Title: A field test investigating the influence of brood stock origin and ploidy on the susceptibility of *Crassostrea virginica* to "triploid mortality" in the Chesapeake Bay

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Abstract

Mass mortalities of cultured triploid *Crassostrea virginica* in late spring, or "triploid mortality," have been reported on farms in the Chesapeake Bay since 2012. Typical causes, such as disease or poor husbandry, were not responsible, and mortalities occurred without clear signs of biological or physical stressors. Previous comparisons of the effects of genetic origin on triploid mortality have been uncontrolled, initiating this investigation of the effect of brood stock source and ploidy on triploid mortality. Four triploid and four diploid crosses, produced by crossing different combinations of brood stock of Virginia, Louisiana, and Maine origin in early 2015, were tested at four commercial oyster farms in Virginia throughout 2016. From February to November, oysters from all crosses and sites were regularly sampled, and growth (shell height), condition (meat weight), and pathology were monitored, as were environmental conditions. Compared to diploids made from Virginia brood stock, diploids with

Maine genetic origin had high mortality and Virginia-Maine diploid hybrids exhibited mid-parent heterosis. A triploid mortality event occurred in late spring at only one site and only affected the triploid crosses. Evidence for substantial disease pressure from *Haplosporidium nelsoni* or *Perkinsus marinus* or of especially stressful environmental conditions based on temperature, salinity, pH, and dissolved oxygen was absent during the triploid mortality event. At 18 months, shell height was similar in the diploids and triploids with the most similar genetic origin. Triploids maintained meat weight through the summer, while meat weight in diploids dropped sharply. Triploids may be especially susceptible to late spring mortality events in the Chesapeake Bay, which justifies their classification as "triploid mortality," and warrants further investigation on traits in triploids that may affect susceptibility.

Keywords: oyster; polyploid; 3n; summer mortality; gametogenesis

1. Introduction

Over the last two decades, the once prolific harvests for the eastern oyster, *Crassostrea virginica*, have been partially restored in the Chesapeake Bay from hatchery-based aquaculture. Pivotal to the rise in hatchery-based aquaculture has been selective breeding, which has made disease resistant and genetically improved oysters available (Dégremont et al., 2015; Frank-Lawale et al., 2014; Ragone Calvo et al., 2003). Production of *C. virginica* from aquaculture in the state of Virginia has grown rapidly in the last fifteen years. Seed production increased from 20 million to 264 million (13-fold) from 2005 to 2016, while the number of cultured oysters sold increased 40-fold (Hudson, 2017; Murray and Oesterling, 2006). Virginia now leads the East Coast of United States in aquaculture production of eastern oysters (Hudson, 2018).

An important genetic improvement in hatchery-based aquaculture in Virginia was the commercialization of triploid oysters. Over the last ten years, oyster aquaculture in Virginia has been

characterized by a near exclusive use of mated triploid oysters, which are triploids produced by crossing tetraploid oysters to diploid oysters. Triploid oysters made up 80 to 95% of oyster crops on Virginia farms between 2009-2017, and in 2015 and 2016, 94% of seed sold from Virginia hatcheries were triploid (Hudson, 2018, 2017; Hudson and Murray, 2016, 2015, 2014; Murray and Hudson, 2013, 2012, 2011; Murray and Oesterling, 2010, 2009). The Virginia industry reports a preference for triploids because they grow faster than diploids and maintain high meat quality during the spawning season (Hudson, 2018). These advantages obtain from the reduced fecundity of triploid oysters, which alters the energy allocation in favor of somatic growth (Purdom, 1972; Stanley et al., 1981). Lack of spawning also improves market condition (Allen and Downing, 1986).

The apparent advantages of growing triploid oysters in the Chesapeake Bay may come with at least one disadvantage, for example, so called "triploid mortality" (Guévélou et al., 2019). Since at least 2012, a number of oyster farms in the Chesapeake Bay have experienced mortality episodes, in which substantial mortalities (> 20% of the crop) occur within a matter of weeks in the late spring, typically between May and July (Guévélou et al., 2019). An acute incidence of triploid mortality in 2014 led to losses as high as 50-80% on some farms (K. Hudson, pers. comm., Guévélou et al. 2019). Farmers reported that oysters growing fastest and close to market size were susceptible. No pathogen was identified in association with these mortalities, nor have any unusual environmental conditions been consistently associated with the events, which made these mortalities unusual for oyster farmers in the Chesapeake Bay (Guévélou et al., 2019). Reports of acute triploid mortality from several farms in Virginia have also been received in 2019, ranging from 30% to 70% loss of crop (K. Hudson, pers. comm.). Unusual mortality episodes involving triploids have also been reported from oyster farms in Maryland, as well as Alabama and Louisiana since 2016 (Wadsworth et al., 2019).

