COMPARING TRIPLOID AND DIPLOID GROWTH AND MORTALITY IN FARMED OYSTERS, *CRASSOSTREA VIRGINICA,* IN THE NORTHERN GULF OF MEXICO

by

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ABSTRACT

The significance of the triploid advantage in terms of growth and survival varies across studies and environmental conditions. A meta-analysis was performed with 30 studies using chemically induced triploids and triploids produced by a diploid x tetraploid cross. The positive response ratios in 110 of the 121 unique experiments suggested in a significant growth advantage of the triploid oyster over the diploid oyster in whole wet weight and shell height. To compare growth and mortality locally, paired triploid and diploid *Crassostrea virginica* were deployed adjacent to commercial oyster farms in Alabama. Growth metrics and infection intensity of *Perkinsus marinus* were significantly different across sites and ploidy and, in all cases, the result was higher in triploid oysters. Triploid oysters experienced significantly higher mortality than diploid oysters at three of four sites and the results highlight the need for a better understanding of the triggers of summer mortality in triploids.

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Introduction

Due to the unreliability of wild oyster stocks, the worldwide oyster industry began shifting towards hatchery produced oysters, starting in 1967 on the west coast of the US (Clark & Langmo 1979), around 1980 in Australia (Maguire & Nell 2005) and around 1999 in France (Buestel et al. 2009). Genetic improvements, such as polyploidy, arose shortly after with the goal to produce a faster growing oyster that could retain meat quality during spawning season and could be resistant to disease related mortality (Barber & Mann, 1991). Groundbreaking experiments by Stanley et al. (1984) with *Crassostrea virginica* and Allen & Downing (1986) with *C. gigas*, were some of the first to show faster growth and greater meat condition in triploid oysters relative to their diploid counterparts.

Triploid oyster production took off on the west coast of the United States, accounting for one-third of aquaculture production in Washington and Oregon during the 1999-2000 growing season (Nell, 2002). Soon after, areas around the Chesapeake Bay adopted triploid aquaculture in response to the collapse of wild oyster stocks from overfishing and habitat loss. In Virginia, for example, triploid oysters consistently made up around 80 to 95% of total oysters grown from 2008 to 2014 (Murray & Hudson, 2015). Today, the aquaculture of triploid oysters for the half shell market is widely adopted in the United States, Europe, and Australia.

There are two main pathways to produce triploid oysters: chemical induction or through mating a diploid and tetraploid oyster. Chemical induction involves either 6-dimethyl-aminopurine (6-DMAP) or cytochalasin B (CB) to retain the first polar body (blocking meiosis I) or the second polar body (blocking meiosis II). CB is a known carcinogen and therefore a more dangerous chemical to work with than 6-DMAP. However, CB has been found to produce a higher percentage of triploids than 6-DMAP (Gérard et a. 1999) and is often the most effective chemical used (Nell 2002). When deciding to block the first or second polar body, there are cost-

benefits to each method. Blocking the first polar body during meiosis I must be done during the first 15 minutes after fertilization and is more difficult to do successfully because it relies on the synchronization of embryotic development (Stanley et al. 1984, Gérard et al. 1999). Blocking the second polar body during meiosis II is done by treating the eggs 15-30 minutes after fertilization. The meiosis I treatment results in higher heterozygosity due to lower recombination rates (Gerard 1999) and increased heterozygosity has been found to improve growth rates (Stanley et al. 1984). On the other hand, Hand et al. (1999) and Stanley et al. (1984) found a higher percentage of triploid oysters and higher larval survival with meiosis II treatment.

However, chemical treatment is not reliable in producing a 100% triploid population and only crossing a tetraploid with a diploid can achieve this (Guo & Allen 1994). Since its inception in 1994, breeding lines for tetraploids are now well developed in the US, France, and Australia (Nell 2002, Chew 2000). Producing a tetraploid depends on the small percentage of triploids which are fecund, as well as female, and can be a painstaking process. It is generally suggested that triploid females are 2% as fecund as diploid females, however this can vary considerably depending on genetic and environmental factors (Normand et al. 2008). Once these reproductive female triploids are found, a tetraploid can be made by fertilizing eggs from a triploid with sperm from a diploid and blocking the first polar body (Guo & Allen 1994). Mated triploids, therefore, receive the third chromosome from the tetraploid male while chemical triploids receive the third chromosome from the diploid female. In this way, chemical triploids obtain two identical chromosomes (pre-meiotic duplication) from the diploid female. The distinction in the origin of the extra set of chromosomes is important to note as it could influence differences in triploid performance (Wang et al. 2002, Callum 2013).

The triploid advantage is a term used to describe the increase in growth and meat condition often observed in triploid oysters compared to diploids. There are several hypothesized reasons why triploids perform better including heterozygosity, energy reallocation, and polypoid gigantism. Heterozygosity is measured across several protein loci and increases from meiosis II induction to meiosis I (Stanley et al 1984, Nell 2002) and from meiosis I induction to triploid induction by mating (Wang et al. 2002). Higher heterozygosity has been found to contribute to faster growth in Eastern oysters (Stanley et al. 1984), Pacific oysters (Yamamoto et al. 1988) and the European flat oysters (Hawkins et al. 1994). Enhanced triploid growth is also thought to occur because triploid oysters exhibit high levels of inter- and intra- individual variation of retarded gametogenesis ranging from a completely retarded state to maturing and mature gametocytes simultaneously or complete maturation and spawning (Normand et al. 2008, Allen et al. 1996, Gardner et al 1996). The sterility allows the energy normally required for spawning to be reallocated for somatic growth and can result in faster growth (Guo & Allen 1994). Lastly, triploid growth may be a result of polyploid gigantism which refers to improved growth due to the increase in cell volume and lack of cell-number compensation (Nell 2002, Wang et al. 2002).

The comparison between triploid and diploid growth and mortality has been found to depend on environmental conditions. Under poor conditions, several studies have cited that triploid survival was similar to (Smith et al. 2000, Garnier-Géré et al. 2002) or less than diploid oysters (Callum 2013, Cheney et al. 2000) and in some circumstances, triploid growth was still faster despite increased mortality (Stanley et al. 1984, Goulletquer et al 1996). Part of this controversy comes from the difficulty in comparing vastly different waterbodies with “poor” water quality given the complexities of acute (e.g., disease and parasites) and chronic stressors (e.g., low dissolved oxygen, high temperature, low salinity, and/or harmful algal blooms).

Additionally, measuring oyster growth through morphology (shell shape) is highly influenced by environmental conditions and poses challenges unlike other shellfish, such as clams or scallops (Harding 2007). Shell shape is comprised of two growth metrics, shell surface area (shell length and width) and biomass (whole, tissue, and shell weight). Shell surface area is influenced by habitat, how the oyster settles on a substrate and, in aquaculture, how densely packed the oysters are or how they are handled (e.g., grading). Biomass, on the other hand, is influenced by food quality, food availability, oyster filtration rate, and fecundity. When determining growth, it is therefore useful to use both biomass and surface area to account for effects of the environmental conditions.

Here, a meta-analysis was conducted to determine whether there is a strong growth advantage of triploid oysters over diploids oysters across a wide range of studies, species, and environmental and physical conditions.

Methods

Literature Search

Studies were obtained from the literature databases (Web of Science, Google Scholar, and Aquatic Sciences and Fisheries Abstracts (ASFA)) using combinations of the following relevant keywords: “diploid”, “triploid”, “oysters” and “growth”. Out of 100 studies identified in Web of Science, 2,540 results in Google Scholar, and 214 results in ASFA the list was reduced to 30 unique studies that reported initial and final shell height and/or whole wet weight to allow the calculation of average growth rate or directly reported growth rates of both diploid and triploid oysters. Studies were separated by whether triploids were chemically induced (blocking either meiosis I or meiosis II during fertilization) or produced through mating a tetraploid and a diploid. Only diploid/triploid oysters of the same species were compared and comparisons were not made across species, such as between diploid *Crassostrea virginica* and triploid *C. ariakensis*.

More than one experimental trial from a single publication was included in the analysis if the trial took place in a unique body of water with different environmental parameters, such as temperature and salinity, given their effects on growth. Initial and final shell height and/or whole wet weight measurements of diploid and triploid oysters were extracted from each study to calculate the growth rate per day (Equation 1). Shell height was defined as the length from the hinge to growing edge (Fig.1, Harding 2007). When data were presented in figures, the relevant information was extracted using ImageJ software (Rasband 2014). In studies with more than one breeding line with significantly different growth, the strains were grouped based on significant difference and compared separately to the triploid line. At a minimum, data associated with sample sizes and focal species were collected. When possible, other parameters, such as

temperature, salinity, tidal height, and grow out gear (e.g., cage, floating bag, and lantern net), were noted.

(Equation 1)

$$\text{Growth rate} = \frac{\text{Final} - \text{Initial}}{\text{Days deployed}}$$

Where final and initial measurements are in grams (whole wet weight) and millimeters (shell height).

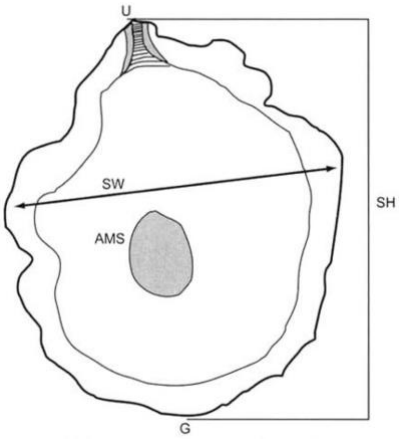

Figure 1: Sketch of growth measurement, shell height (SH) as well as the shell width (SW), umbo (U) and abductor muscle scar (AMS) for reference (Harding 2007).

Effect Size Calculations

All calculations were conducted using the *metaphor* package (Viechtbauer 2010) in the statistical software program, R (R Core Team, 2014). The natural log transformed ratio of means, also called the response ratio (Equation 2, Hedges et al. 1999), was chosen to quantify the difference between triploid and diploid oyster growth rates. Response ratios compare the mean difference between experimental treatment (triploid oyster) and control treatment (diploid oyster) in a unitless ratio and are commonly used in ecology due to the ease of interpretation and strong

statistical properties. A response ratio of zero would indicate no difference in growth between triploid and diploid oysters.

(Equation 2)

$$RR = \ln(\frac{X_T}{X_D})$$

Where X_T and X_D are the growth rate means of the triploid and diploid oysters, respectively.

As most studies included only initial and final measurements, error estimates for growth rate were not available. Instead, studies were weighted based on an estimated sampling variance using the equation described by Adams et al. 1997 (Equation 3).

(Equation 3)

$$v_i = \frac{N^T + N^D}{N^T N^D} + \frac{RR^2}{2(N^T + N^D)}$$

Where N^T and N^D are the sample sizes for the triploid and diploid groups, respectively, and *RR* is the natural log transformed response ratio.

A sample size weighted, random-effects model was used to calculate the mean effect size for both types of growth rate responses. The random-effect model was chosen because it assumes that each study has a unique effect size and takes into account biological and environmental variation across studies (Nakagawa & Santos 2011). To determine whether heterogeneity was due to the influence of moderators, a mixed-effects model was used for the following variables, including taxa, initial size, length of experiment, and sample size. Length of experiment was compared by grouping experiments into 0, 0.5, 1, or 2 years, where 0 years = 0-181 days, 0.5 years = 182-364 days, 1 year = 365-729 days, 2 years = 730-1095 days.

To determine whether there was significant publication bias, the Rosenberg method was used to calculate a fail-safe number for each induction technique and growth measure. A fail-safe number measures the number of unpublished or nonsignificant studies that would make a significant outcome nonsignificant if added to the meta-analysis. The Rosenberg method was a more practical method to use compared to original methods laid out by Rosenthal (1979) and Orwin (1983) because the calculations take into account random-effects models and effect sizes weighted by variance (Rosenberg 2005). If the resulting fail-safe numbers were large relative to the number of observations, then publication bias likely did not have a significant influence.

Results

Comparison of Response Ratios between Diploid and Triploid Growth Rates

A total of 121 effect sizes were calculated across 30 studies, consisting of 68 experimental trials measured in shell height (mm/day) and 53 experimental trials measured in whole wet weight (g/day). In terms of triploid induction technique, there were 62 experimental trials using chemical induction (29 in g/day, 33 in mm/day) and 59 experimental trials using diploid x tetraploid mating (24 in g/day, 35 in mm/day). Out of 16 studies using chemical induction, 14 studies used cytochalasin B (CB) and the remaining studies used 6-DMAP (Mallia et al. 2006) or did not specify (Garnier-Géré et al. 2002). In the following results, studies using chemically induced triploids will hereafter be referred to as chemical induction while studies using diploid x tetraploid mated triploids will be referred to as mated induction.

There was a significant growth advantage of the triploid oyster over the diploid for chemical and mated induction techniques and using both growth measurements ($p < 0.01$; Table 1). For chemical induction experiments, the mean response ratio for growth in g/day was 0.28 and more than twice that of the response ratio for growth in mm/day, 0.12 (Fig. 3). A similar pattern was observed for mated induction experiments; the mean response ratio for growth in g/day was 0.40 and the mean response ratio for growth in mm/day was 0.18 (Fig. 3). Back transformed to a linear scale, this suggests that, on average, chemically induced triploids grow 32% faster than diploids and mated triploids grow 48% faster in terms of whole wet weight. In terms of shell length, chemically induced triploids grow 13% faster than diploids and mated triploids grow 20% faster.

A Pearson correlation was used to compare the two growth measures, shell height (mm/day) and whole wet weight (g/day), within each induction method (Fig. 2). To compare the

growth measures, only the experiments that reported growth in both shell height (mm/day) and whole wet weight (g/day) were used. This consisted of 21 experiments using mated induction and 22 experiments using chemical induction out the total 121 experiments. There was a strong positive correlation (R = 0.90) for chemical induction experiments and a slightly lesser positive correlation (R = 0.78) for mated induction experiments.

Growth rates in shell height and whole wet weight were highly varied across studies with greater variation in whole wet weight than shell height and greater variation among experiments using chemical induction than experiments using mated induction (Table 1). The measure of heterogeneity (I^2), as stated by Higgins & Thompson (2002), indicated considerable between-study variation for mated induction experiments, with 87.4% variation for g/day measurements and 58.3% variation for mm/day measurements that could be explained by variables. Conversely, for chemical induction experiments the low I^2 values (17.12% and 0.00% for g/day and mm/day respectively) suggests that most of the variability is due to sampling error (Table 1).

In terms of the effect of publication bias, measured by the fail-safe number, it is generally considered that a fail-safe number greater than $5n + 10$ (where n is the number of studies) indicates a robust analysis (Rosenthal 1991). Chemical induction response ratios for growth in mm/day had a fail-safe number of 51 and suggested presence of publication bias as the number was less than $5n + 10$, where n is equal to 33 experiments. While chemical induction response ratios for growth in g/day and mated induction response ratios for growth in g/day and mm/day suggested that the analysis was robust, with fail-safe numbers of 380, 1079 and 526 respectively.

Table 1: The mean growth rates for diploid and triploid oysters as cited in the 30 studies and the results of random effects model using the response ratio [ln (3*n*/2*n*)] for diploid and triploid growth measured in whole wet weight (g/day) and shell height (mm/day). RR denotes the natural log response ratio. Variance was calculated using Equation 2. The triploid advantage refers to the improved growth in triploids relative to diploids, calculated by back transforming the response ratio (exp[ln(3*n*/2*n*)]).

		Diploid		**Triploid**						
Induction	**Measure**	**Mean** ± SD	**Range***	**Mean** ± SD	**Range***	**Mean RR**	**Triploid Advantage**	**I^2**	**Q-value**	**P-value**
Chemical	**g/day**	0.133 ± 0.184	0.006[a] - 0.781[b]	0.167 - 0.107	0.006[a] - 1.229[b]	0.278	32%	17.12 %	31.53	<.01
	mm/day	0.167 ± 0.107	0.056[f] - 0.456[i]	0.189 - 0.122	0.057[f] - 0.490[i]	0.115	12.2%	0.00 %	12.58	<.01
Mated	**g/day**	0.103 ± 0.076	0.010[g] - 0.278[h]	0.158 - 0.116	0.013[g] - 0.480[h]	0.395	48.4%	84.51%	155.69	<.01
	mm/day	0.148 ± 0.048	0.052[c] - 0.243[d]	0.178 ± 0.032	0.075[c] - 0.273[e]	0.178	20%	58.29%	73.90	<.01

* Range values as cited in the following: (a) Troup et al. 2005, (b) Shpigel et al. 1992, (c) Harding et al. 2007, (d) Ibarra et al. 2017, (e) Stone et al. 2013, (g) Guo et al. 1996, (h) Walton et al. 2013, (f) Smith et al. 2000, (i) Walton & Murphy 2011.

