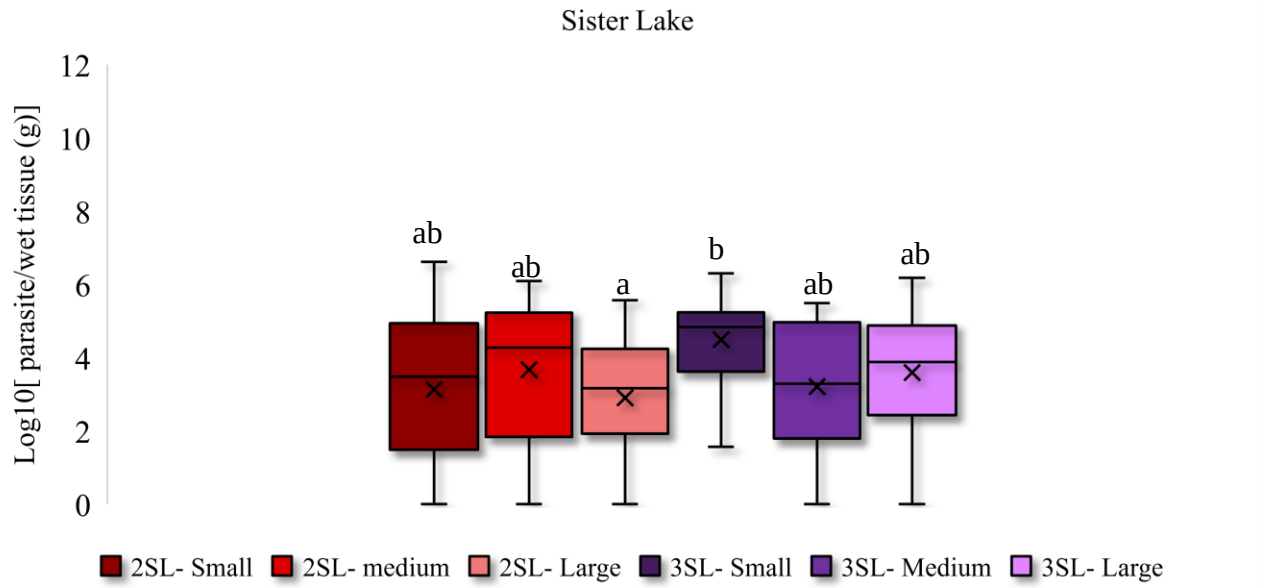

Figure 26: *Perkinsus marinus* infection intensity [$\mathrm{Log}_{10}(\ \mathrm{g}^{-1}$wet tissue)] for Sister Lake (SL) oysters in November 2020. Superscripts denote statistical differences ($p < 0.05$ Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

Perkinsus marinus infection intensities for CL oysters were significantly affected by both oyster size category ($p<0.01$) and ploidy ($p<0.01$) with no interaction between these factors ($p=0.11$, Figure 27). For the effect of size category, medium sized oysters had significantly higher infection than large sized oysters ($p<0.01$) with no other significant differences ($p\geq0.17$). For the effect of ploidy, CL triploids had significantly lower infection intensities than diploids ($p<0.01$).