Often, mass mortality events of oysters involve a pathogen, such as a protozoan parasite (e.g. *Perkinsus* spp., *Haplospiridium* spp., *Bonamia* spp.), bacterium (e.g. *Vibrio* spp.), or virus (e.g. OsHV-1).

Of most recent importance in terms of global oyster aquaculture are mortalities caused from a certain genotype of Ostreid herpesvirus type I (OsHV-1), OsHV-1 µVar (Segarra et al., 2010). The µVar genotype was first identified following widespread mortalities on farms in France in the summer of 2008 and is now associated with mortalities during summer in other western European countries (Peeler et al., 2012), New Zealand (Keeling et al., 2014), and Australia (Paul-Pont, 2014). The severe economic impact of these mass mortalities has made resistance to OsHV-1 µVar a focus of applied research, including breeding research, which has demonstrated high potential for increased resistance through selection (Camara et al., 2017; Dégremont et al., 2015; Kube et al., 2018).

Mass mortalities of oysters have often occurred without an attributable pathogen or specific environmental stressor. Similar to *triploid* mortalities in Virginia, many cases of the long-reported *summer* mortalities of *Crassostrea gigas* involve only adult oysters and are not ascribed to a specific pathogen or environmental condition (Cotter et al., 2010; Glude, 1975; Koganezawa, 1975; Maurer and Comps, 1986; Samain and McCombie, 2008; Wendling and Wegner, 2013). Since the 1940s, possibly even 1912 (Takeuchi et al., 1960), episodes of mortalities of cultured *C. gigas* ranging from 10% up to 70% have been reported in summer in Japan (Koganezawa, 1975; Ventilla, 1984). Similar mortalities have been reported on the West Coast of the United States starting in the 1950s (Cheney, 2000; Glude, 1975; Perdue et al., 1981) and France in the 1970s (Goulletquer et al., 1998; Parache, 1989; Samain and McCombie, 2008). The conclusion in Koganezawa (1975) that summer mortalities were due to a physiological "disorder" induced by a suite of environmental conditions has been supported from follow up research on another continent (e.g. Samain and McCombie, 2008).

In Virginia, nearly all the farms affected by triploid mortality in 2014 were on the Chesapeake Bay-side of Eastern Shore of Virginia, and many farmers in the area were growing the "Northern cross" (Guévélou et al., 2019). The Northern cross was produced by mating tetraploids selectively bred from mid-Atlantic stocks with a proprietary line of diploids developed from New England stocks. Thus, a

hypothesis was developed that genes passed to the triploid by the "northern diploid" may contribute to late spring mortality in triploids. The initial investigation into triploid mortality in the Chesapeake Bay by Guévélou et al. (2019) included a version of this "Northern cross," but the opportunistic nature of the field trial meant certain variables, like genetic provenance of the tested groups, could not be controlled.

This study is intended to be a more controlled complement to Guévélou et al. (2019) and shares many of the same objectives: testing whether genetic origin affects triploid mortality, measuring the survival of diploids during a triploid mortality event, examining the relationship between size and mortality, and investigating associations between triploid mortality and environmental conditions. Unique to this study are tetraploid brood stock of varying genetic origin. Considering tetraploids contribute two thirds of the chromosomes to the triploid genome, the genetics of the tetraploid parent may be especially influential of the triploid phenotype. Four genetically distinct diploid and triploid crosses were produced and deployed in a field test from February 2016 to November 2016. Triploid crosses were designed to produce a range of genotypes – origins in Virginia, Louisiana, and Maine – that would putatively affect susceptibility to triploid mortality. The diploid crosses comprised those of Virginia origin, which were produced for comparison with the triploids, as well as those of Maine origin, which were produced to further examine the performance of New England derived genotypes in the Chesapeake Bay. Special care was taken to mimic growing conditions in which previous triploid mortality events had been observed, specifically, early season spawns that yielded market sized adults at the cusp of the triploid mortality window 16-18 months after spawning (May – June).