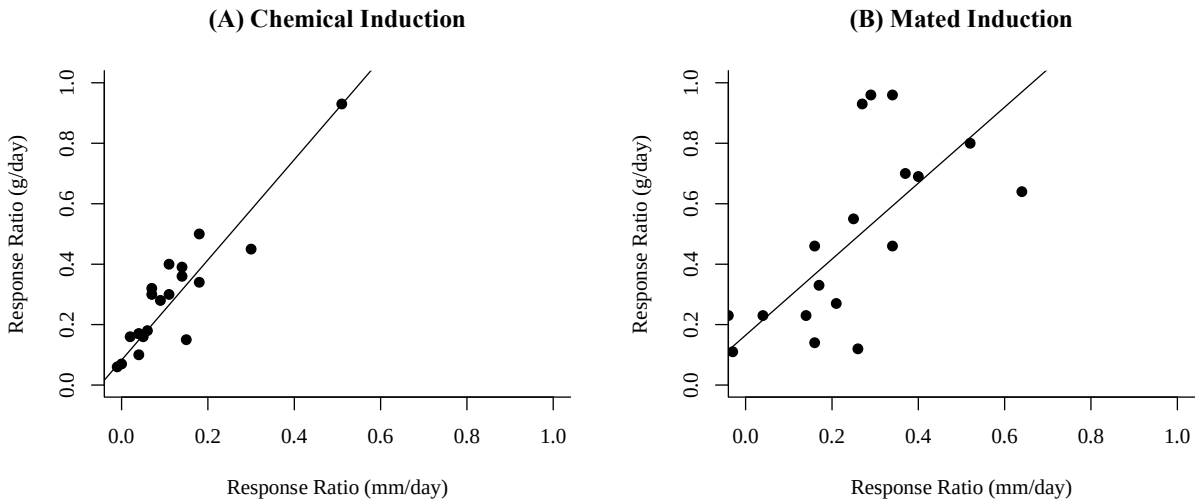

Figure 2: Pearson correlation for response ratios [ln (triploid/diploid growth)] when growth is measured in g/day and mm/day. (A) Pearson correlation (R = 0.90) for studies using chemically induced triploids. (B) Pearson correlation (R = 0.78) for studies using mated triploids.

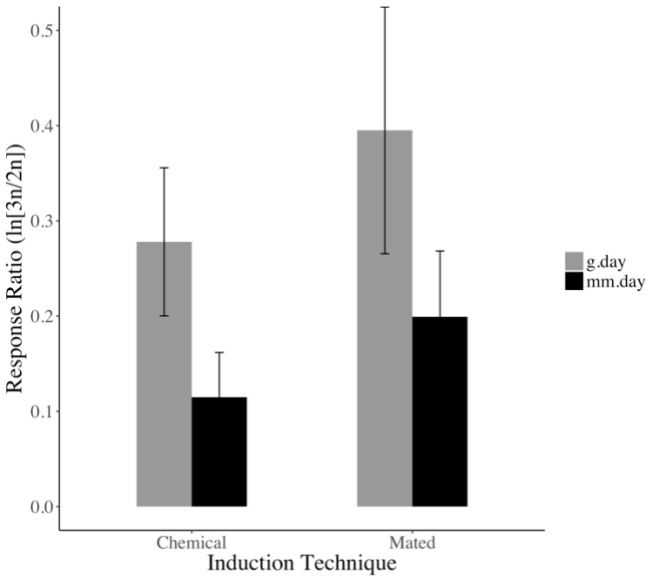

Figure 3: Mean response ratios [ln (triploid/diploid growth)] and 95% confidence interval for experiments using chemically induced triploids and mated triploids, and measuring growth rate in g/day and mm/day. All mean response ratios were significantly different ($p < 0.05$).

Effect of Species on Response Ratios

The influence of species on the difference in growth rate between triploids and diploids varied depending on the growth measurement, triploid induction and number of studies on each species (Fig. 4). The mixed effect model showed no significant difference ($p > 0.05$) in the response ratio between species for chemical induction experiments (measuring growth in mm/day and g/day) and mated induction experiments (measuring growth in mm/day). For mated induction experiments measuring growth in g/day, there was a slight significant difference, $p = 0.05$. The significance is likely due to a single study by Neil & Perkins (2005), which involved *C. gigas* and observed exceptionally fast triploid growth, with response ratios greater than 0.90. Studies using chemically induced triploids consisted of considerably more species than studies using mated triploids. The most commonly used species was *C. virginica*, used in 28 out of 35 mated induction response ratios for mm/day, 14 out of 24 mated induction response ratios for g/day, and 15 out of 33 chemical induction response ratios for mm/day. Among mated induction experiments, *C. gigas* had a higher response ratio (mean ± SE) for growth measured in mm/day (0.25 ± 0.08 compared to 0.16 ± 0.03 for *C. virginica*) and for growth measured in g/day (0.59 ± 0.12 compared to 0.33 ± 0.08 for *C. virginica* and 0.29 for *S. glomerata*) (Fig. 4). Only chemical induction response ratios for g/day appeared to be more evenly distributed across taxa, with 5 out of 29 response ratios using *C. virginica* and 8 out of 29 response ratios using *C. gigas*. Overall, there was no clear relationship between the response ratio and species, likely due limited number of studies per species.

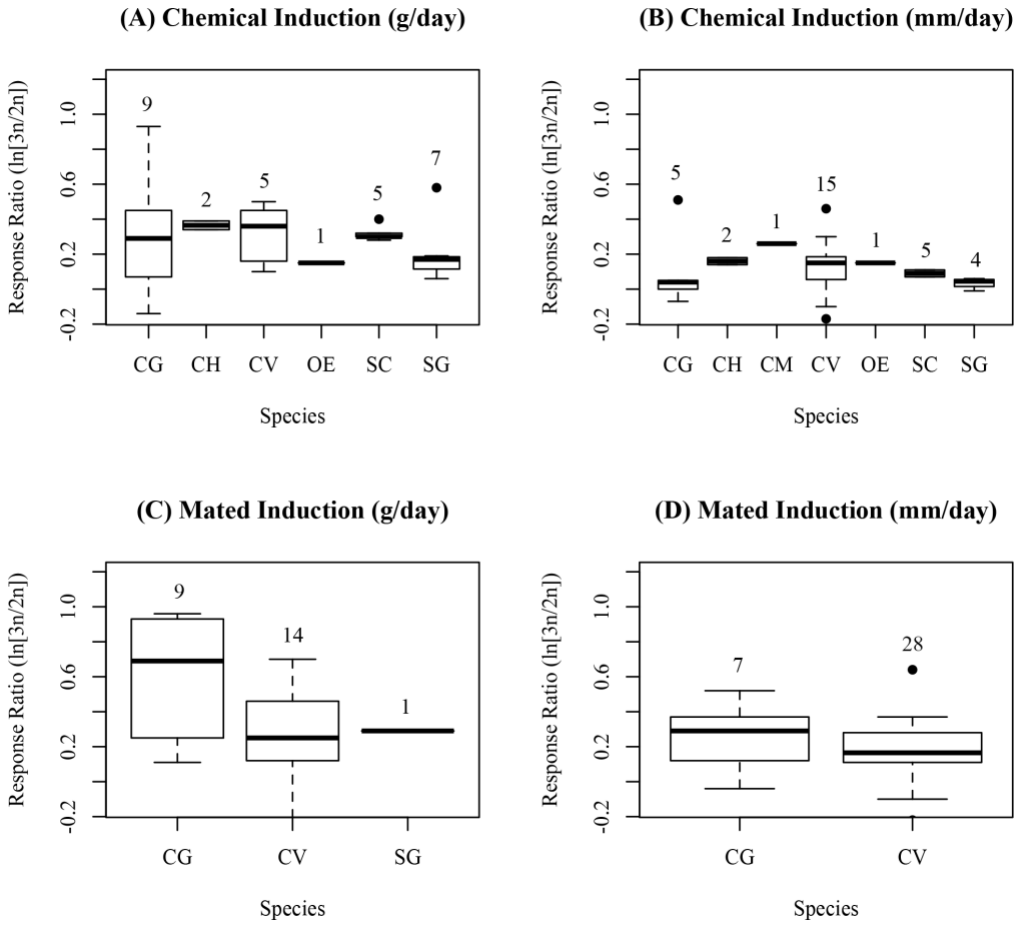

Figure 4: Comparison of response ratios [ln (triploid/diploid growth)] across the measured species for studies using measuring growth rate in g/day (A) and mm/day (B) and mated triploids measuring growth rate in g/day (C) and mm/day (D). The number of experiments per species is displayed above each box and whiskers. Boxes include 25th to 75th percentiles and whiskers extend to the 10th and 90th percentiles. CG = *Crassostrea gigas*, CV = *Crassostrea virginica*, SC = *Saccostrea commercialis*, SG = *Sassostrea glomerata*, OE = *Ostrea edulis*, CH = *Crassostrea hongkongensis*, CM = *Crassostrea madrasensis.*

3.2 Effect of Moderators on Response Ratios

Several moderators, including sample size, study length, and initial size were considered to determine the effect on triploid and diploid growth. The sample size, length of study and size of oyster at initial deployment did not significantly influence the response ratio ($p > 0.05$) for all chemical induction experiments (g/day and mm/day) and mated induction experiments measured in mm/day. For mated induction studies measuring growth in g/day, there was a significant difference in the response ratios based on length of study ($p = 0.01$), where experiments lasting 365-729 days had the greatest response ratio (mean ± SE) of 0.68 ± 0.12 compared to 0.38 ± 0.09 for 0-181 days, 0.25 ± 0.08 for 182-364 days and 0.29 for 730-1095 days (Fig. 5). The significance is likely due to the study by Neil & Perkins (2005) which involved three experiments with response ratios greater than 0.90 and oysters that were deployed for 579 days.

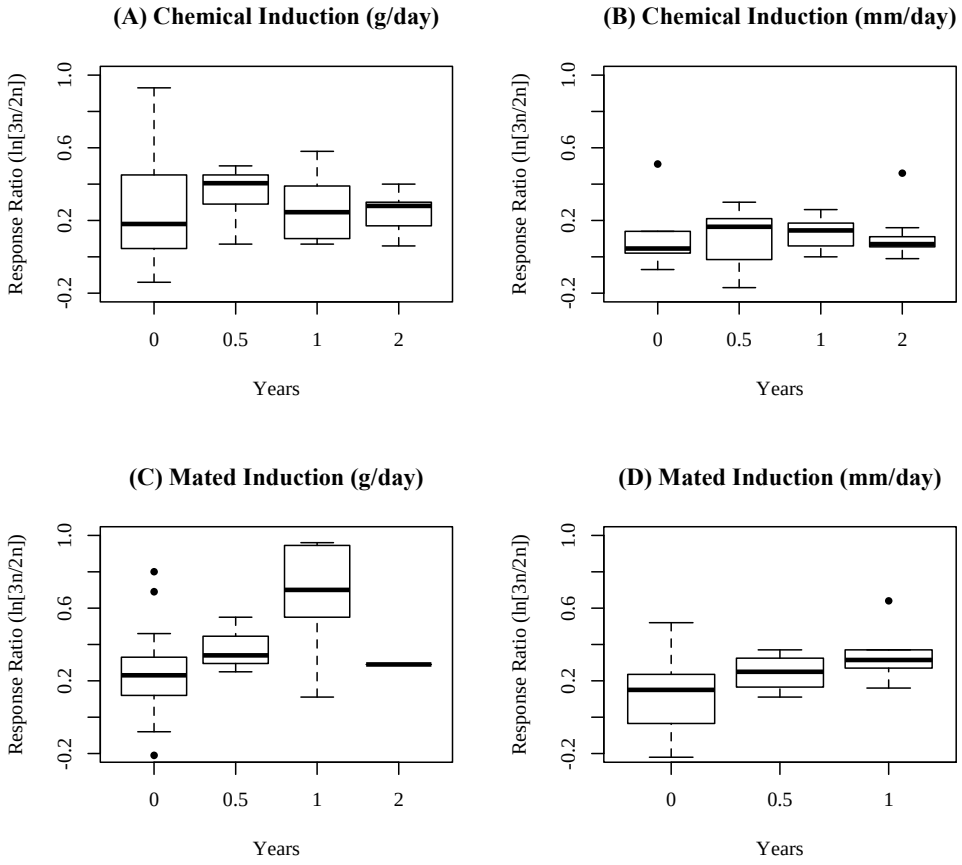

Figure 5: Response ratios [ln (triploid/diploid growth)] compared to length of experiment (years deployed) for studies using chemically induced triploids measuring growth rate in g/day (A) and mm/day (B) and mated triploids measuring growth rate in g/day (C) and mm/day (D). Boxes include 25th to 75th percentiles and whiskers extend to the 10th and 90th percentiles. Length of experiment was grouped as follows: 0 years = 0-181 days, 0.5 years = 182-364 days, 1 year = 365-729 days, 2 years = 730-1095 days.

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Discussion

This study is the first quantitative synthesis to show that triploid oysters significantly grow faster than diploids (Table 1). The comparison between response ratios suggests that in terms of whole wet weight, chemically induced triploid oysters grow an average of 43% faster than diploids and mated triploids grow an average of 49% faster than diploids. In terms of shell height, the improved growth is less impressive but still significant with an average 13% faster growth when using chemically induced triploids and an average 20% faster growth when using mated triploids. These results suggest that triploids produced through mating a diploid x tetraploid grow 6% faster in whole wet weight and 7% faster in shell height than chemically induced triploids, though this difference is not statistically significant. Wang et al. (2002) similarly observed that mated triploids were 10% larger than chemically induced triploids and attributed the growth to increased heterozygosity in mated triploids.

As both measurement techniques (whole wet weight and shell height) are used to evaluate the same outcome, growth, one would expect a strong correlation in response ratios. A Pearson correlation indicated a positive relationship, $R = 0.90$, for chemical induction comparisons and $R = 0.78$ for mated induction comparisons (Fig. 2). While there is a clear correlation, the difference between triploid and diploids growth rates appears to be greater in terms of whole weight wet, where triploids grow twice as fast in whole wet weight than shell height. This could simply be a result of scaling as shell height is a linear measure and whole wet weight is a volumetric measure. The difference between growth measures could also be indicative of the partial sterility of triploids oysters where the energy and biomass normally required for spawning is reallocated to somatic growth (Guo & Allen 1994) and results in a higher glycogen content (Allen & Downing 1986).

Only experiments using mated triploids and measuring growth in whole wet weight resulted in a significant difference in the triploid advantage between species (greater mean response ratios in *C. gigas*) and length of experiment (greater mean response ratio in experiments lasting between 1-2 years). The overall minimal effect of moderators (length of study, size at initial deployment and sample size) in the remaining experiments, suggests that triploid oysters can grow faster than diploid oysters in shell height (Chemically induced and mated triploids) and whole wet weight (chemically induced triploids only) throughout all stages of growth post-settlement. Several studies showed increased growth in triploid oysters starting at an initial size less than <25 mm and the length of study less than 6 months (Matthiessen & Davis 1992, Guo et al. 1996 and Troup et al. 2005).

The similar response ratios across species, with the exception of mated induction experiments measuring growth in g/day, suggests that the production of triploid can be applied to a variety of species globally. Given the high proportion of studies using *C. virginica*, however, more studies are needed to confirm species level comparisons. When comparing the range of species used for both growth measurements and triploid induction techniques, there was a greater variety of species in studies used chemical induction than mated induction (Fig. 3). This reflects some of the potential challenges with diploid x tetraploid mated triploids, including accessibility to tetraploids and the production of a tetraploid line. Additionally, the techniques to produce triploids through chemical induction were developed around ten years prior to discovery of mated triploids (Allen & Downing 1986, Stanley et al. 1984, Guo & Allen 1994). Triploid production through tetraploids is likely to increase in the future due to the health risks of chemical induction and variable triploid success rate (Nell 2002).

The results of this study support current oyster aquaculture practices and assumptions

with triploid and diploid oyster growth. It is generally accepted that triploid oysters can have improved marketability relative to diploid oysters, particularly during the reproductive season, as consumer acceptance is dependent on the weight and meat quality of the oysters (Nell et al. 1994). The significant growth advantage in whole wet weight in triploids relative to diploids found in this study supports the market goals of superior meat quality. In addition, the significantly faster growth in whole wet weight and shell height, as well as, the correlation between the measures supports the assumption that triploids can have a shorter growing season and can reach market size faster than diploids.