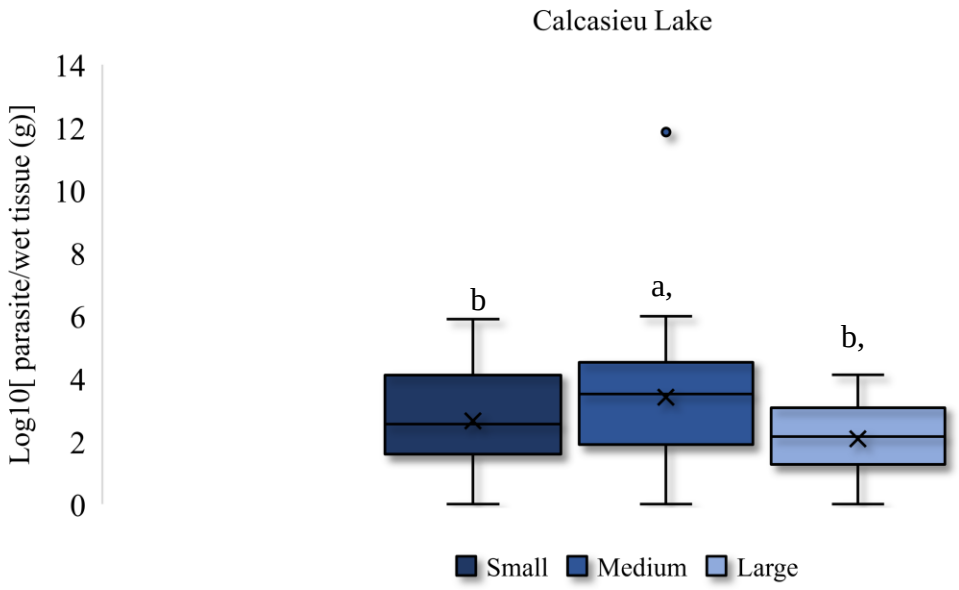

Figure 27: *Perkinsus marinus* infection intensity [$\mathrm{Log}_{10}(\ \mathrm{g}^{-1}\mathrm{wet\ tissue})$] for Calcasieu Lake (CL) oysters in November 2020. Superscripts denote statistical differences ($p \leq 0.05$). Boxes include 25th and 75th percentiles and whiskers extend to 10th and 90th percentiles.

Discussion

To address concerns about mortalities associated with triploid oysters along the Gulf coast, this study was conducted to determine if maternal broodstock collected from estuaries with historically different salinity regimes (low, high and variable) would influence mortality. Additionally, the effect of size (as a proxy for growth) on mortality patterns was examined. In both cases, the intent was to provide commercial oyster farmers guidance on management to reduce losses, while maximizing growth.

The three study sites were selected to represent three different environmental conditions: low, high, and variable salinity. MOB was observed to the low salinity site as expected (5.62 ± 5.30ppt), however, despite expectations DI had the highest variability (20.8 ± 5.77ppt). DI and GBOP were both relatively high salinity sites (Table 3), GBOP data concluded it was less variable than DI (19.2 ± 5.42ppt, Table 3).

Does Maternal Broodstock Mitigate Mortalities?

Despite the maternal genetic contribution from three broodstock groups (presumably subjected to different salinity regimes), broodstock origin had no effect on cumulative mortality at any of the three study sites ($p \geq 0.18$), nor were there any significant interactions of broodstock origin with ploidy ($p \geq 0.12$). Triploids, however, experienced significantly higher cumulative mortality at all three sites ($p < 0.01$). The timing of most of these mortalities occurred March to April at all three sites, with an additional pulse of mortality from August to September at DI and GBOP (Figures 8 and 9).

Broodstock origin did not appear to differentially affect mortality of triploid and diploids, however, differences appeared during specific intervals. During the March to April interval at MOB (a very low salinity period) and DI, both ploidy and broodstock origin affected mortality rates (with no interaction); triploids suffered higher mortality than diploids at both sites. The effect of broodstock origin on mortality during this interval differed between sites; at the low salinity site (MOB), the CL line (collected from a high salinity site) tended to suffer higher mortalities than the other two lines, while the VB line (collected from a variable salinity site) suffered lower mortality than the other two lines at DI (the most variable salinity study site). These results suggest that broodstock origin did affect mortalities during these intervals but did not interact with ploidy (and did not persist across the duration of the study, given the lack of broodstock origin effects on cumulative mortality). Additionally, at GBOP during the March to April interval, there was no effect of broodstock origin nor an interaction with ploidy, with triploids suffering higher mortality. Regardless of broodstock origin triploids at DI from August to September experienced higher interval mortality, while GBOP had no significant differences among the treatments ($p \geq 0.28$). Wadsworth et al. (2019), observed similar patterns at DI and

GBOP, with triploids experiencing high mortality in early summer (June to July) and early fall (September-October).