2. Methods

2.1 Brood stock

Two lines of diploid and two lines of tetraploid *C. virginica* were used to produce diploid and triploid crosses (Figure 1). The diploid brood stock consisted of the Virginia Institute of Marine Science

(VIMS) DEBY line (Ragone Calvo et al., 2003) and a proprietary commercial line from Mook SeaFarms, Walpole, Maine. The tetraploid brood stock consisted of VIMS GEN and VIMS VBOY. The GEN line has been the principal source of tetraploids for the vast majority of commercial triploid production in the Chesapeake and has been propagated by the Aquaculture Genetics and Breeding Technology Center (ABC) since 2003. The VBOY line originated from the creation of a tetraploid line for the Oyster Research Lab of Louisiana State University (LSU). VBOY was made as a hybrid between a diploid line (OBOY, developed by Jerome LaPeyre, LSU) from Louisiana and the GEN line developed in Virginia. VBOY has also been held by ABC since 2013.

2.2 Crosses

All crosses were spawned simultaneously at the VIMS research hatchery in Gloucester Point, VA in February of 2015. Male tetraploids from the Virginia GEN line (chromosome set contribution: VV) and VBOY line with some Louisiana origin (chromosome set contribution: LL) were crossed to female diploids from the ABC DEBY line (chromosome set contribution: V) or the Mook line from Maine (chromosome set contribution: M) in a 2x2 matrix to produce triploid crosses (VVV, VVM, LLV, LLM). Eggs from the same V and M female diploids were also crossed to V and M male diploids in a 2x2 matrix to produce a diploid control group to gauge triploid mortality (VV), reciprocal Virginia-Maine hybrids (VM, MV), and Maine diploids (MM).

All crosses were conducted via strip spawning (Allen and Bushek, 1992). For each of the two diploid brood stocks (V & M), five to eight females were stripped and eggs were pooled. Each pool was then split into four aliquots. One aliquot of the egg pool from either V or M was allocated for each of the sperm sources (2N – V and M; 4N – VV and LL) (Figure 1). For each sperm source, five males were used, so each aliquot of diploid eggs was further subdivided five ways so that an individual male was fertilizing one fifth of the eggs from each egg source. After each subdivided batch of eggs had been allowed to complete fertilization, they were re-pooled into the eight major crosses depicted in Figure 1.

The early (February) spawn in 2015 was necessary to emulate the commercial experience of farms that had seen triploid mortality in near market size oysters in late spring.

2.3 Larvae and Seed

Larvae were reared at two hatcheries to ensure success in producing pediveliger larvae: the VIMS research hatchery (Gloucester Point, VA, USA) and a commercial hatchery, Oyster Seed Holdings (Gwynn's Island, VA, USA). Half of the embryos from each cross were transferred directly into tanks at the VIMS hatchery, and half were transported in a 50-milliliter centrifuge tube to the commercial hatchery, 50 kilometers away. Pediveliger larvae were successfully produced for all crosses at both hatcheries. Competent pediveligers were collected from each cross from both hatcheries were set on finely crushed oyster shell at the VIMS hatchery.

Several times throughout the spring, seed were graded to ensure fast growth to promote the earliest possible deployment to the field, which emulates the commercial process that gave rise to triploid mortality. By June of 2015, when enough seed were larger than 19 mm, 10-15 haphazardly selected individuals from each cross were measured for shell height (maximum dimension from the hinge to the bill), and a few thousand seed from each cross were deployed to four experimental field sites. At all sites, seed were reared as a bulk deployment in the intertidal zone in bags nested within single tier cages.

2.4 Sites and Experimental Deployment

All four experimental sites were commercial oyster farms. Three farms were on the bayside of the Eastern Shore of Virginia and one was on the western side of the Chesapeake Bay. For the three eastern sites – Nandua Creek (ND), Pungoteague (PG), Occohannock Creek (OC) – triploid mortality had been observed. For the western site – Rappahannock River (RR) – no such mortalities have been observed (Figure 2). Deployment of the experiment took place between February 29 and March 3 of 2016, in which oysters from all eight crosses were split into replicate bags at each site. From each cross

at each site, 450 oysters were haphazardly selected and equally split into 3 bags (150 x 3). At RR, ND, and OC, oysters were reared in single-tier cages in the subtidal zone, and each bag was randomly assigned a slot within the cages. Bags and cages were held in their assigned position throughout the experiment. For PG, oysters were reared in triple-tier cages in the subtidal zone. Bags and cages were rotated during the trial, but cages remained in the same area during the experiment.