It is important to note that at sites with less than ideal environmental conditions, the advantage of triploids might not be as noticeable, if at all. Several studies included in the meta-analysis observed differences across sites due to the varying environmental parameters such as, temperature, salinity, dissolved oxygen and primary productivity, and 11 experiments observed similar to or faster growth in diploid oysters. For example, at temperate sites (18.8 – 31.5˚C), Ibarra et al. (2017) found that triploid *C. gigas* had 4% slower growth in shell height and only had a 6% greater weight gain, whereas at tropical sites (18.8 – 31.5˚C) the oysters had slower growth overall and triploids gained 70% and 64% more weight per month than diploids. Similarly, at sites with particularly low salinity (6-13 ppt), Callum (2013) found reduced growth of *C. virginica* for both ploidies and a slower growth rate in triploid oysters compared to diploids.

The influence of environmental conditions on triploid and diploid growth rates stresses the impact of water quality on oyster growth and the importance of taking these measurements during growth studies. About a third of the studies included in this meta-analysis did not report temperature or salinity averages. Lack of information on the environmental conditions made it

difficult to accurately compare the growth measurements and could account for the high heterogeneity, as well as the lack of a relationship between moderators. Other potential influencing factors, such as tidal height and culture gear, could not be compared due to lack of data. While culture gear was typically mentioned, the gear types were too varied (e.g., upweller, floating bags, fixed bag on rack, cage, lantern net, and bottom tray) with an insufficient number of studies using each gear for an accurate comparison.

The outcomes of this study demonstrate the growth advantage of producing triploid oysters compared to diploid oysters. Specifically, triploid oysters were found to grow around 40-50% faster in terms of whole wet weight and around 15-20% faster in terms of shell height. While the results found marginally faster growth in mated triploids compared to chemically induced triploids and differences among species and length of study, more studies are needed to provide a clearer understanding of the differences. Additionally, there is no question that environmental conditions have a significant effect on growth and further research should emphasize the role of these factors to a greater extent.

Chapter 2:

Comparing Triploid and Diploid Growth and Mortality in Farmed Oysters, *Crassostrea virginica*, in the northern Gulf of Mexico

Introduction

Summer mortality in oysters is not a new phenomenon; studies in the US Pacific Northwest and France have documented summer mortality events for decades (Soletchnik et al. 2007, Gagnaire et al. 2006, Cheney et al. 2000, Burge et al. 2007). Summer mortality impacting older, reproductively mature oysters generally happens over a prolonged period (Cheney et al. 2000), while summer mortality observed in juvenile oysters can occur over 1-2 weeks (Burge et al. 2007, Degremont et al. 2010). The particular cause of mortality tends to be inconclusive and highly site specific. The general consensus is that the mortality events likely occur due to a combination of stressors, including temperature, salinity, pathogens, aquaculture practices and predation (Cheney et al. 2000, Gagnaire et al. 2006, Soletchnik et al. 2007, Pernet et al. 2012).

High summer temperatures typically coincide with sexual maturation and suggest that as oysters use energy for gametogenesis there is less energy available for immune defense (Gagnaire et al. 2006). Because triploid oysters have retarded gametogenesis, it is expected that triploids would experience lower mortality than diploids during the summer. However, a number of studies comparing mortality rates in triploid and diploid oysters have come to contrasting conclusions. When investigating the summer mortality events in France, for example, Degremont (2010) noted no survival advantage of juvenile triploid *C. gigas* oysters under laboratory conditions, while Boudry et al. (2008) found higher survival rates in triploids at field sites and Guolletquer et al. (1996) found lower survival.

In terms of disease resistance, triploid oysters have also had varying success. Hand et al. (1998) found that triploid Sydney rock oysters (*Saccostrea commercialis*) performed better against the parasite *Mikrocytos roughleyi*, the cause of winter mortality. Similar studies on winter mortality with *S. commercialis* and *S. glomerata* found no difference in mortality rates

between ploidy (Nell et al. 1994, Smith et al. 2000 respectively). Survival against the ostreid herpesvirus OSHV-1 in *C. gigas* was improved in triploid oysters during the summer and fall but not the spring (Pernet et al. 2012). With the eastern oyster, *C. virginica*, Mathiessen & Davis (1992) observed that triploids had increased resistance to the parasite *Haplosporidium nelsoni* (MSX) and Callum (2013) found increased resistance to the parasite, *Perkinsus marinus* (Dermo), while Barber & Mann (1991) and Degremont et al. (2012) found no difference in Dermo infection intensity between ploidy.

Similar to mortality, the growth of triploid and diploid oysters can vary depending on a number of environmental factors. Under poor environmental conditions, diploids have reduced reproductive output and use energy reserves for somatic growth as a triploid would. A study by Davis (1994), was one of the first to show that triploid and diploid *C. gigas* growth was comparable in sites less suited for germinal production, due to environmental or genetic factors. When multiple triploid and diploid *C. virginica* lines were compared, triploid oysters were found to perform worse than all diploid lines at the low salinity sites and similar to selected diploid lines at moderate salinity sites (Callum 2013). Similarly, Degremont et al. (2010) found that diploid *C. gigas* lines selected for resistance to heat stress outperformed unselected triploids as well as selected triploids with a heat resistant diploid parent. However, when immune parameters are considered, triploids appear to have higher haemocyte parameters (Gagnaire et al. 2006) and less varied THC (Duchemin et al 2007), which could suggest less sensitivity to environmental stressors.

Coastal ecosystems in the Gulf of Mexico provide an interesting environment for this study, having nutrient rich waters, large salinity fluctuations and high summer temperatures. In these ecosystems, salinity appears to be the dominating factor in determining oyster population

dynamics (La Peyre et al. 2009). A recent study in Louisiana found that prolonged periods of low salinity (<5 ppt), regardless of temperature, resulted in high mortality rates and especially impacted adult oyster populations (Rybovich et al. 2016). Similarly, low mortality rates were observed at salinities between 9-13 ppt regardless of temperature (Cases et al. 2015) and salinities above 15 ppt resulted in higher growth rates than salinities below 10 ppt (La Peyre et al. 2016).

Although the Gulf of Mexico has a long history of wild oyster harvesting, off-bottom oyster farming is relatively new and private industries started around six years ago (Davis 2013). As of 2016, there were 14 registered oyster aquaculture operations in Alabama, 4 operations in Louisiana and over a dozen oyster farms in Florida (Russel Grice, per. comm.) In Alabama alone, over 2.7 million oysters were sold in 2016 and amounted to a wholesale value of $1.96 million (Grice & Walton 2017). The vast majority of the oysters grown are triploid, and if diploid oysters are grown at all, it is often at a farm that grows both triploids and diploids. Commercial oyster sales at the Auburn Shellfish Hatchery this past season, 2017, reported a ratio of around 200 triploid oysters for every diploid oyster (Scott Rikard, per. comm.). This reliance on triploids put farmers at risk if triploids are found to be more vulnerable to management practices or environmental conditions.

Methods

Obtaining Broodstock & Spawning

All oyster spawning, larval culture and the nursery phase took place at the Auburn University Shellfish Laboratory in Dauphin Island, Alabama. Spawning took place on May 5th 2016 for diploids and on June 15th 2016 for triploids. Thermal heat shock was used to stimulate spawning. For the diploid spawn, eggs were obtained from 13 females and fertilized with sperm from 13 males. Diploid broodstock were a combination of several genetic lines from Florida and Alabama. For the triploid spawn, eggs were obtained from 26 female diploids ('A-select' line) and fertilized with sperm from 11 tetraploid males following the methods of Guo et al. (1996). Tetraploid broodstock were a genetic line (4MGNL) from Louisiana Sea Grant Program's Oyster Research Laboratory at Grand Isle, LA. Sperm from each tetraploid male was verified by flow cytometer prior to fertilization.

Nursery Culture

On July 8th both triploids and diploids were deployed at the Auburn University Oyster Research and Demonstration Farm in Portersville Bay (30°21'11.56"N, 88°11'28.45"W). The oysters were kept in 2 mm mesh bags with 5,000 oysters per bag and kept in floating OysterGro ™ cages (Ketcham Supply, New Bedford, MA). About two weeks later, the oysters were graded into 4.5 mm bags at a density of 2,500 oysters per bag. On August 26, the oysters were graded again into 12 mm bags at a density of 600 oysters per bag. The oysters remained at the farm until the start of the experiment on November 30th, 2016.

Ploidy Determination

Triploids were ploidy verified at the larval, seed and adult stages of development using flow cytometry (Chaiton & Allen 1985). Between individual seed and adult oysters, the dissecting forceps and scissors were sterilized with 95% alcohol and fire to avoid cross contamination. All oysters were determined to be the assigned ploidy.

At the larval stage, the number of larvae needed for an accurate reading depends on the stage of development. Early stages require around 5,000 larvae, while for late stages around 1,000 larvae are needed. For this study, verification was done four days post fertilization and around 5,000 larvae were used. The larvae were condensed into a 1.5 ml microcentrifuge tube and centrifuged for 5 minutes at 3-4000 rpm to form a pellet. Once condensed, ~ 1ml of a DAPI/DMSO solution is added and vortexed for 15 seconds. From this solution, 1 ml was taken with a sterilized syringe into a 3.5 ml Röhen tube (Sarstedt, Germany) with 1 ml deionized (DI) water and inserted into the flow cytometer.

At 2 weeks post-set, the triploid seed was verified by finely crushing each oyster in a 1 ml of 0.75-1.00 ml of DAPI/DMSO solution for a total of 50 oysters. Then 1 ml of the mixture was filtered through a 20 µm screen with a sterilized syringe into a 3.5 ml Röhen tube (Sarstedt, Germany) with 1 ml of DI water and inserted into the flow cytometer.

After 9 months of the study, ploidy was verified in both triploid and diploid oysters in three oysters from each sampling basket (n = 12) per ploidy per site. A sample of the gill (~2-3 mm^2) was taken from each oyster and placed into a solution of ~ 1 ml DAPI. Then 1 ml of the mixture was filtered through a 20 µm screen with a sterilized syringe into a 3.5 ml Röhen tube (Sarstedt, Germany) with 1 ml of DI water and inserted into the flow cytometer.

Experimental Sites & Design

The field study took place in Mobile Bay, Alabama estuary which is a shallow, river discharge dominated estuarine system. Typical depth of Mobile Bay is 3 m with a narrow ship channel, 120 m wide and 12-14 m deep (Fig. 1). The Mobile River system (dominated by Mobile and Tensaw rivers) can vary in mean daily discharge from 2,656 m^3s^{-1} in the spring to 816 m^3s^{-1} in the fall (Dzwonkowski et al. 2014). While the freshwater input typically dictates prolonged changes in salinity, freshwater along with wind and tidal forcing, can result in sudden changes in salinity (Wiseman et al. 1988).

A total of 1,680 diploid oysters and 1,680 triploid oysters were deployed across 4 sites, each associated with a nearby (<50 m) commercial oyster farms, using the adjustable long-line system (ALS) with SEAPA® baskets. Oysters were stocked at a density of 60 oysters per basket with 7 baskets per ploidy/site. While the recommended stocking density for the ALS in the northern Gulf of Mexico is 75 oysters per basket (Maxwell & Supan 2010, Davis 2013), the lower density in this study (60 oysters per basket) ensured that any negative effects of crowding were avoided. Four of the seven baskets per ploidy/site were used for monthly sampling while the remaining three were used to replace mortality each month. The baskets were deployed on November 30th, 2016 underneath working piers at each site, hanging approximately 1 meter above the sea floor. Prior to the start of the experiment, 20 oysters were taken from each ploidy for initial condition indices.

Due to an unexpected storm in late April, 2017, several sampling baskets were lost including one diploid basket at Grand Bay, two diploid baskets and one triploid basket at Mobile Bay, and three diploid baskets at Dauphin Island. At Grand Bay and Mobile Bay, the extra baskets were used to replace the lost sampling baskets. At Navy Cove and Dauphin Island,

additional oysters (diploids and triploids) from the same initial spawns and grown on site were incorporated in new sampling baskets.

Environmental conditions (temperature, salinity and dissolved oxygen) were monitored by using a combination of in-water loggers, long-term monitoring stations and handheld measurements taken during site visits. In Grand Bay, an In-Situ Inc. Aqua TROLL 600 Multi-Parameter Sonde was deployed on site for the duration of the experiment (11/30/16 – 10/7/17). Dauphin Island and Navy Cove sites were monitored with YSI 6600 V2 Multi-Parameter Sondes from 5/19/17 to 8/22/17 (Yellow Springs Instruments, Yellow Springs, Ohio). Additional data on environmental conditions for the duration of the experiments at Dauphin Island and Navy Cove were taken from long-term monitoring stations (Katrina Cut and Dauphin Island Sea Lab respectively) maintained by the Mobile Bay National Estuary Program and the Dauphin Island Sea lab (Fig. 1). Mobile Bay site was monitored by a portable YSI Pro30 Conductivity, Salinity Instrument daily from 2/7/17 to 10/7/17 (Yellow Springs Instruments, Yellow Springs, Ohio).

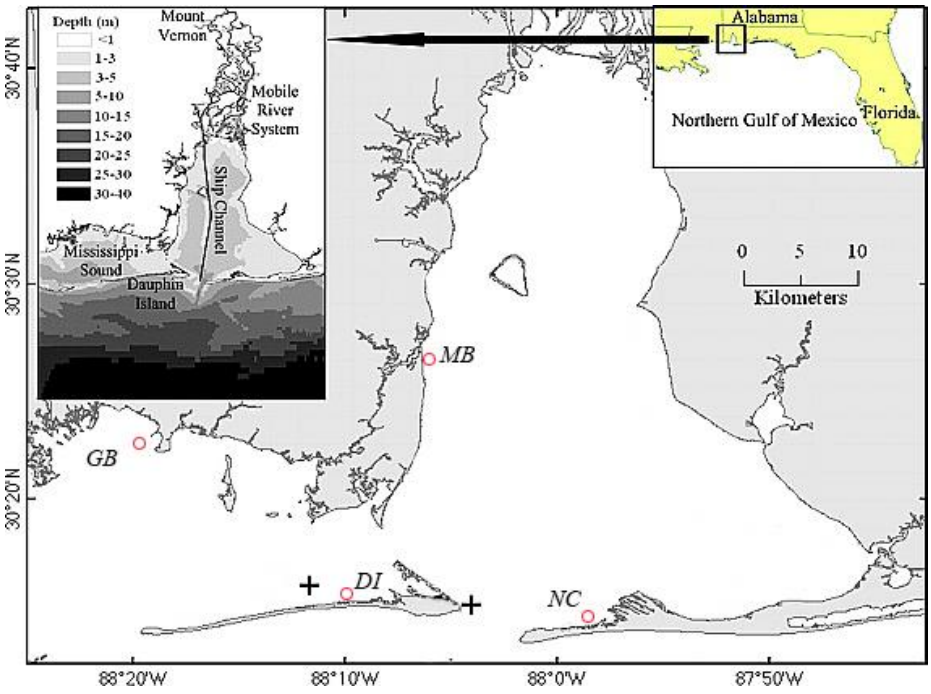

Figure 1: A map of Mobile Bay and the Mississippi Sound (adapted from Kim et al. 2010). Red open circle denotes deployment sites: Grand Bay (GB, 30°22'58.9"N 88°18'47.6"W), Dauphin Island (DI, 30°15'10.1"N 88°10'04.8"W), Mobile Bay (MB, 30°26'44.5"N 88°06'18.3"W) and Navy Cove (NC, 30°13'59.1"N 87°58'43.4"W). Crosses represent Mobile Bay National Estuary Program monitoring stations, Dauphin Island (30° 15.075' N, 88° 04.470'W) and Katrina Cut (30° 15'30" N, 88° 12'47" W). The map on the top left shows the depth profile and location of ship channel.

Data Collection

Every month, 12 oysters from 4 assigned baskets per ploidy/site were measured for shell height (Fig. 2) to the nearest 0.01 mm using Mitutoyo IP67 ABS coolant proof calipers. The total numbers of live and dead oysters were recorded for each basket. All dead oysters were discarded after each sampling period and replaced with live oysters from one of the 3 extra baskets so that each basket had 60 oysters at the beginning of each month. All baskets were replaced monthly to

reduce biofouling and improve water flow. Growth rate, interval mortality and cumulative mortality was calculated for each basket, each month (Equations 1-3). At the end of the study (October 2017), the triploid advantage was evaluated for each site using the mean growth across the 4 baskets for each ploidy (Equation 4):

(Equation 1)

Growth rate = (Current month mean shell height – Previous month's mean shell height) ÷ No. of days since last measurement

(Equation 2)

Interval mortality = No. of dead oysters in current month ÷ Total No. of oysters

(Equation 3)

Cumulative mortality = Interval mortality + Previous month's cumulative mortality

(Equation 4)

Triploid advantage = (3*n* final shell height – 2*n* final shell height) ÷ 2*n* final shell length * 100

where a positive value signifies faster growth in triploids and a negative value signifies faster growth in diploids (Callum 2013).