The results of the current study agree with Wadsworth et al. (2019) that across several sites in Alabama triploids experienced significantly higher mortality than diploids. Matt et al. (2020) described similar results, for one study site in Virginia, where triploids died regardless of distinctly different genetic lineages (including different tetraploid broodstock) between experimental oysters. Also, like the findings of Wadsworth et al. (2019), cumulative mortalities differed substantially among sites. In this study, triploids and diploids at MOB had the highest cumulative mortalities of 95.8% (+0.0019) and 78.1% (+0.0034), respectively. In comparison, overall cumulative mortality was relatively low at DI and GBOP (Figure 6). DI triploids experienced 12.1% (+0.02) cumulative mortality, while diploids only experienced 3.9% (+0.0077). At GBOP triploids experienced 17.5% (+0.022), and diploids experienced 5.1% (+0.0083). Again, this was a pattern observed by Wadsworth et al. (2019) that triploids had a disproportional susceptibility to mortality at a very low salinity site (<5 ppt).

Triploids could be at a disadvantage under stressful conditions like these, and their ability to adapt to water chemistry changes could be compromised (Casas et al., 2018a; Gainey & Greenburg 1977; van Winkle 1972). Because triploids have increase genomic content this can potentially disproportionately increase cell volumes and surface areas and lead to inefficient cell volume regulation that allows oysters to cope with highly variable salinities (Guo and Allen 1994). Upon shell closure under low salinities, oysters engaged in anaerobic metabolic pathways to sustain themselves until conditions improve; however, there is speculation about the ability of triploids to do so (Andrews 1982; Andrews 1959). It has been observed in some fish species that triploids have a reduced ability to engage in anaerobic pathways and can be further impaired at

high temperatures leading to higher mortality (Maxime 2008; Hyndman et al., 2003). Mortality at MOB appears to be best explained by high temperature and low salinity for a prolonged period (> 3 weeks). Past studies have recorded effects of temperature and salinity in the survival of oysters exposed to these stressors (Jones et al. 2019, Casas et al. 2018, Cheney et al. 2000, Calvo et al. 1999). In the current study, monthly salinity averages did not exceed 5ppt from February until mid/late June, while temperature was rising, simultaneously. This site is also characterized as shallow most time of the year, so temperatures remained high. Together the interaction of salinity and temperature had larger effect than either variable did individually (Jones, 2018, Soniat et al. 1985). Cheney et al. (2000) also found that triploid mortality increased with temperature increase but did not credit temperature individually to be fatal. Dissolved oxygen could be an additional factor, but it was not monitored throughout this study. Importantly, in both this study and Wadsworth et al. (2019), it is worth noting that while triploids died at a faster rate at MOB, essentially all oysters, regardless of ploidy, died at the very low salinity site by June.

At the two higher salinity sites (DI and GBOP), though, triploids also suffered higher mortality than diploids (regardless of broodstock origin), albeit at much lower rates than at MOB. Wadsworth et al. (2019), observed similar patterns at DI and GBOP, with triploids experiencing high interval mortality in early summer (June to July) and early fall (September-October) compared to the pulses of mortality in spring (March to April) and late summer (August to September). Like the current study, salinities were above 10 ppt, so this elevation in mortality cannot be attributed to low salinity stress and could be contributed to other environmental conditions such as dissolved oxygen, food availability, rising temperature (Wadsworth et al., 2019). Future studies should focus on the variability of these conditions

throughout the growing season to better understand potential causes of elevated spring/summer mortality.

While stress imposed by low salinity or changes in salinity (associated with temperature increases) are known to affect oysters, it is not clear that low salinity stress provides a broad explanation for spring/summer mortality of triploids. While the magnitude of mortality varied, this study documents higher mortality in triploids than diploids across three sites. Critically, the tested maternal broodstock had no effect on cumulative oyster mortality and did not interact with ploidy, suggesting that the tested broodstock would not lend itself to selection for improved survival of triploid lines.

Does Broodstock Origin and/or Ploidy Affect Oyster Performance?