2.5 Ploidy Verification

Oysters were sampled twice to verify ploidy via flow cytometry (FCM) (Allen and Bushek, 1992). All FCM measurements were made with a Sysmex-Partex Cyflow Space flow cytometer (Partec GmbH, Münster, Germany) using DAPI as a stain. The first sample occurred in April of 2015 when 25 two-month-old seed from each cross were haphazardly selected. The second ploidy verification was repeated at deployment (February/March 2016) by sampling 15 haphazardly selected individuals of each cross from each site.

2.6 Site Visits and Sampling

All sites were visited throughout the spring, summer, and fall of 2016. Sites were visited within a week of each other monthly except in May and June when they were visited twice a month. Live oysters were sampled, without replacement, at experimental deployment and during all site visits in the spring and summer of 2016 (April-August). For all sampling times after deployment, oysters were randomly sampled. Random sampling consisted of ordering oysters in piles of 10, selecting a pile with a random number generator, ordering the oysters from the pile, and then selecting the individual oysters with a random number generator. Five oysters from each bag were selected in April, July, and August. In May and June, five oysters were selected from each bag of diploids, and seven were selected from each bag of triploids. Shell height and meat weight were measured for all live samples. Meat weight was measured after shucking oysters, removing the somatic tissue from the shell, and letting the meat drain on a mesh net for a few minutes.

193 **2.7 Mortality**

194 Mortality within bags was assessed during each site visit. Live oysters and empty shells were
195 counted from each bag and empty shells removed. Oysters that failed to seal their shell commissure
196 were deemed moribund and were also counted and removed. For the first mortality assessment in
197 April, the percent cumulative mortality per bag was calculated with the following equation:

198 $$\text{Cumulative mortality} = \frac{\text{Live}}{\text{Deployed}}$$

199 Where Live is the number of live oysters counted and Deployed is the sum of dead, live, and moribund
200 oysters. For all subsequent sampling times, cumulative mortality per bag was calculated with the
201 following equation:

202 $$\text{Cumulative mortality}_t = \frac{\text{Live}_t}{\text{Live}_{t-1} - \text{Samples Removed}_{t-1}} \text{ x Cumulative mortality}_{t-1}$$

203 where t indicates sampling time and Samples Removed are the number of live oysters sampled without
204 replacement. The number deployed, assessed during counts in April, was uniform for most replicates,
205 ranging from 146 to 155 oysters per bag. Two bags out of the 96 deployed had 171 and 198 oysters
206 instead of 150. Mean cumulative mortality for each cross was calculated by averaging the cumulative
207 mortalities among the three bags. Shell height was measured for empty shells and moribund oysters.

208 Mid-parent heterosis was the phenotypic difference between the mean of the parental crosses
209 and the mean of the hybrids, defined as heterosis by Falconer (1981), and was measured for mortality
210 between the Virginia and Maine diploids and the Virginia-Maine hybrids with the following equation:

211 $$\text{H} = \frac{(\text{VV} + \text{MM})}{2} - \frac{(\text{VM} + \text{MV})}{2}$$

212 where VV, MM, VM, and MV refer to the mortality of the corresponding crosses.

213

214 **2.8 Histology and Pathology**

Live samples were preserved for histology. For all live samples, the excised sample (slab) consisted of a 4 mm section of tissue cut perpendicular to the anterior –posterior axis, slightly ventral of the labial palps. Slabs were fixed in Davidson's solution for 48 hours, then stored in 70% ethanol. Some fixed samples were selected *post-hoc* for pathology based on mortality results and were processed for histology by standard methods used at the VIMS Shellfish Pathology Laboratory (Carnegie and Burreson, 2011).