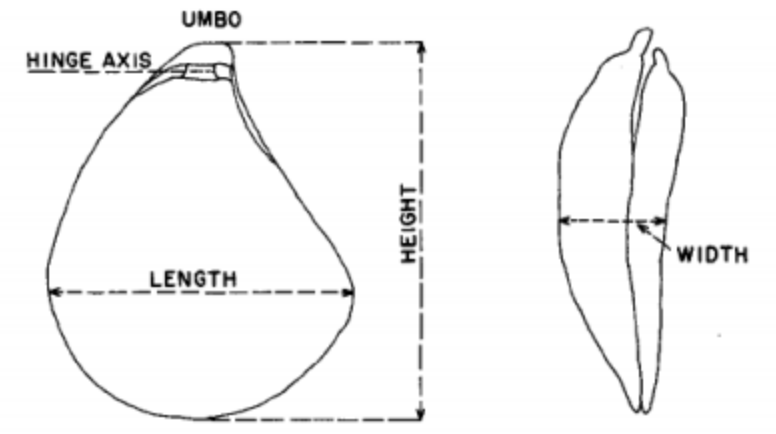

Figure 2: Shell metrics used for determining growth (from Galtsoff, 1964).

Bimonthly, 10 oysters per ploidy/site were removed from one of the extra baskets to determine whole wet weight, dry shell weight, dry tissue weight, condition index and presence of *Perkinsus marinus* (Dermo). Measurements (Fig. 2) were used to calculate growth in shell height. Oysters were then individually weighed on a Metler Toledo AL204 balance to measure whole wet weight to the nearest 0.001 g. Once opened, a ~5mm^2 sample of the mantle in front of the labial palps was taken for Dermo analysis. Instruments to remove the mantle tissue were dipped in a 90% alcohol solution and fire sterilized to ensure there was no cross contamination between oysters. The remaining tissue was then removed from the shells and placed in individually labeled cups. Shells were air dried for 48 h and weighed to obtain a dry shell weight measure. Dry tissue weight was obtained by drying the tissue for 48 h in a Fisher Scientific Isotemp oven set at 80°C. Condition index was calculated following the equation described by Abbe & Albright (2003) (Equation 5):

(Equation 5)

$$\text{Condition index} = [(\text{dry tissue weight}) / (\text{whole wet weight} - \text{dry shell weight})] * 100$$

P. marinus processing

RTFM tissue assay

The bi-monthly infection intensity and prevalence of *Perkinsus markisus* (Dermo) was determined using Ray's fluid thioglycollate medium (RTFM). The media was prepared by adding 20 g of NaCl and 29 g of thioglycollate to 1 L of deionized water then lightly heated and mixed. Once fully dissolved, 10 ml of the media was dispensed into screw cap culture tubes and autoclaved for 15 minutes with the caps loosely on. The tubes were then cooled and the caps tightened before storing in the dark. To inoculate the tubes 0.05 ml of a Chloromycetin/Nystatin Working Solution (antibiotic) was added to each tube. Then the portion of mantle tissue (~5mm^2) above the labial palps was added to the tube and inverted to mix. The tubes were stored for a week in a dark area. To read the samples the oyster tissue was carefully removed using a sterilized (90% alcohol and fire) inoculating needle and placed onto a glass slide. An iodine solution (2-3 drops) was added to the tissue and then the tissue was macerated with a blunt probe to ensure the tissue was well-stained. Then a cover slip was pressed over the tissue and read at a 40x to 100x magnification. The level of parasitism was determined on a zero-to-five-point scale using the Mackin (1962) scale, modified by Craig et al. (1989) (Table 1). Once the number of hypnospores has been estimated, infection intensity (Craig et al. 1989), prevalence and weighed prevalence (e.g. Kim & Powell 2015) was calculated for each sampling (Equations 6-8). Weighted prevalence was chosen as the preferred metric of comparison as the values include low infection intensities that would otherwise be ignored (Powell 2017).

(Equation 6)

Prevalence = no. infected/no. assayed

(Equation 7)

Infection intensity = ∑(disease code ÷ no. infected)

(Equation 8)

Weighted prevalence = prevalence x infection intensity

Table 1: Disease code scale and corresponding infection intensity to measure the level of parasitism (Craig et al. 1989).

Infection intensity	Code	Number of hypnospores
Negative	0	0
Very light	0.33	1-10
	0.67	11-74
Light	1.00	75-125
	1.33	>125, but less than <25% of the tissue is hypnospores
	1.67	<25% of the tissue is hypnospores
Light/moderate	2.00	25% of the tissue is hypnospores
	2.33	>25%, but much less than 50% of the tissue
	2.67	>25%, but <50% of the tissue is hypnospores
Moderate	3.00	50% of the tissue is hypnospores
	3.33	>50%, but much less than 75% of the tissue
	3.67	>50%, but <75% of the tissue is hypnospores
Moderately heavy	4.00	75% of the tissue is hypnospores
	4.33	>75%, but much less than 100%
Heavy	4.67	>75% of the tissue is hypnospores
	5.00	Nearly 100% of the tissue is hypnospores

ARFTM body burden assay

Dermo body burden was determined at three time points during the summer (June, August and October) using the following methods laid out by Fisher & Oliver (1996) and later modified by La Peyre et al. (2003). Media preparation and analysis was done at the Louisiana State University Animal and Food Science lab.

To prepare the media, alternative Ray's fluid thioglycollate media (ARFTM) was supplemented with lipid and chloramphenicol acid at a ratio of 949 ml ARFTM, 50 ml of a

1000x lipid mixture (5% final concentration) and 1 ml Chloromphenicol stock solution. Each oyster was shucked and the tissue removed, leaving the adductor muscle on the shell. The tissue was added to a 50 ml test tube of sterile artificial seawater at a volume (ml) equal to twice the mass (g) of the tissue. The salinity of the artificial seawater was adjusted according to the salinity at the farm when the oysters were removed. The tissue and seawater mixture was then homogenized using a Biospec biohomogenizer for about 5-10 seconds until the tissue was well minced. The homogenized tissue (1 ml) was added to a test tube of 9 ml the supplemented ARFTM solution. Then to the top of the solution, a 0.05 ml layer of the antibiotic, nystatin, was added and the tubes were kept in the dark for 1 week. Each tube was centrifuged at 1,500x g for 10 minutes and the ARFTM supernatant was discarded. To digest the tissue, 10 ml of 2N NaOH was added to the tube and incubated in a 60°C water bath for 2-6 hours. The tube was again centrifuged at 1,500x g for 10 minutes to remove the NAOH supernatant. The pellet was then suspended thoroughly, washed once with 10 ml deionized water, and centrifuged at 1,500 x g for 10 minutes. The pellet was washed twice with 10 ml phosphate buffer/BSA solution (0.1 M, pH 7.8, 0.5 mg/ml BSA). About 50 µl of each sample was serial diluted in a phosphate buffer/BSA solution and spread across 96 well plates. Lugol's working solution (50 µl) was added and left for 10 minutes to stain the hypnospores. The well plate was first centrifuged for 5 minutes at 200 x g and then the number of hypnospores was counted in the well. Based on the count, the number of hypnospores per oyster and per gram of oyster tissue was calculated. The infection intensity was classified as negative (0 parasites), light ($<10^4$ parasites g^{-1} wet tissue), moderate (1×10^4 to 5×10^5 parasites g^{-1} wet tissue) or heavy ($>5 \times 10^5$ parasites g^{-1} wet tissue) (Bushek et al. 1994).

Farm Sites

To supplement the results of the experimental data, a subsample of oysters from four commercial oyster farms was chosen for comparison. The farm sites were sampled during the summer as this is when farmers had previously seen mortality events (Bill Walton, pers. comm.). Each site consisted of 3 replicates per ploidy and/or lineage when available and were handled according to the individual farmer's practices (Table 2). Sites were sampled at three intervals throughout the 2017 summer in June, August and October. At each sampling period, the number of live and dead oysters were counted from each replicate bag, 10 oysters were removed for condition index and 15 oysters were removed for Dermo analysis using the body burden assay. Results for growth and survival were compared to the experimental sites.

Table 2: Description of farm sites sampled during the summer, 2017. Lineage refers to where the oysters were initially spawned, AU = Auburn University Shellfish Hatchery, LSG = Louisiana Sea Grant's Grand Isle Oyster Research Lab, SC = Southern Cross Sea Farms.

Farm	Location	Lineage	Ploidy	Spawn Date	Deployment	Oysters per replicate	Gear Type
Pensacola Bay Oyster Co.	Pensacola Bay, Fl	AU	T	Oct. 2016	Dec. 2016	282	OysterGro™
Navy Cove Oysters	Fort Morgan, Al	LSG	T	May 2016	June 2016	89	OysterGro™
Navy Cove Oysters	Fort Morgan, Al	AU	T	Aug. 2016	Oct. 2016	171	OysterGro™
OysterMom	Apalachee Bay, Fl	SC	T	Sept. 2015	June 2016	67	Adjustable Long-line
Triple N Oyster Farm	Grand Isle, LA	AU	T	May 2016	June 2016	46	Floating bag
Triple N Oyster Farm	Grand Isle, LA	AU	D	Sept. 2016	Dec. 2016	150	Floating bag

Data Analysis

All analyses were done using the statistical software program, R and the packages *ggplo*t, *multcomp* and *xts* (R Development Core Team, 2014). Data from the experimental deployments in mid-summer 2017 were analyzed by site (3 df), ploidy (1 df) and the interaction between the two (4 df) for the following response variables calculated for each basket: change in shell height, change in whole wet weight, change in dry tissue weight, change in dry shell weight, condition index, cumulative mortality, interval mortality, and Dermo infection intensity (RFTM tissue assay and ARFTM body burden assay). Normality and homogeneity of variance were determined using the Shapiro-Wilk test, where data were considered normally distributed when $p > 0.05$. Statistical significance between site, ploidy, and each response ratio was determined using analysis of variance (ANOVA). For mid-summer interval mortality, the data was non-normal and a binomial generalized model was used to determine significance. Significant interactions ($p < 0.05$) were compared using Tukey's honest significant test.

Results

Environmental Parameters

The environmental conditions varied among experimental sites and commercial farms (Table 3). At the experimental sites, salinity generally decreased from winter to summer as the temperature increased (Fig. 3). Mobile Bay had the lowest average salinity (mean ± SE) of the four sites (6.38 ± 3.5 ppt) and 52 days where the salinity dropped below 5 ppt. During 88% of the days with low salinity, the temperature was also above 25°C. Navy Cove had the second lowest salinity (15.03 ± 5.55 ppt) with 14 days where the salinity dropped below 5 ppt and 13 days where the temperature was also above 25°C. Grand Bay experienced a similar environment with an average salinity of 18.56 ± 7.01 ppt and all 13 of the days with salinity < 5 ppt also had temperatures above 25°C. Oysters at the Dauphin Island site did not experience any days with salinity < 5 ppt.

Table 3: Average ± SE for water temperature, salinity and dissolved oxygen (DO) over the sampling periods 11/30/16 – 10/7/17 (experimental sites) and 5/8/17 – 10/16/17 (commercial farms). *Water quality data for Mobile Bay spans 2/9/17 – 10/7/17 due to limited data at the beginning of the year. **Water quality data for Pensacola Bay spans 5/8/17 – 7/19/17, at which point the site was terminated.

	Temperature (˚C)	Salinity (ppt)	DO (mg/L)
Experimental Sites			
Dauphin Island	21.2 ± 0.31	22.3 ± 0.28	5.9 ± 0.13
Grand Bay	23.5 ± 0.29	18.7 ± 0.08	8.7 ± 0.32
Mobile Bay*	26.0 ± 0.32	6.8 ±.24	--
Navy Cove	22.8 ± 0.35	15.0 ± 0.32	7.8 ± 0.12
Commercial Farms			
Navy Cove Oyster Farm	22.8 ± 0.35	15.0 ± 0.32	7.8 ± 0.12
Oyster Mom	26.7 ± 0.48	19.7 ± 0.49	6.4 ± 0.13
Pensacola Bay Oyster Co.**	26.6 ± 1.12	9.7 ± 1.54	7.8 ± 0.35
Triple N Oyster Farm	24.3 ± 0.29	15.4 ± 0.32	--

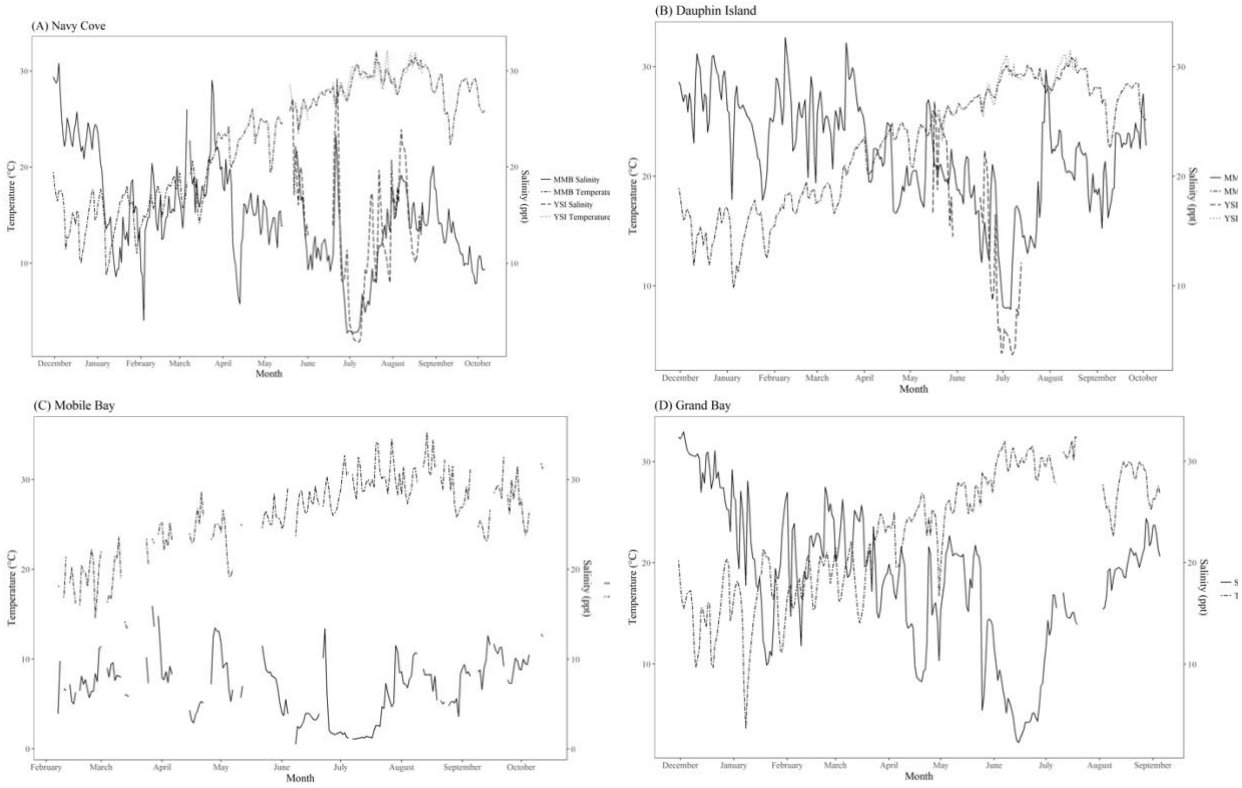

Figure 3, A-D: Daily water temperature and salinity at the four experimental sites from 11/30/16 to 10/7/17. Navy Cove (A) and Dauphin Island (B) conditions were logged by My Mobile Bay (MMB) monitoring stations and onsite YSI 6600 V2 Multi-Parameter Sondes during the summer. Mobile Bay (C) conditions were taken from a portable YSI instrument. Grand Bay (D) conditions were logged an onsite In-Situ Inc. Aqua TROLL 600 Multi-Parameter Sonde.

Mid-Summer Growth

In the middle of the summer, July 2017 (13-14 months post-fertilization) there were clear differences in shell height across sites and ploidies. Deployment site and ploidy had a significant effect on shell height ($p < 0.01$, $p < 0.01$ respectively, Table 5), while there was no significant site x ploidy interaction ($p = 0.47$, Table 5). On average (mean ± SE), across the four sites, triploid oysters grew significantly faster than diploids, with a mean shell height 71.1 ± 1.9 mm compared to 65.8 ± 2.0 mm for diploid oysters (Table 4). Among sites, Navy Cove produced oysters with the greatest mean shell height for both triploids and diploids (78.3 ± 0.6 mm, 74.0 ± 1.8 mm respectively, Table 4) and these were significantly greater than oysters grown at Grand Bay and Mobile Bay ($p < 0.01$, $p < 0.01$ respectively). Shell heights of oysters grown at Dauphin Island (73.9 ± 0.5 mm triploids, 69.8 ± 1.7 mm diploids, Table 4) ranged between the heights of oyster grown at Navy Cove and Grand Bay (71.7 ± 0.8 mm triploids, 62.3 ± 1.1 mm diploids, Table 4) and was statistically similar to both sites ($p = 0.39$, $p = 0.09$ respectively). Oysters from Mobile Bay were significantly smaller than the other three sites, with a mean shell height of 60.5 ± 3.2 mm for triploids and 57.1 ± 1.4 mm for diploids (Table 4).