At both MOB and DI, broodstock origin and ploidy had an interactive effect on growth rates over the course of the study, whereas only broodstock origin affected growth rates at GBOP. At MOB, overall growth rates of all oysters were very low (even negative presumably due to shell loss or larger oysters dying at a higher rate). Both SL triploids and diploids had the highest growth rates (along with CL diploids), which were significantly higher than VB diploids (with no other significant differences). Interestingly, through the April to May interval, there appeared to be some growth advantage for triploid SL relative to their diploid counterparts, but this pattern reversed in the May to June interval. This complex result is not easily explained but agrees with the conclusion of Callam et al. (2016) that triploid growth advantages are usually not observed in low salinity conditions (Callam et al., 2016) or sites with unfavorable environmental conditions (summarized in Wadsworth et al. 2019b). Past studies have documented that overall growth can decrease as well as triploid advantage under high temperature climates (Ibarra et al., 2017; Shatkin, 1992).

At DI, the highest growth was observed in SL and VB triploids, which were significantly higher growth rates than their diploid counterparts. There was no advantage of ploidy within the CL oysters, however (Figure 12). These results are interesting because CL oysters were collected from an area that historically experienced higher salinity that were expected to thrive in these site conditions, while SL oysters are low salinity progeny that might have not done well under these conditions. While noting that diploid oysters tended to start smaller than triploid oysters at the start of the study, diploid SL were the only experimental treatment at DI that, on average, did not reach market size (≥75mm) by the final collection in September (Table 4). 3VB had the highest average shell height by September of 83.3 ± 0.79mm (Table 4).

Growth rates at GBOP were significantly affected by only broodstock origin ($p<0.01$), and not ploidy ($p=0.30$), nor any interaction with ploidy. Among broodstock origins, SL oysters had a significantly higher growth rate than both CL and VB oysters (Figure 3). Again, this was unexpected as the SL broodstock were from a low salinity environment, while the CL and VB broodstock did not confer any advantage, and were, in fact, substantially slower growing. Again, noting that triploid seed started this experiment at a larger size than diploids, diploid VB were the only experimental treatment at GBOP that did not reach market size by final collection in September (Table 4). Despite SL having the highest average growth rate, triploid CL oysters had the highest average shell height (mm) at final sampling in September of 81.7 ± 0.64mm (Table 9).

Condition index increased from March to June at MOB, however from March to September at DI and GBOP it decreased (Figures 14-16). Condition index was not significantly impacted by ploidy ($p=0.29$); however, it was affected by broodstock origin ($p= 0.11$). SL had significantly higher condition index than CL ($p=0.01$), while VB did not differ from either

($p \geq 0.06$). This is most likely a result of stress on the oysters from extreme conditions at this site triggering energy allocation to somatic growth instead of gametogenesis (Davis 1994), which was observed by Wadsworth (2018) as well.

At DI, September was the only sample month that had an observed broodstock origin and ploidy effect on condition index. Expectedly, triploids had significantly higher condition index than diploids ($p<0.01$). At GBOP in June and September triploids had higher condition index than diploids ($p \leq 0.01$), however, broodstock origin effect was only observed during September ($p= 0.04$). Low salinity progenies (SL) had significantly higher condition index than high salinity progeny, while VB did not differ from either. Casas et al. (2017) reported stock and sampling time effect at DI, and only sampling time effect at Sand Bay (GBOP), despite the current study having no sample time interaction, Figures 15 & 16 show similar patterns, of condition index decreasing over time. Higher triploid condition index is most likely due to reduced gonadal developments allowing triploids to allocate energy towards growth and not gametogenesis.