When looking at growth in terms of whole wet weight, there were significant effects of site ($p < 0.01$) and ploidy ($p = 0.01$, Table 5), but no interaction between these factors ($p = 0.54$, Table 5). Mobile Bay was excluded from the analysis as the high summer mortality resulted in too few animals to process. Across the three sites, triploid oysters had a significantly greater mean whole wet weight, 74.2 ± 3.5 g, than diploid oysters, 62.3 ± 3.8 g (Table 4). Among sites, the whole wet weight of oysters grown at Navy Cove and Dauphin Island were statistically similar ($p = 0.94$), and had significantly greater whole wet weight than oysters at Grand Bay ($p <$

0.01). Navy Cove had the greatest mean whole wet weight for triploid oysters, 79.7 ± 5.6 g and Dauphin Island had greatest mean whole wet weight for diploid oysters, 72.4 ± 7.8 g (Table 4).

Dry tissue weight and dry shell weight had similar effects as whole wet weight, with significant differences among sites and between ploidy ($p < 0.01$, Table 5) but no difference in the interaction, site x ploidy ($p = 0.56$, $p = 0.51$ respectively, Table 5). Mobile Bay was excluded from the analysis as the high summer mortality resulted in too few animals to process. Across the three sites, triploids were significantly heavier than diploids with an average (± SE) dry tissue weight of 2.5 ± 0.15 g for triploids, 1.5 ± 0.15 g for diploids and an average dry shell weight of 45.0 ± 2.4 g for triploids and 38.8 ± 2.3 g for diploids (Table 4). In terms of dry tissue weight, all sites were significantly different from one another ($p < 0.01$). Dauphin Island oysters had significantly greater dry tissue weight (3.1 ± 0.3 g triploids, 2.2 ± 0.2 g diploids) than Navy Cove (2.6 ± 0.2 g triploids, 1.4 ± 0.1 g diploids) ($p = 0.01$). Grand Bay had significantly lower dry tissue weight (2.0 ± 0.6 g triploids and 0.6 ± 0.03 g diploids) than Dauphin Island ($p < 0.01$) and Navy Cove ($p < 0.01$). In terms of dry shell weight, Navy Cove and Dauphin Island were statistically similar ($p = 0.70$) and significantly greater than Grand Bay ($p < 0.01$, $p = 0.03$ respectively, Table 5). Among the triploid oysters, Navy Cove oysters had the greatest mean dry shell weight of 49.2 ± 4.0 g, while among the diploid oysters, Navy Cove and Dauphin Island oysters were relatively equal (42.9 ± 1.8 g and 43.1 ± 4.8 g respectively) (Table 4).

The condition index of triploid and diploid oysters was significantly different between ploidy and among sites ($p < 0.01$, Table 5), but not different in the interaction site x ploidy ($p = 0.10$, Table 5). Mobile Bay was excluded from the analysis as the high summer mortality resulted in too few animals to process. Triploid oysters, across the three sites, had a significantly higher average condition index (8.9 ± 0.3) than diploid oysters (6.0 ± 0.3) (Table 4). Dauphin

Island had significantly greater conditions indices than Navy Cove and Grand Bay ($p = 0.01$, $p < 0.01$, respectively), ranging from a mean of 9.5 ± 0.5 for triploids to 7.5 ± 0.3 for diploids (Table 4). The condition indices for oysters at Navy Cove (8.7 ± 0.6 triploids, 5.6 ± 0.1 diploids) and Grand Bay (8.5 ± 0.6 triploids, 4.7 ± 0.2 diploids) did not differ significantly ($p = 0.36$, Table 5). Mobile Bay was excluded from the analysis as the high summer mortality resulted in too few animals to process.

Table 4: Mean (± SE) shell height (SH), whole wet weight (WWW), dry tissue weight (DTW), dry shell weight (DSW) and condition index (CI) for the four experimental sites at the mid-summer sampling point. Due to the high summer mortality in triploids at Mobile Bay, there were too few animals to measure WWW, DTW, DSW and CI.

Site	Ploidy	SH (mm)	WWW (g)	DTW (g)	DSW (g)	CI
Dauphin Island	T	73.9 ± 0.5	79.5 ± 7.3	3.1 ± 0.3	46.5 ± 5.0	9.5 ± 0.5
	D	69.8 ± 1.7	72.4 ± 7.8	2.2 ± 0.2	43.1 ± 4.8	7.5 ± 0.3
Grand Bay	T	71.7 ± 0.8	63.3 ± 3.1	2.0 ± 0.1	39.2 ± 2.3	8.5 ± 0.6
	D	62.3 ± 1.1	43.6 ± 2.2	0.6 ± 0.0	29.5 ± 1.6	4.7 ± 0.2
Mobile Bay	T	60.5 ± 3.2	--	--	--	--
	D	57.1 ± 1.4	--	--	--	--
Navy Cove	T	78.3 ± 0.6	79.7 ± 5.6	2.6 ± 0.2	49.2 ± 4.0	8.7 ± 0.6
	D	74.0 ± 1.8	68.7 ± 2.6	1.4 ± 0.1	42.9 ± 1.8	5.6 ± 0.1

Table 5: Analysis of variance for mean shell height, whole wet weight, dry tissue weight and dry shell weight mid-summer, 2017, across the four experimental sites (Mobile Bay, Navy Cove, Grand Bay and Dauphin Island) and two ploidies (triploid and diploid). *Mobile Bay triploids were not included in the analysis due to their unusually high mortality rate which resulted in too few animals to measure. ** Dry shell weight was log_{10} transformed for normality.

	Growth Mid-Summer 2017			
	df	MS	F	P
Measure				
Shell Height				
Site	3	446.2	25.757	<0.001
Ploidy	1	212.4	12.264	0.002
Site x Ploidy	3	15.1	0.874	0.469
Error	22	17.3		
Whole Wet Weight*				
Site	2	3246	9.422	0.015
Ploidy	1	2186	6.345	<0.001
Site x Ploidy	2	213	0.617	0.543
Error	56	345		
Dry Tissue Weight*				
Site	2	9.435	23.436	<0.001
Ploidy	1	19.468	48.360	<0.001
Site x Ploidy	2	.240	0.595	0.555
Error	56	0.403		
Dry Shell Weight***				
Site	2	0.09170	5.908	0.005
Ploidy	1	0.07303	4.706	0.034
Site x Ploidy	2	0.01043	0.672	0.515
Error	56	0.01552		

Mid-Summer Mortality

Mid-summer cumulative mortality was significantly different across ploidy ($p < 0.01$), site ($p = 0.05$) and the site x ploidy interaction ($p = 0.01$) (Table 6). Triploids had significantly greater cumulative mortality at Navy Cove ($p < 0.01$), Dauphin Island ($p = 0.04$) and Mobile Bay ($p < 0.01$). The Grand Bay site, however, had no significant difference in cumulative mortality between diploids and triploids ($p = 1.00$). Mobile Bay had the greatest average cumulative mortality in triploids, 0.6 ± 0.1, while Grand Bay had the greatest cumulative mortality for diploids, 0.2 ± 0.1 (Fig. 4, A).

Interval mortality was significantly different across ploidy ($p < 0.01$), site ($p < 0.01$), and the site x ploidy interaction ($p < 0.01$) (Table 6). Triploids had significantly greater interval mortality at Mobile Bay ($p < 0.01$), while there was no significant difference at Navy Cove ($p = 0.10$), Dauphin Island ($p = 0.06$) and Grand Bay ($p = 0.32$) Mobile Bay triploids suffered an average of 53% mortality between June and July, 40% more than the second highest observed interval mortality (11% mortality in Navy Cove triploids) (Fig. 4, B).

Table 6: Analysis of variance for cumulative and interval mortality mid-summer 2017 across the four sites (Mobile Bay, Navy Cove, Grand Bay and Dauphin Island) and two ploidies (triploid and diploid). *Arcsin transformed for normality. **non-normal distribution was analyzed by a generalized linear binomial distribution.

	Mortality Mid-Summer 2017			
	df	MS	F	P
Measure				
Cumulative Mortality*				
Site	3	0.0559	3.011	0.05
Ploidy	1	1.0142	54.680	<0.001
Site x Ploidy	3	0.0954	5.144	0.007
Error	24	0.0185		
Interval Mortality**				
Site	3			<0.0001
Ploidy	1			<0.0001
Site x Ploidy	3			0.005

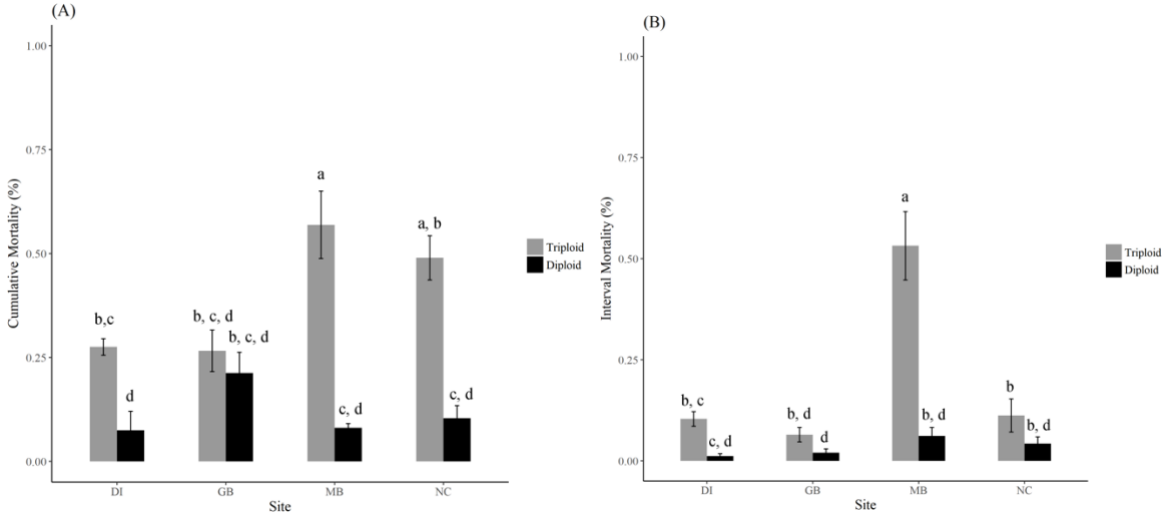

Figure 4: Cumulative mortality (A) and interval mortality (B) during the mid-summer sampling point at the four experimental sites: Dauphin Island (DI), Grand Bay (GB), Mobile Bay (MB) and Navy Cove (NC). Error bars represent standard error. Different letters indicate a significant difference ($p < 0.05$).

Shell height over time

At deployment, diploid oysters were an average of 35.6 ± 2.4 mm in shell height, while triploid oysters were an average of 37.1 ± 1.7 mm (p = 0.97). Beginning in the spring (April), triploids consistently had a greater shell height at all sites (Fig. 6). Growth measurements at Mobile Bay ended in July (triploids) and August (diploids) due to the high mortality rate. Oysters at Navy Cove has the fastest mean growth rate of 0.18 mm/day for triploids and 0.16 mm/day for diploids (Fig. 5). Mobile Bay and Grand Bay oysters had similar growth rates for each ploidy (0.12 mm/day and 0.14 mm/day for triploids, 0.09 mm/day and 0.09 mm/day for diploids respectively) (Fig. 5). At the end of the experiment, October 2017, oysters at Grand Bay had smaller mean shell heights (78.1 ± 1.4 mm and 65.3 ± 1.3 mm for triploid and diploid oysters respectively) than Navy Cove (88.9 ± 1.4 mm triploids, 85.1 ± 1.4 mm diploids) and Dauphin Island (81.9 ± 2.3 mm triploids, 76.6 diploids).

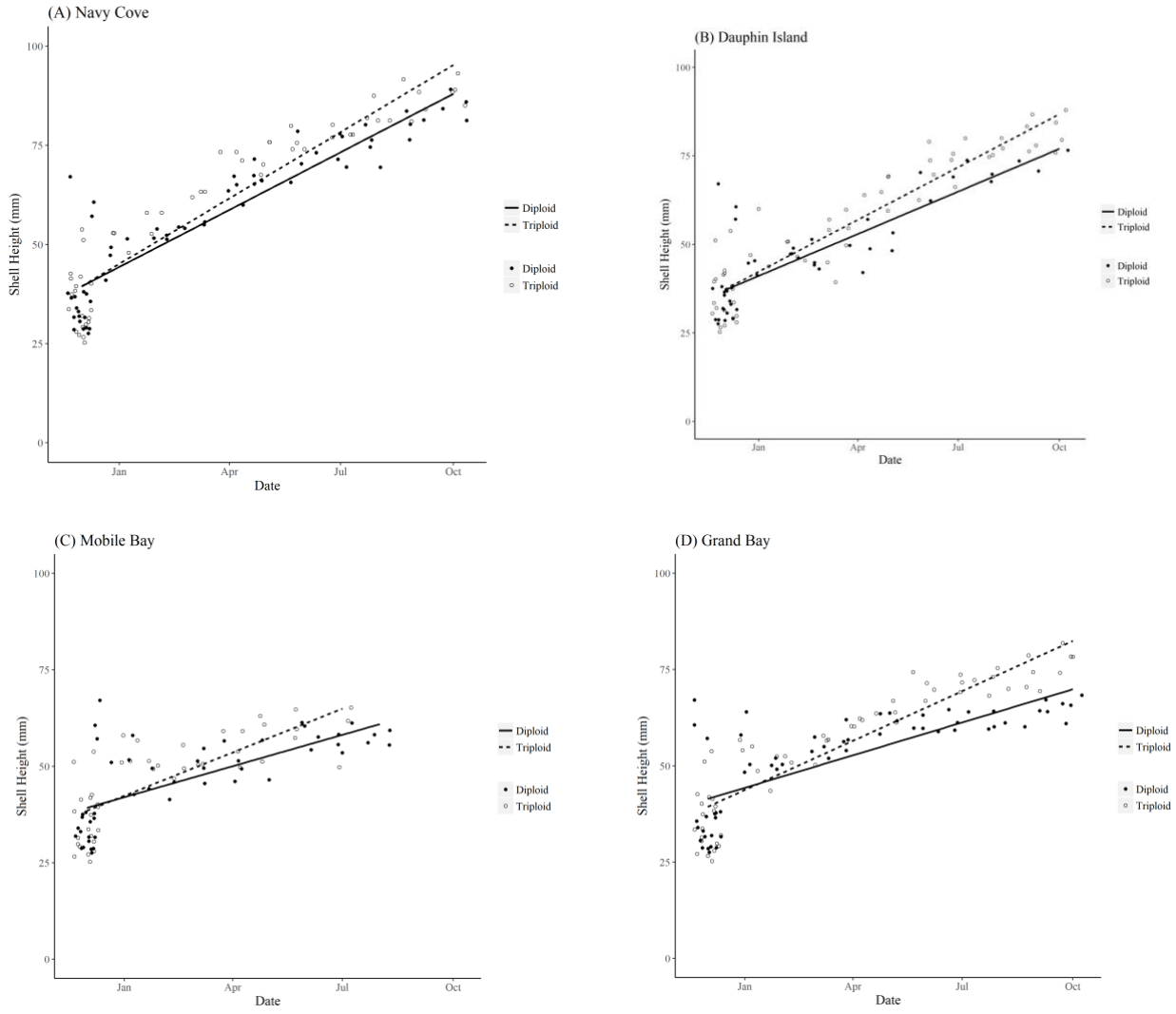

Figure 5: Linear regression for shell height (mm) of diploid (red) and triploid (blue) oysters across 11 months (December 2016 – October 2017) at four experimental sites: (A) Navy Cove: triploid ($y = 0.18x – 3105.7$, $R^2 = 0.89$), diploid ($y = 0.16x – 2699.3$, $R^2 = 0.84$); (B) Dauphin Island: triploid ($y = 0.16x -2755.8$, $R^2 = 0.87$), diploid ($y = 0.13x -2216.2$, $R^2 = 0.69$); (C) Mobile Bay: triploid ($y = 0.13x – 2098.5$, $R^2 = 0.58$), diploid ($y =0.09x – 1489.5$, $R^2 = 0.46$) and (D) Grand Bay: triploid ($y=0.14x -2388.3$, $R^2 = 0.83$), diploid ($y = 0.09x -1563.9$, $R^2 = 0.58$).