In this study, *Perkinsus marinus* infection intensities increased over time at all three sites (Figures 17-19) but do not appear to have reached levels that would explain mortalities. La Peyre et al. (2019), found that overall Sister Lake cohorts had significantly higher infection intensities than Calcasieu Lake, as well as higher cumulative mortality (reaching 60% by the second grow out year), which was attributed to the higher infection intensities. By the second grow out year in October 2016, Sister Lake oysters had a mean infection intensity of approximately 5.5 $\mathrm{Log}_{10}[\text{parasite g}^{-1}\text{wet tissue weight}]$ or higher (La Peyre et al., 2019). Infection intensities in this study saw significant increases at sampling times, and more so during the second year (La Peyre et al., 2019). Furthermore, the effects of broodstock and ploidy differed among sites. At MOB in March, broodstock origin and ploidy had no effect on infection intensities (with no

additional sampling due to high mortality). At DI, broodstock origin and/or ploidy had no effect on infection intensities during all three sampling months. By June at GBOP, however, broodstock origin had a significant impact on infection intensities ($p= 0.02$). SL oysters had significantly lower infection intensities than both CL and VB, which did not significantly differ. There was an interaction between broodstock origin x ploidy in September at GBOP; triploid VB oysters had significantly higher infection intensities than triploid CL ($p= 0.04$). While broodstock from the relatively high salinity site (CL) might be expected to have developed resistance to *P. marinus,* this was not observed in diploid CL.

Previous studies at these sites found similar infection intensity temporal patterns, with an increase over time and early fall months having the highest intensities (Wadsworth et al., 2019; Casas et al., 2017). Dittman et al. (2001) found a trend of increasing intensities when spawning season was coming to an end. However, Wadsworth et al. (2019) reported triploids having higher infection intensities than diploids, but this is not the case in the current study. On the contrary, past studies have reported ploidy has no effect on infection intensities (Dégremont et al., 2012; Barber and Mann, 1991; Meyers et al., 1991).

Despite the general lack of effect of broodstock origin or interaction with ploidy on mortality, these results suggest that broodstock origin, ploidy and their interaction can affect several metrics of oyster performance (growth rate, condition index, *P. marinus* infection intensity) in a variety of ways. While these results were not consistent across site or metric, triploids generally perform better or no worse than diploids. Additionally, broodstock origin does seem to offer some potential to improve performance, though there was not a clear alignment between broodstock salinity regimes and grow-out performance. Suggesting, triploid mortality is not readily explained through broodstock contribution but rather other factors such as molecular

processes related to the abnormal gonad development of triploids, which needs to be further investigated.

Does Size Category and Ploidy Affect Oyster Performance?

Based on qualitative observations that many of the dead oysters collected during each interval by Wadsworth et al. (2019) appeared to be the largest coupled with feedback from commercial growers, it was hypothesized that the fastest growing oysters might be more susceptible to mortality. As a proxy for growth, this study divided two different lines (SL and CL progeny) into three size categories as an indicator of past growth rates at least. In this study, there was no significant effect of size category on cumulative mortality response in SL or CL oysters, nor any interaction ($p \geq 0.08$). There was, however, a significant effect of ploidy ($p \leq 0.01$). For both SL and CL oysters, triploids had significantly higher cumulative mortality than diploids regardless of size category ($p \leq 0.01$). Looking at interval mortality, there was no effect or interaction with ploidy of size category within the SL progeny during the March to April pulse of mortality. However, within the CL progeny, there was a significant effect of size where small oysters had significantly higher mortality than medium oysters (with large oysters not differing from either). Certainly, this study provides no evidence that large oysters suffered higher mortality.

For growth rates of SL progeny, growth was significantly impacted by size category as well as ploidy (Table 8). Regardless of size category, triploids grew faster than diploids ($p \leq 0.04$). For size categories, growth rates were negatively correlated with size category. For CL, growth rates were affected by the interaction between size category and ploidy. Overall, within a ploidy, growth rates were negatively correlated with size structure. Intriguingly, the measured

growth rates suggest that the fastest growing oysters were the smaller size category where some increased March to April interval mortality in the CL progeny was observed.

Condition index of SL oysters was significantly affected by both size category and ploidy ($p< 0.01$). Small sized oysters had higher condition index (12.4 ± 0.34) than both medium (10.9 ± 0.26) and large size (9.23 ± 0.33). Triploids for both SL and CL oysters had significantly higher condition index than diploids ($p<0.001$). CL small sized oysters had significantly higher condition index (12.2 ± 042) than medium and large, however, there were no differences between medium (9.89 ± 0.41) and large sized oysters (9.65 ± 0.40).