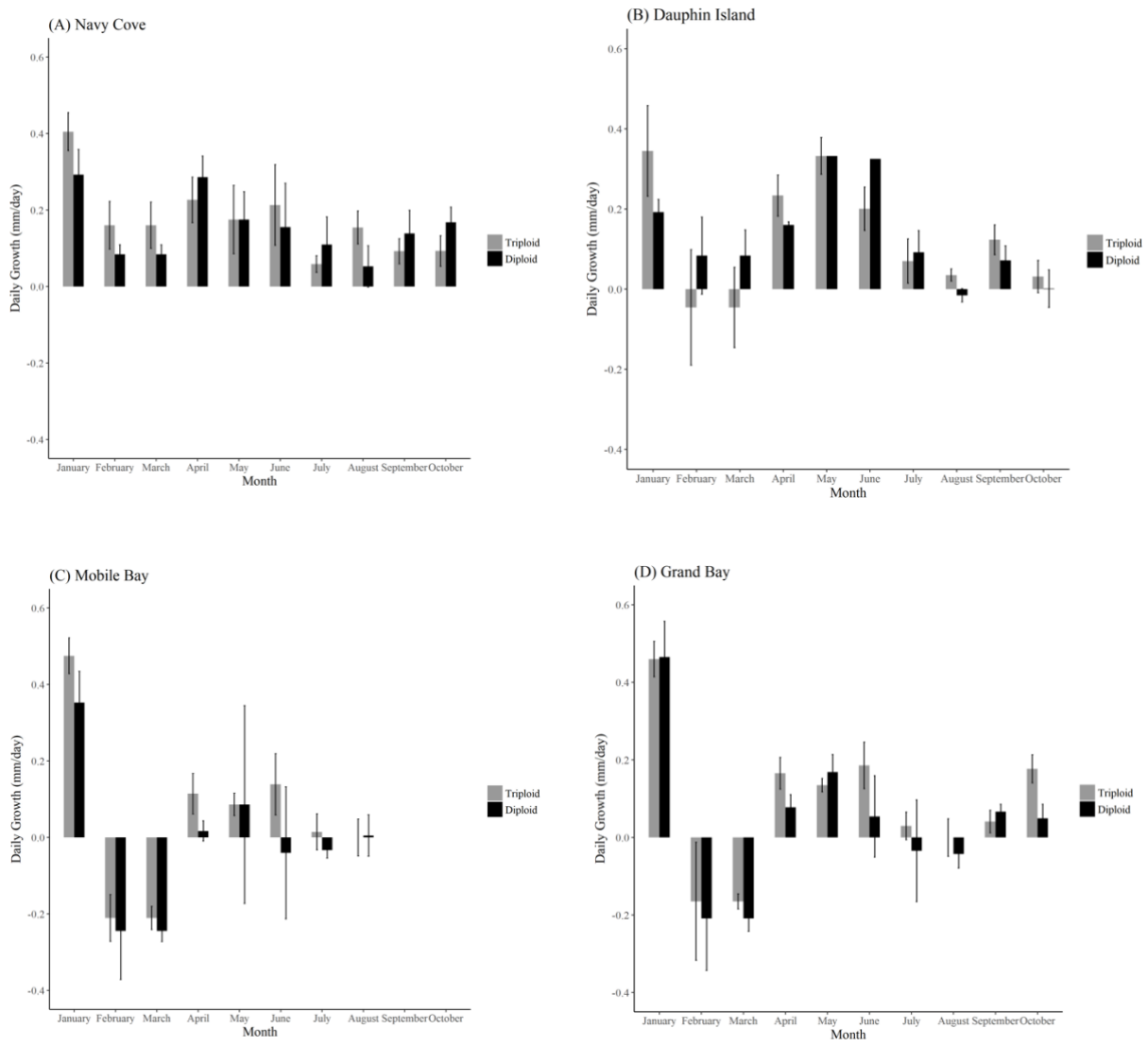

Figure 6: Daily growth (mm/day) of diploid (red) and triploid (blue) oysters across 11 months (December 2016 – October 2017) at four experimental sites: (A) Navy Cove, (B) Dauphin Island, (C) Mobile Bay and (D) Grand Bay. Error bars represent standard error.

Dry tissue weight over time

From December 2016 to October 2017, the dry tissue weight varied across sites and ploidy (Fig. 7). Initial triploid and diploid oysters had a dry tissue weight (mean ± SE) of 0.19 ± 0.03 g and 0.21 ± 0.05 g respectively ($p = 0.52$). Triploid oysters had similar to or greater dry tissue weights than diploids at each sampling period with the exception of Mobile Bay where diploid oysters were heavier until April. Dry tissue weight at Grand Bay plateaued in February for diploids and in April for triploids. At Navy Cove, diploid dry tissue weight plateaued and slightly decreased in April while triploid oysters dropped in dry tissue only during the July and August sampling. At Dauphin Island, diploid dry tissue weight similarly began to decrease in April while triploid weight continued to increase. Mobile Bay sampling ceased in August due to high mortality rates, however, triploid oysters appeared to consistently increase in dry tissue weight while diploid oyster weight decreased from the June to August sampling period.

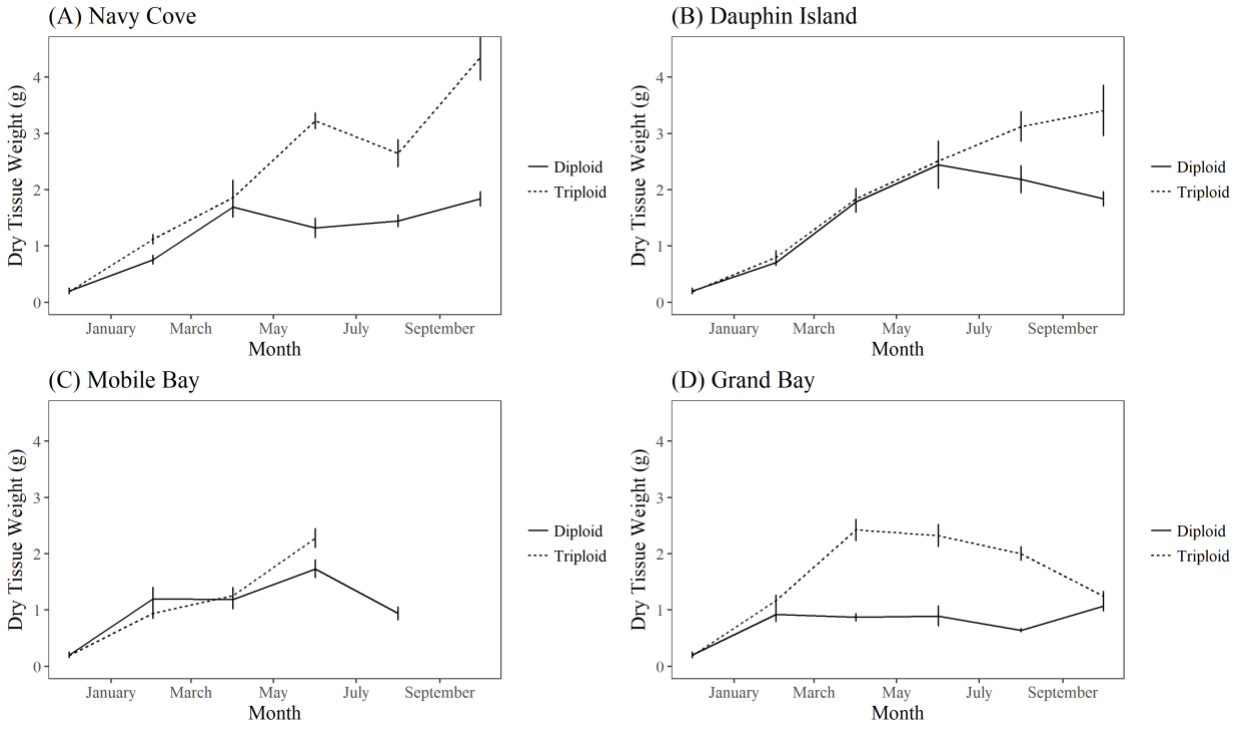

Figure 7: Change in dry tissue weight (g) in diploid and triploid oysters from December 2016 to October 2017 at four experimental sites: (A) Navy Cove, (B) Dauphin Island, (C) Mobile Bay and (D) Grand Bay. Error bars represent standard error.

Condition index over time

Initial condition indices (mean ± SE) of triploid and diploid oysters were 8.5 ± 0.4 and 6.4 ± 0.5 respectively ($p = 0.02$). Oysters at all sites experienced an increase in condition index from December to February (Fig. 8). In April, the condition index of triploid and diploid oysters at Navy Cove and diploid oysters at Grand Bay began to decrease. By June, all oysters began to decrease in condition and continued to decrease until October where the condition improved in oysters at Navy Cove and diploid oysters at Grand Bay. With the exception of Mobile Bay and February sampling at Grand Bay, triploid oysters had consistently higher condition indices and

generally followed the same increasing/decreasing pattern as diploid oysters. Diploids at Mobile Bay appeared to have a greater condition index than the triploids during the spring and early summer sampling periods (April, June).

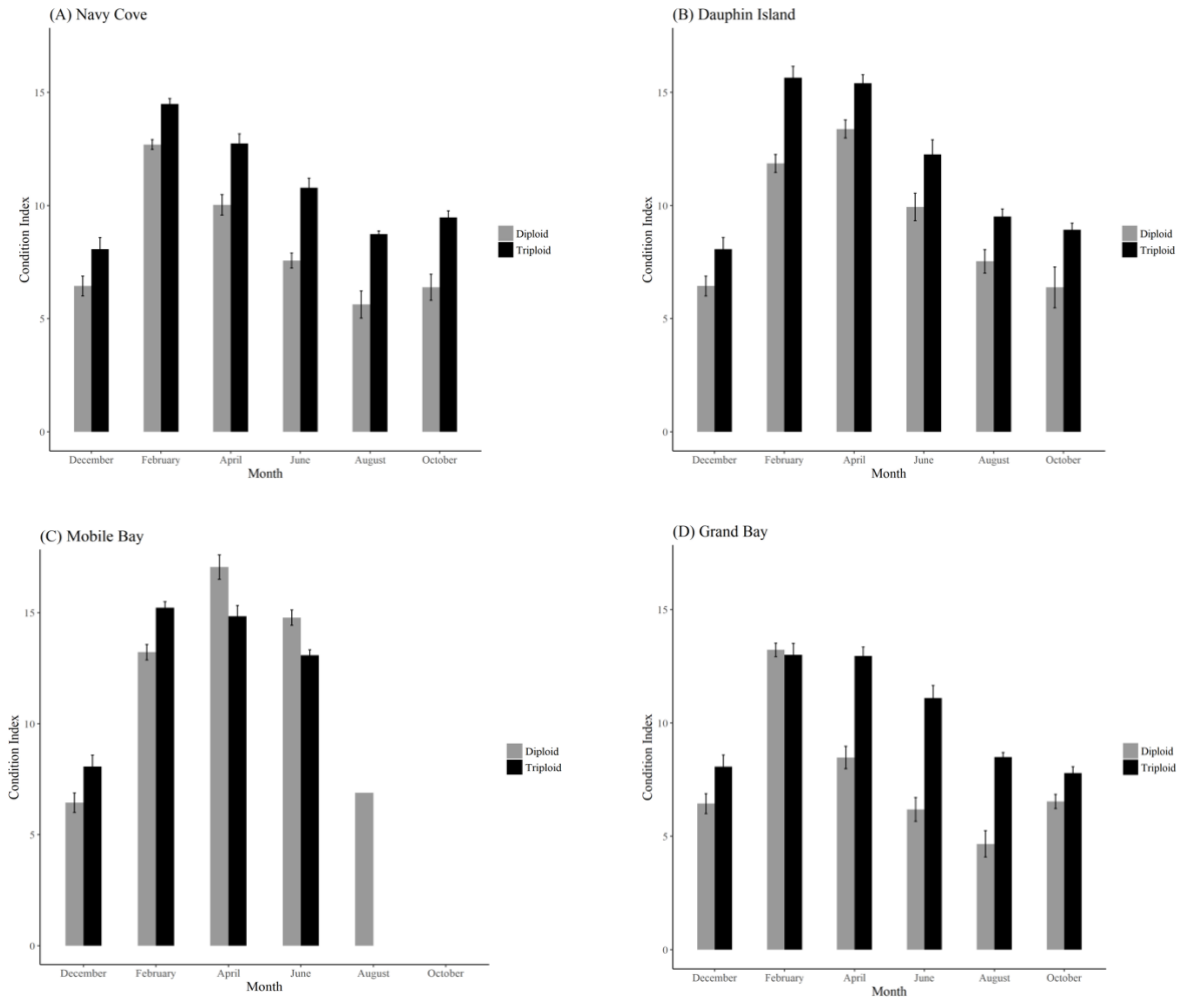

Figure 8: Condition index [dry tissue weight / (whole wet weight –dry shell weight)] in diploid and triploid oysters from December 2016 to October 2017 at four experimental sites: (A) Navy Cove, (B) Dauphin Island, (C) Mobile Bay and (D) Grand Bay. Error bars represent standard error.

Mortality over time

At all sites, mortality was relatively negligible during the first few months (December 2016 to March 2017) of the experiment. The first spike in mortality was observed in April at both Navy Cove (triploids only) and Grand Bay in both triploids and diploids (Fig. 10). Mortality at Grand Bay leveled off thereafter, while Navy Cove triploids continued to increase. Diploid oysters across all sites except Mobile Bay, experienced mortality from April to May and minimal mortality (0-10% per month) thereafter. Mobile Bay triploids suffered the highest cumulative mortality of 157% cumulative by August while Navy Cove triploids suffered the second highest cumulative mortality, reaching 125% by October (Fig. 9). Cumulative mortalities exceeding 100% is due to the fact that at each sampling period, the dead oysters were replaced so that each basket (n = 4/ploidy/site) had 60 live oysters. The majority of the mortality (mean % interval mortality) at Mobile Bay occurred in July (53% for triploids) and August (100% for triploids, 67% for diploids) (Fig. 10). Navy Cove triploids also experienced a spike in mortality in August, with a 48% mean interval mortality (Fig. 10). Dauphin Island triploids experienced surprisingly high mortality towards the end of study; an average of 20% mortality was observed between August to September and an additional 30% mortality was observed from September to October.

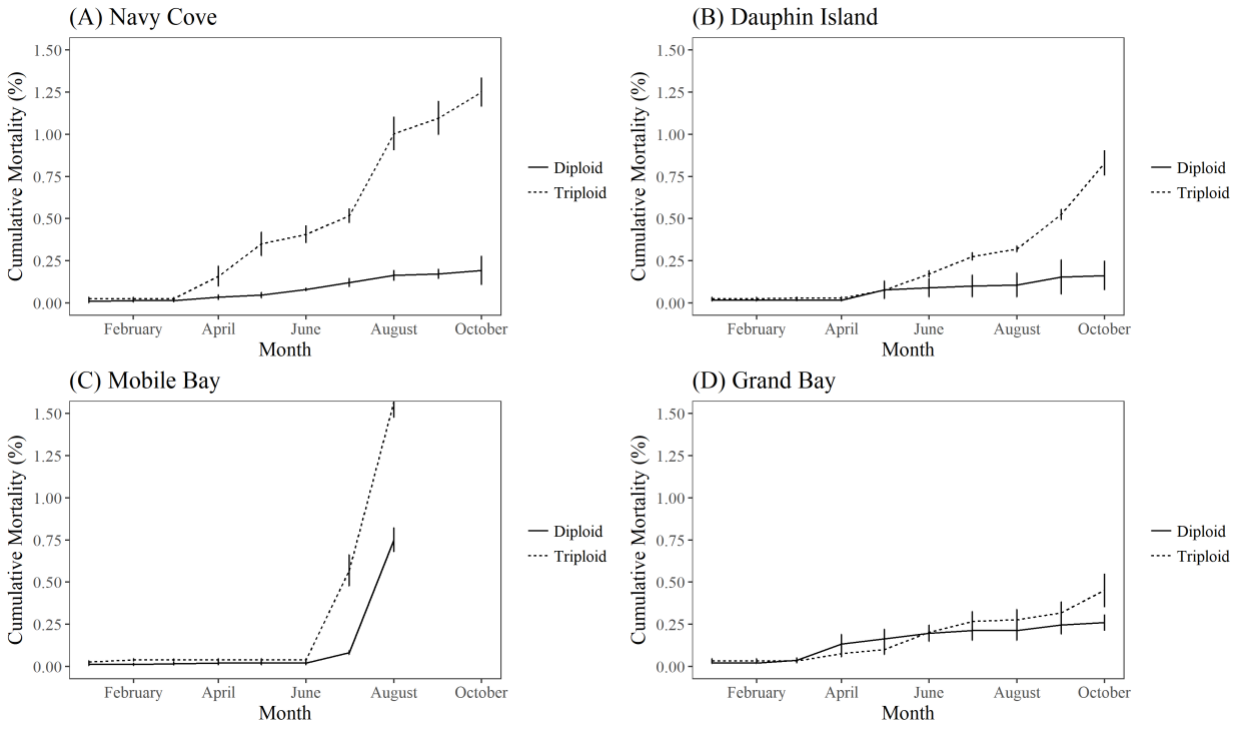

Figure 9: Cumulative mortality (%) in diploid and triploid oysters from December 2016 to October 2017 at four experimental sites: (A) Navy Cove, (B) Dauphin Island, (C) Mobile Bay and (D) Grand Bay. Error bars represent standard error. At each sampling period, the dead oysters were replaced so that each basket (n = 4/ploidy/site) had 60 live oysters and resulted in cumulative mortalities that can exceed 100%.

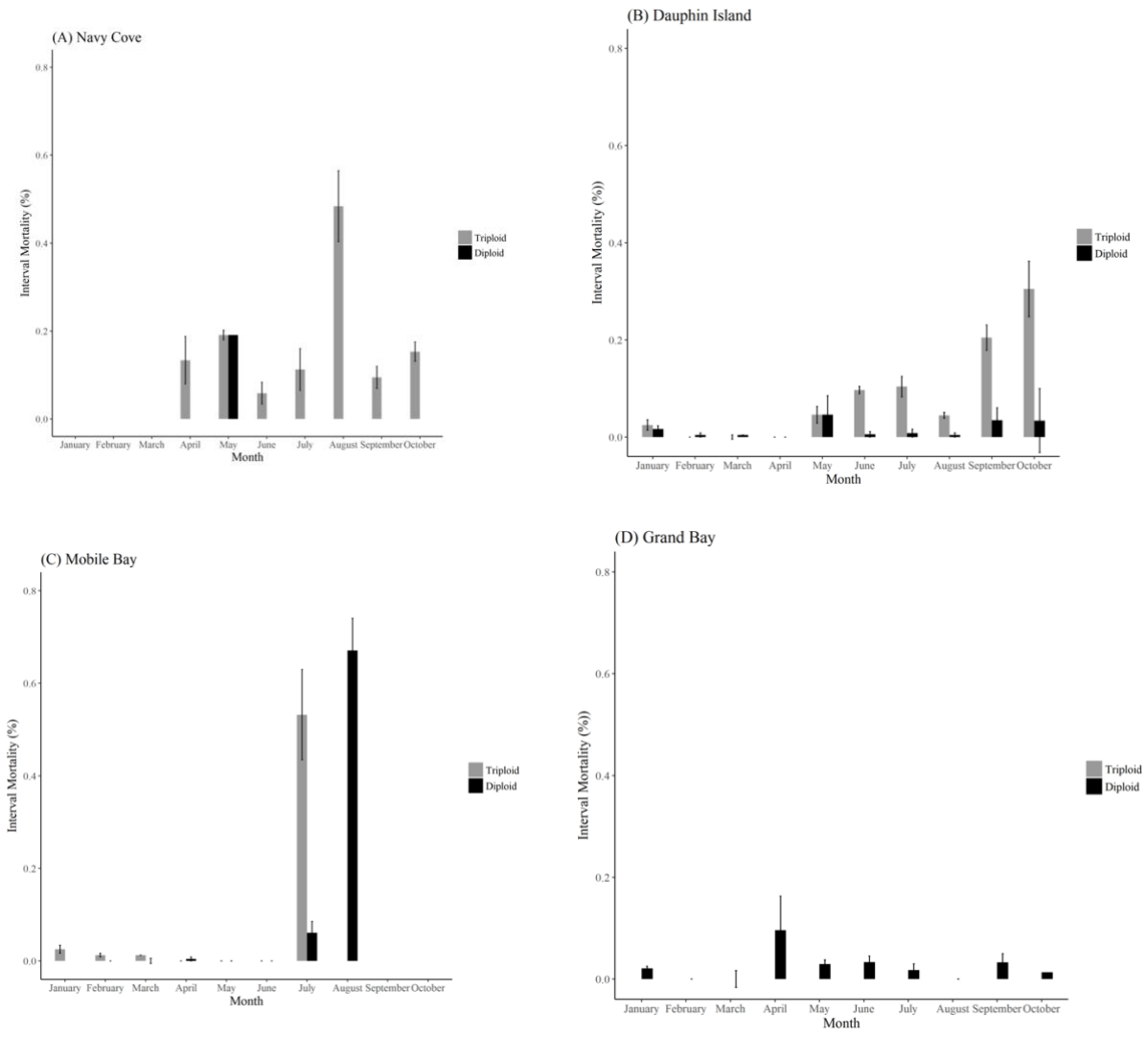

Figure 10: Interval mortality (%) in diploid and triploid oysters from December 2016 to October 2017 at four experimental sites: (A) Navy Cove, (B) Dauphin Island, (C) Mobile Bay and (D) Grand Bay. Error bars represent standard error.

Intensity, Prevalence and Body Burden of P. marinus Infection

As expected, the prevalence and intensity of *Perkinsus marinus* increased at all experimental sites from February to October as the temperature increased and was greatest at the higher salinity sites (Fig. 11, Table 7). At the mid-summer sampling (August), there was a significant difference in body burden (Log_{10} parasites g^{-1} wet tissue) across ploidy (p =0.05) and site ($p < 0.01$) but not the interaction between site x ploidy ($p = 0.84$). Overall, triploids had the greatest Dermo infection, with an average weighted prevalence of 0.8 and 4.6 Log^{10} parasites g^{-1} wet tissue, compared to diploids with an average weighted prevalence of 0.4 and 3.6 Log^{10} parasites g^{-1} wet tissue. Dauphin Island oysters had the highest infection intensity in June and August sampling while Navy Cove had the highest infection intensity in October. There were considerable differences in the infection intensity classification using the tissue assay and body burden assay, where the body burden assay consistently classified the infection heavier.

Table 7: Results of *P. marinus* infection in triploid and diploid oysters at the four experimental sites using the Ray's fluid thioglycollate medium (RFTM) tissue assay (n = 10) and the alternative Ray's fluid thioglycollate medium (ARFTM) body burden assay (n = 15). Samples were taken at three time points (June, August and October) during summer 2017. Mobile Bay was not included in October sampling due to high mortality. Using the body burden assay, infection intensity was classified as follows, light: $<10^4$ parasites g^{-1} wet tissue, moderate: $1x\ 10^4$ to 5×10^5 parasites g^{-1} wet tissue, heavy: $> 5 \times 10^5$ parasites g^{-1} wet tissue.

		June			
		RFTM tissue assay		ARFTM body burden assay	
	Ploidy	Weighted Prevalence	Infection Intensity	Log^{10} parasites g^{-1} wet tissue	Infection Intensity
Grand Bay	Triploid	0.5	Light	5.1	Moderate
	Diploid	0.4	Light	3.7	Light
Dauphin Island	Triploid	0.3	Light	4.2	Moderate
	Diploid	0.7	Light	5.7	Heavy
Mobile Bay	Triploid	0.0	Negative	2.9	Light
	Diploid	0.1	Light	2.3	Light
Navy Cove	Triploid	0.2	Light	5.5	Moderate
	Diploid	0.2	Light	3.8	Light

		August			
		RFTM tissue assay		ARFTM body burden assay	
	Ploidy	Weighted Prevalence	Infection Intensity	Log^{10} parasites g^{-1} wet tissue	Infection Intensity
Grand Bay	Triploid	0.3	Light	4.4	Moderate
	Diploid	0.1	Light	2.4	Light
Dauphin Island	Triploid	1.4	Light/Moderate	5.8	Heavy
	Diploid	0.8	Light	5.6	Moderate
Mobile Bay	Triploid	-	-	-	-
	Diploid	0.03	Light	1.9	Light
Navy Cove	Triploid	0.6	Light	3.6	Light
	Diploid	0.4	Light	2.9	Light

		October			
		RFTM tissue assay		ARFTM body burden assay	
	Ploidy	Weighted Prevalence	Infection Intensity	Log^{10} parasites g^{-1} wet tissue	Infection Intensity
Grand Bay	Triploid	1.7	Light/Moderate	5.9	Heavy
	Diploid	0.5	Light	4.8	Moderate
Dauphin Island	Triploid	2.2	Light/Moderate	6.0	Heavy
	Diploid	1.3	Light	5.5	Moderate
Mobile Bay	Triploid	-	-	-	-
	Diploid	-	-	-	-
Navy Cove	Triploid	1.2	Light	6.1	Heavy
	Diploid	0.8	Light	6.2	Heavy

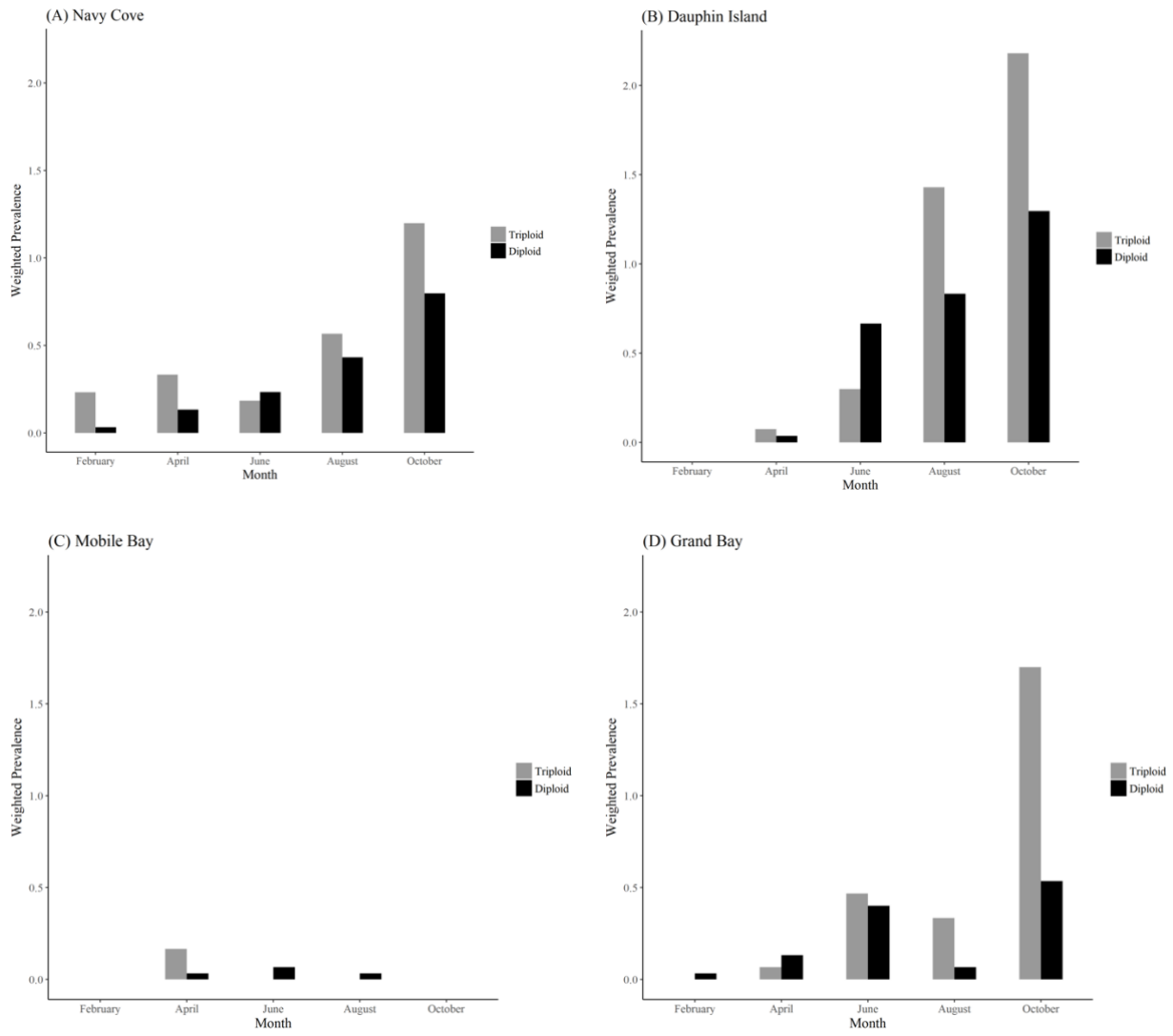

Figure 11: Weighted prevalence of *P. marinus* in triploid and diploid oysters using the RFTM tissue assay measured at five sampling points from February 2017 to October 2017 at four experimental sites (NC) Navy Cove, (DI) Dauphin Island, (MB) Mobile Bay and (GB) Grand Bay. Mobile Bay was not included in October sampling due to high mortality.

Observations of Growth, Mortality and P. marinus infection at Commercial Farm Sites

The oysters at four commercial farm sites experienced varying mortality and growth rates throughout the summer (Table 8). Pensacola Bay Oyster farm grew at an average of 0.1 mm/day from June to July and experienced the highest mortality rate of the four farms, reaching 100% in July. The Louisiana Sea Grant oysters at Navy Cove had the second highest mortality by August, 91% and no growth was observed. Auburn University oysters at Navy Cove survived better in August; however, mortality was still exceptionally high (69%) and growth was an average of 0.1 mm/day from May to October. Oyster Mom oysters had the highest survival out of the triploid groups with an average mortality of 19% from May to October and growth of 0.1 mm/day from May to August. At the Triple N Oyster farm, diploid oysters had a lower average mortality rate (9%) than triploid oysters (50%) from May to October and twice the growth rate (0.2 mm/day compared to 0.1 mm/day respectively).

The presence and intensity of *P. marinus* at the commercial farms were consistent with the results from the experimental sites (Table 9). Throughout the summer, the infection intensity was moderate or heavy at all commercial farms, with the exception of Louisiana Sea Grant seed at Navy Cove and diploid oysters at the Triple N Oyster Farm in May (2.0, 1.9 Log^{10} parasites g^{-1} wet tissue respectively). The commercial farms, OysterMom and Triple N Oyster Farm, had the heaviest Dermo infections across the three sampling points, with an average of 5.6 and 4.9 Log^{10} parasites g^{-1} wet tissue respectively.

Table 8: Average interval mortality ([no. dead / no. total]*100) and growth rate (mm/day) observed at four commercial farms across three sampling periods throughout the summer. PB = Pensacola Bay Oyster Farm, NC-LSG = Navy Cove Oysters, Louisiana Sea Grant seed, NC-AU = Navy Cove Oysters, Auburn University seed, OM = OysterMom, TN = Triple N Oyster Farm. Initial (May 2017) mortality estimates for Triple N Oyster farm were unavailable.

		Interval Mortality			Growth (mm/day)		
Site	Ploidy	May	August	October	Initial SH (mm)	August	October
NC-LSG	Triploid	34.6%	90.9%	--	66.0 (± 7.9)	-0.1	--
NC-AU	Triploid	8.3%	68.7%	46.7%	53.5 (± 6.8)	0.1	0.1
OM	Triploid	10.8%	11.0%	34.8%	56.5 (± 8.2)	0.1	0.1
PB	Triploid	1.4%	100%	--	64.6 (± 7.4)	0.1	--
TN	Triploid	--	41.9%	57.6%	78.9 (± 6.2)	0.1	0.0
TN	Diploid	--	5.9%	12.5%	48.3 (± 4.4)	0.2	0.2

Table 9: Average *P. marinus* body burden (Log^{10} parasites g^{-1} wet tissue) and infection intensity observed at four commercial farms across three sampling periods throughout the summer. Infection intensity was classified as follows, light: $<10^4$ parasites g^{-1} wet tissue, moderate: $1x\ 10^4$ to 5×10^5 parasites g^{-1} wet tissue, heavy: $> 5 \times 10^5$ parasites g^{-1} wet tissue. PB = Pensacola Bay Oyster Farm, NC-LSG = Navy Cove Oysters, Louisiana Sea Grant seed, NC-AU = Navy Cove Oysters, Auburn University seed, OM = OysterMom, TN = Triple N Oyster Farm.