Conclusion

Despite broodstock origin selection being advantageous for selected lines in terms of growth rates and other measures of performance, the high mortality of triploid oysters when exposed to three different environmental conditions bred from those three historically different broodstock lines from Louisiana suggests that this is not a promising solution to address triploid mortality. As suggested by Matt et al. (2020), selection of paternal tetraploid lines may offer an alternative breeding strategy to reduce triploid mortality though no difference was found between the two tetraploid lines tested in that study. Survival and disease susceptibility should be further examined, to determine vulnerabilities of oysters that are different sizes in different environments and to understand the triggers of mortality events. Furthermore, the response of growth rate may deserve further exploration because our results show that broodstock and size can affect their performance.

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CHAPTER THREE:
ADDRESSING MORTALITIES IN TRIPLOID OYSTERS

Importance

Triploid oysters are a mainstay for farmers along the Gulf Coast for their many desirable characteristics (Wadsworth et al., 2019). Triploids typically grow significantly faster than diploids, which is ideal for farmers to get their products to market size quicker. Triploids are also often observed to have better summer meat condition compared to diploids that are "spawned out" by mid/late-summer. However, some oyster farmers on the Gulf Coast have also reported mortalities in the spring and summer, especially among their triploid oyster crops. In some years at some locations, these mortalities can be very high and make it a challenge for even the hardest working oyster farmers to succeed.

How Are Triploids Produced?

Production of triploid oysters in aquaculture has been established for several decades, used in at least both the Eastern oyster on the US Gulf and East Coasts and the Pacific oyster on the US West Coast. Regardless of species, triploids are used because they tend to grow faster and keep meat quality throughout the year. Unlike typical oysters which have two copies of DNA, triploids have three copies of DNA (all from the same species, but with an extra copy), which makes these oysters non-reproductive. How are triploids produced? Today, in commercial hatcheries, typical temperature-shock methods are used to breed typical oysters (with two copies of DNA) with special broodstock that has four copies of DNA (again, all DNA from the same species) to produce oyster seed that have those three copies and are triploids.

'Summer' Mortalities and Triploids

Dating back to the 1940's, 'summer' mortality, where large, unusual oyster die-offs occur, has been a global concern spanning from Japan to France and to the US. Causes for this mortality are typically not clear and not associated with a disease and have been suggested to be

caused by a combination of increased temperatures and low salinities, and a reduced physiological ability of oysters to adapt to these changes. In the past decade, farmers on the East Coast in the Chesapeake Bay reported unusual mortality in their oyster crops without an explanation (as high as 85%), though it was noted that it affected triploids and was called a triploid mortality. Similarly, growers on the Gulf Coast noted very high unexplained losses of triploid oysters beginning in 2016. These die-offs prompted several research studies in the Chesapeake Bay and on the Gulf Coast; the results suggest that triploid can die at higher rate than diploids at some sites in some years, with mortality typically occurring in the spring and summer. These mortality events varied in degree, in some cases reaching the very high levels reported by farmers. This suggests that triploids may be more sensitive to certain stressors (salinity, temperature, etc.) and more vulnerable to these spring/summer mortalities.

What Can Growers Do?

Considering the mortalities that growers have observed, growers have several options.

- First, any grower may choose to raise diploids and not take on the risk of triploid mortalities.
- Second, growers may explore other sites, as some of the mortalities appear to occur more frequently and/or at a higher rate at certain sites.
- Third, growers may modify their growing practices to reduce additional stressors on their triploid crop, especially during warmer months.
- Fourth, hatcheries may use selective breeding to develop lines of triploids that are better adapted to local conditions and exhibit improved survival.