		Body Burden (Log^{10} parasites g^{-1}) & Infection Intensity		
Site	Ploidy	May	August	October
NC-LSG	Triploid	5.6 Moderate	5.2 Moderate	--
NC-AU	Triploid	2.0 Light	4.8 Moderate	5.9 Heavy
OM	Triploid	5.3 Moderate	6.2 Heavy	5.3 Moderate
PB	Triploid	4.3 Moderate	--	--
TN	Triploid	5.4 Moderate	5.9 Heavy	5.5 Moderate
TN	Diploid	1.9 Light	4.5 Moderate	6.0 Heavy

Discussion

As expected (Wadsworth et al., In Prep.), oysters at the experimental sites demonstrated a significant growth advantage of triploid oysters in all observed growth metrics (shell height, whole wet weight, dry tissue weight and dry shell weight), relative to paired diploids. At the end of the 11-month field trial (16-17 months post fertilization for triploids/diploids), the triploid advantage in shell height was highest at Grand Bay (19.8%) and lower at Dauphin Island (6.9%) and Navy Cove (4.5%). The results at Grand Bay was consistent with a past study in Grand Bay (referred to as Sandy Bay) by Walton et al. (2013), that reported a triploid advantage of 16.7% after 17 months post fertilization. The minimal triploid advantage at Dauphin Island and Navy Cove could be related to the high cumulative mortality experienced at the sites compared to Grand Bay. Growth in shell height of triploid oysters has been observed similar to or less than diploid oysters under stressful environmental conditions (Callum 2013). Overall, the majority of oysters surpassed market size (≥ 75 mm) by October, with the exception of Grand Bay diploids and oysters at Mobile Bay. This is consistent with the literature, as oysters along the northern Gulf typically reach 60 mm within a year (Casas et al. 2015) and market size (≥ 75 mm) in 15-18 months post-fertilization (Walton et al. 2013).

Both ploidies followed the same trend in condition index, increasing during the winter (December to February) and decreasing from the spring to summer (April to August). This pattern is expected for diploids as they undergo gonad maturation and spawning (Normand et al. 2008). Triploids, on average, had a significantly higher condition index than diploid oysters at the peak of the summer, August, but an overall decline from June to October. Furthermore, the dry tissue weight in triploids reflects the condition index, with significantly greater dry tissue weight in august and a slight decrease between June and August at Navy Cove (from 1.79 g to

1.61 g) and Grand Bay (from 2.32 g to 2.00 g). This is representative of the slower reduction in glycogen common in triploid oysters, and related to retarded gametogenesis (Allen & Downing 1986). Mobile Bay diploid oysters had a higher condition index than triploids during the summer and suggests that the diploid oysters were stressed and likely allocated energy for somatic growth instead of gametogenesis (Davis 1994).

Despite the advantages in growth and condition index, at three of the four sites, triploid oysters had significantly higher mortality than diploid oysters and suggests a clear difference in vulnerability to stressors between ploidy. At the affected sites, mortality rates in triploid oysters appeared to be the highest during the summer, with significant spikes in mortality between June and August at Navy Cove and Mobile Bay. This summer mortality event was also observed in triploids at some of the commercial farm sites, specifically at Pensacola Bay Oyster Co., Navy Cove Oyster farm and Triple N Oyster Farm. There are a number of potential stressors that could have influenced the increased summer mortality including salinity, temperature, food supply, flow rate, disease presence, gametogenesis and age of the oyster. While any combination of these stressors could have increased mortality, this study will only discuss the influence of age of the oyster, temperature, salinity, and presence of Dermo.

In the warmer waters of the Gulf of Mexico, oysters that are spawned in the early summer (May/June) will be market size by the following summer, just as the environmental conditions (temperature, salinity, dissolved oxygen) begin to reach extremes. As a result, the mortality rate at these sites could be dependent on when the oysters reach market size. Older and larger oysters have been found to be more susceptible to mortality events since the 1940's in a study in Japan (Koganezawa 1974 as cited in Cheney et al. 2000) and since the late 1950's on the west coast of the US (Glude 1975). It is, therefore, possible that oysters spawned in the fall or

early winter could better overcome these summer mortality events. This would not explain the differences in mortality rates between the oysters at the experimental sites as all the oysters were the same age. However, at the Navy Cove Oysters farm, the triploids spawned in May, 2016 (LSG line) had an average of 26.3% and 22.2% greater mortality than the triploids spawned in August, 2016 (AU line) at the May and August sampling points respectively.

Oysters are able to withstand large variations in both temperature and salinity by closing their shell and converting to anaerobic metabolism. Studies on *C. virginica* found that the oysters can survive long periods of low salinity when the temperature is also low (La Peyre et al. 2009, Munroe et al. 2013) because the oysters are able to maintain a "sparing hypometabolic state" (Heilmayer et al. 2008). However, when salinity is low and temperature is high, it causes a buildup of carbon dioxide in the tissue which eventually leads to death (La Peyre et al. 2009, Munroe et al. 2013, Lombardi et al. 2013).

In the summer of 2017, there was an exceptional amount of rainfall along coastal Alabama and the Florida Panhandle leading to drop in salinity in coastal waters. According to the National Weather Service, Mobile, AL received 14.36 inches of rain during the month of June, only 2 inches less than the 2004 record while Pensacola, AL received 20.73 inches in June and surpassed the 2012 record by 2 inches (w2.weather.gov, accessed 12/5/17). This prolonged freshwater event and corresponding low salinity, in combination with high water temperatures, likely had an influence on mortality, especially on triploids. The site with the greatest number of days below 5 ppt and above 25 ºC (Mobile Bay, 52 days) also had the highest cumulative mortality in triploid and diploid oysters (151% and 66% respectively). However, salinity does not fully explain the high triploid mortality (138%) at Navy Cove compared to Grand Bay (35% cumulative mortality) as both Navy Cove and Grand Bay had similar salinity profiles with 13

days of salinity below 5 ppt and temperatures above 25 ºC. Diploid oyster mortality also did not follow the salinity hypothesis as Dauphin Island had the second highest diploid cumulative mortality (36%) after Mobile Bay diploids and no days with salinity less than 5 ppt.

Mortality events in triploid oysters during the summer were also observed at some of the commercial farm sites, Pensacola Bay Oyster Co. and Navy Cove Oyster farm (LSG triploids) at rates of 91-100% mortality. At all sites experiencing this high triploid mortality, experimental and commercial, the majority of the mortality occurred over a short period of time (a few weeks in early July). Degremont et al. (2010) noted in laboratory and field experiments that the significant summer mortality events in France related to heat stress typically occurs within a week, with 80% of the cumulative mortality occurring in four days. While the circumstances leading to mass mortalities in this study are vastly different, there is reason to believe as Degremont et al (2010) did, that the sudden spike in mortality is influenced by pathogens more than environmental conditions alone.

The prevalence and intensity of Dermo (*P. marinus*) was consistent with past reports for the Alabama coast. Casas et al. (2017) found infection intensity at Grand Bay (referred to as Sandy Bay) to be 3.7 Log_{10} parasites g^{-1} wet tissue in July and increased to 4.5 Log_{10} parasites g^{-1} wet tissue in September. This study found the mean infection intensity (in Log_{10} parasites g^{-1} wet tissue) at Grand Bay to be 5.1 and 3.7 in June, and 4.4 and 2.4 in August, and 5.9 and 4.8 in October for triploids and diploids respectively. The decrease in intensity from June to August is likely a result of a freshwater event from May 29th to July 30th where the salinity at Grand Bay was below 15ppt, the lower limit for optimum *P. marinus* proliferation. Dauphin Island, on the other hand, only experienced 10 days (July 1st to July 10th) where the salinity was below 15 ppt and the infection intensity increased as a result. The moderate to heavy infection intensity from

June to October at Dauphin Island compared to the other sites likely influenced the high mortality rate observed later in the summer: 20% mortality in triploids from August to September and an additional 30% mortality from September to October (Fig. 10). Between ploidy, there was a significantly greater infection intensity in triploids which is inconsistent with past studies. Callum (2013) found increased resistance to Dermo, while Barber & Mann (1991) and Degremont et al. (2012) observed a similar prevalence of Dermo in diploid and triploids oysters during the summer. However, Barber & Mann (1991) noted similar cumulative mortality across ploidy while Degremont et al. (2012) found increased survival in triploids.

The differences between the *P. marinus* infection intensity determined through RFTM tissue assay and ARFTM body burden assay highlights the sensitivity of the body burden assay and limitations of the tissue assay. At all three sampling times during the summer (June, August, October), oysters at Dauphin Island were classified with a light to moderate by the tissue assay method and classified as moderate to heavy with the body burden assay. The body burden assay also identified small infections in Mobile Bay triploids that would otherwise have been reported as negative. The differences between assays has been noted previously in the literature. Bushek et al. (1994) suggests that the tissue assay should be used for monitoring seasonal patterns in Dermo intensity, while the body burden assay should be used for quantitative comparisons.

Despite the significantly faster growth in triploid oysters across the four sites the significantly higher mortality brings to light the weaknesses of triploid oysters under less than ideal environmental conditions. Developing improved genetic lines with greater tolerance to low salinity and high temperatures could be the solution. Callum (2013) tested several diploid "superlines" bred for salinity tolerance that preformed as well as, or better than triploid oysters at low salinity sites. Additionally, Degremont et al. (2010) compared diploid lines selected for

resistance to summer mortality to triploid lines in which the diploid parent had been selected for resistance. While both selected lines in the study had a positive response to survival, the selected diploid line still survived better than the selected triploid line. Improvements in triploid selection, therefore, need to involve both parents (diploid and tetraploid) selected for resistance.

Chapter 3: Summary for industry

Background

In the Gulf of Mexico, oyster farmers have a number of options available when purchasing oyster seed including size, genetic line, and ploidy (triploid or diploid). Triploid oysters have three sets of chromosomes instead of two sets, as a diploid oyster would. The odd number of chromosome sets makes triploids generally sterile, as the chromosomes rarely successfully combine. Triploid sterility, among other traits, results in many benefits to oyster farmers over diploid oysters. For example, triploids can grow faster than diploids as the energy typically required for spawning is used for growth instead and triploids can have a premium meat quality during the summer as diploids typically have a thin, 'watery' appearance after spawning. Recent experimental work, however, suggests that growers should be aware of potential risks with triploid oysters and understand what can be done to minimize those risks.

Advantages

One of the main reasons that oyster farmers opt for triploids is to have plump oysters in the middle of summer, and not risk selling 'spawned out', thin diploid oysters (Fig. 1). This is a clear benefit to producers. In addition to the summer meat condition, a number of growers choose to raise triploid oysters because they are reported to grow faster. From a survey of the scientific literature, it is clear that triploids *can*, and generally do, grow faster than diploids, both in terms of shell height and whole wet weight. Most commercially available triploid oyster seed are from mating tetraploid broodstock with diploid broodstock and research suggests that these 'mated triploids' perform better than chemically-induced diploids (Fig. 2). As might be expected, the increase in whole wet weight seen in mated triploids (compared to diploids) differed across oyster species and how long the oysters were grown. Interestingly, the growth benefits in shell

height seen in triploids (relative to diploids) were comparable across a range of studies, suggesting that oyster farmers can see the growth advantage of triploids at all stages of production and regardless of the size of the farm or production cycle.

Figure 1: Optimum meat quality and fullness in triploid oysters in July, 2017 at Navy Cove Oyster Farm.

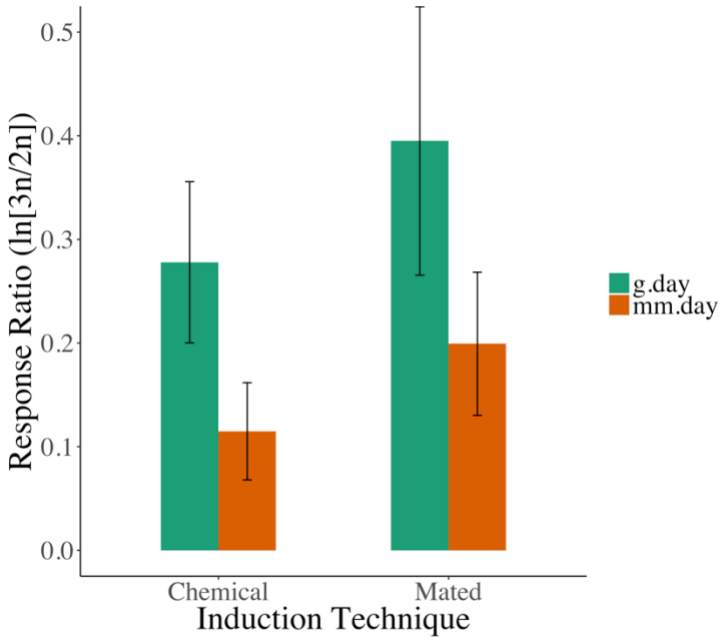

Figure 2: Mean response ratios [ln (triploid/diploid growth)] and 95% confidence interval for 121 experiments from 30 studies using chemically induced triploids and mated triploids, and measuring growth rate in g/day and mm/day. All mean response ratios were significantly different ($p < 0.05$).

Concerns

Despite the potential benefits of summer meat conditions and higher growth rates, recent experimental work has suggested that triploids appear to be more vulnerable to summer mortality (Fig. 3). The results of this study found that triploid oysters in coastal Alabama died at a faster rate than diploid oysters at a number of sites, and the majority of this mortality occurred in the summer (Fig. 4). Mortality appeared to be triggered by a number of stressors, including high water temperature, low salinity and possibly disease, such as Dermo (*Perkinsus marinsus*). The age of the oysters also could have played a role in mortality; a group of oysters reaching market

size during the most extreme summer conditions had higher mortality than the oysters that reached market towards the end of the summer or early fall.

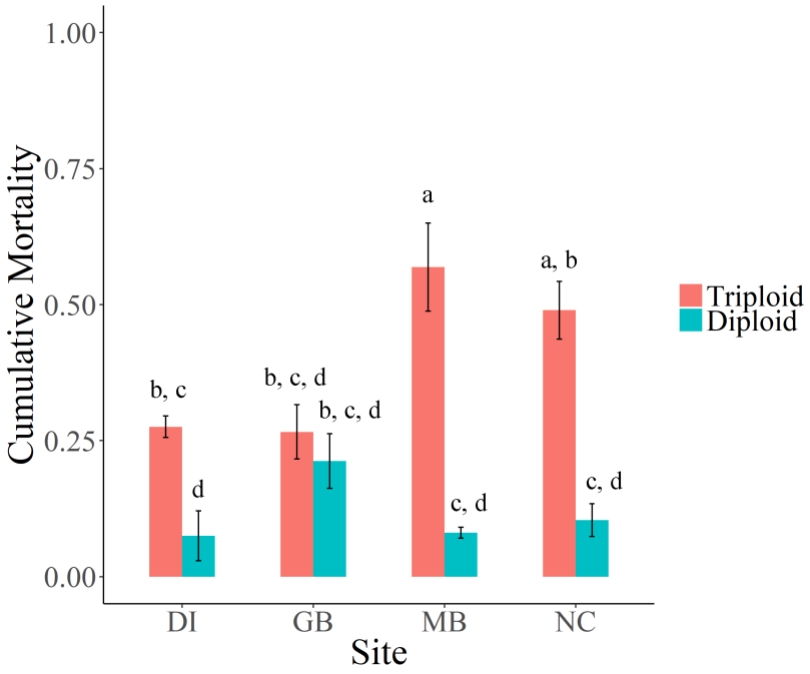

Figure 3: Cumulative mortality in July at the four experimental sites: Dauphin Island (DI), Grand Bay (GB), Mobile Bay (MB) and Navy Cove (NC). Error bars represent standard error. Different letters indicate a significant difference ($p < 0.05$).

Figure 4: Moribund oysters with gaping valves taken after the July mortality event.

Precautions and Recommendations

Given these observations, what are reasonable precautions for oyster farmers to take?

- First, oyster farmers should be aware that summer may be a particularly vulnerable time of the year for triploids, and strive to reduce stress when handling their oysters. This includes reducing the time out of the water, reducing tumbling, and maintaining optimum stocking density.
- Second, farmers may want to include a crop of diploids in the farm production to reduce the reliance on triploids. This is especially important in areas with a greater potential for stressors that an oyster farmer cannot control, such as prolonged periods of low salinity.
- Third, farmers may want to consider harvesting triploids prior to the onset of summer. While this reduces the risk of raising triploids in the summer, it inherently loses the benefit of having triploid oysters available for summer harvest and sale.
- Finally, farmers should work with their seed suppliers to explore different genetic lines of triploid oysters, selected for improved survival under different environmental stressors.

In summary, triploid oysters offer a number of potential advantages to oyster farmers in the Gulf of Mexico and elsewhere, but the risk for increased mortality needs to be understood by oyster farmers. These risks, once understood, can be minimized, allowing regional oyster farmers to have productive and profitable oyster farms.

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