In this study of the potential for selective breeding, wild broodstock were collected from three different wild populations with historical differences at each site in terms of temperature

and salinity. These oysters were spawned to create three diploid lines, as well as three triploid half-sibling lines (crossing eggs from the wild broodstock with sperm from tetraploids). These six lines were grown at three different sites in Alabama (of varying salinities) in aluminum floating cages. Through monthly sampling, performance was assessed in terms of survival, growth, condition, and disease infection intensities, and environmental data was recorded daily at each site for the duration of the study.

What were the results? Triploids suffered higher mortality than diploids at each site with a pulse of mortality in the spring, but the amount of mortality varied widely among sites. The very low salinity site, MOB in the graph, experienced almost complete mortality, while mortality was lower at the other two sites (Figure 1).

While there were differences among the lines in terms of growth and condition index, there was no differences among the seed produced from the different lines. This suggests that the tested broodstock selection did not improve survival of triploids across the range of environments tested. Of course, there is still the potential for other broodstock to demonstrate benefits, as well as the selection for improved tetraploid lines.

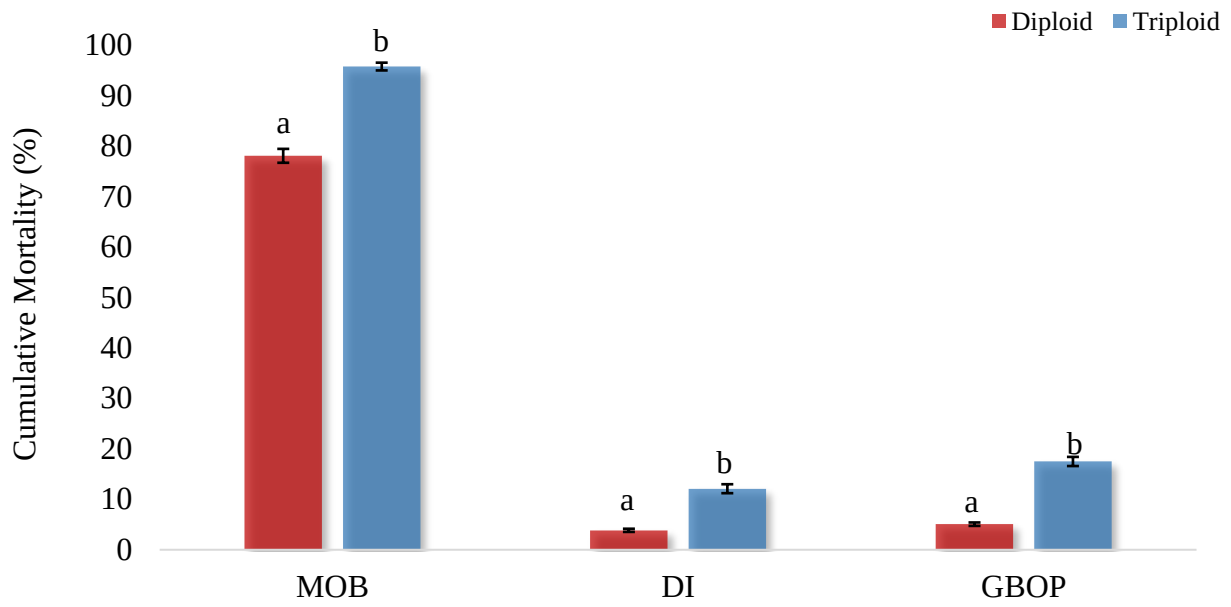

Figure 1: Average (mean ± SE) cumulative mortality of diploid and triploid oysters at each site. Superscripts denote statistical differences between ploidy within each site ($p \leq 0.05$). Error bars represent standard error.

Conclusion

Successful oyster farmers need to be aware of the risks and opportunities presented by different lines of oysters, including triploids. It is widely acknowledged that stress factors like temperature, salinity, or pathogen prevalence, during the spring and summer months can impact the performance of oysters and potentially more so for triploids. Selective breeding has been a global topic of discussion in shellfish aquaculture, ideally providing benefits to farms in terms of higher survival, growth, and/or condition, but this may not be a solution in all cases. Farmers need to consider the trade-offs for their own operations and adopt practices that reduce their vulnerability to spring/summer mortality events.

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