Infections were described by prevalence (percent of infected individuals) and weighted prevalence, which is based on the intensities of the infections. Intensities of *Haplosporidium nelsoni* infections were rated according to Carnegie and Burreson (2011), and intensities of *Perkinsus marinus* infections were rated according to Mann et al. (2014). Weighted prevalence (WP) was calculated with the following equation:

$$WP=\frac{5(\text{Heavy}) + 4(\text{Moderate to Heavy}) + 3(\text{Moderate}) + 2(\text{Light to Moderate}) + 1(\text{Rare or Light}) + 0(\text{Absent})}{\text{Number of samples}}$$

where Heavy, Moderate to Heavy, Moderate, Light to Moderate, and Rare or Light represent the number of oysters with these qualitative ratings of infection intensity. All pathology, via visual examination of histology slides, was completed by the VIMS Shellfish Pathology Laboratory.

2.9 Environmental Conditions

Temperature and salinity were monitored at each field site during the experiment by HOBO® conductivity logger (Onset Computer Corporation, Bourne, MA, USA) attached to a cage in the field trial at each site from January 1 to August 10, 2016. Some mechanical failure of the loggers produced gaps in the data. Calibration readings for conductivity were taken with a portable conductivity meter during each site visit, and conductivity data were converted to salinity in parts per thousand (ppt) using HOBOware Conductivity Assistant (Onset Computer Corporation, Bourne, MA, USA). For RR, salinity

data was taken from daily recordings at a nearby VIMS research hatchery on Locklies Creek (VA, USA). Average daily values were calculated for temperature and salinity at each site.

Conductivity, dissolved oxygen concentration, pH, chlorophyll *a* concentration, and turbidity were monitored at ND and PG during the time triploid mortality was expected (May-July). Measurements were taken in 15-minute intervals using the 6600 V2-4 Multi-Parameter Water Quality Sonde (YSI Inc. / Xylem Inc., Yellow Springs, OH), between May 10 and July 12. In cases where data from the conductivity logger and sonde were available at ND or PG, sonde data was reported.

2.10 Statistical Analysis

Mortality was analyzed using a mixed effects logistic regression. To compare "triploid mortality," the following model was used for mortality from deployment to the end of spring (February 29-June 28):

$$\text{logit}\,(\pi_i) = \log(\frac{\pi_i}{1-\pi_i}) = \mu + \alpha_i + \beta_j + \alpha_i\beta_j + \gamma_{k(i,j)} + \varepsilon_{ijk}$$

where survival is a binary response variable (live or dead), μ is the mean probability of survival, α is the effect of cross (i is VV, VVV, VVM, LLV, and LLM), β is the effect of site (j is RR, ND, PG, and OC), $\alpha\beta$ is the interaction of cross and site, γ is the effect of bag nested within cross and site, and $\varepsilon_{ijk}$is the residual error. The mortality of the Virginia diploids, Virginia-Maine hybrids, and Maine diploids was analyzed with the same model except the data were from deployment to the end of the field trial (Februrary 29-November 16) and i was VV, VM, MV, and MM. For both models, cross and site were fixed effects and bag was a random effect. Pair-wise differences were examined post-hoc using Tukey's Honest Significant Difference (HSD) test. Mixed effects logistic regression was performed using the lme4 package in R (Bates et al., 2015).

Shell height in August of 2016 was analyzed using a two-way ANOVA:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i\beta_j + \varepsilon_{ijk}$$

where Y_{ijk} is shell height of individual oysters in August 2016, μ is the overall mean, α is the effect of cross (i is VV, VM, MV,MM, VVV, VVM, LLV, and LLM), β is the effect of site (j is RR, ND, PG, and OC), $\alpha\beta$ is the interaction of cross and site, and $\varepsilon_{ijk}$is the residual error. Pair-wise differences were examined post-hoc using Tukey's HSD test. To determine if there was a difference in size among surviving triploids and triploids that died during the mortality event, shell heights of live triploids and dead triploids were analyzed using a nested ANOVA:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{k(j)} + \alpha_i\beta_j + \varepsilon_{ijkl}$$

where Y_{ijk} is height of shells of live and dead triploids measured on June 6, 2016 at ND, μ is the overall mean, $\alpha$is the effect of status (i is live or dead), β is the effect of cross (*j* is VVV, VVM, LLV, LLM), γ is the effect of bag nested within cross (*k* is replicate 1, 2, 3), $\alpha_i\beta_j$ is the interaction between status and cross, and ε_{ijk} is the residual error.

Shell height data were visually assessed for normality and homogeneity of variance among factors, and residuals from models were visually examined for normality and homoscedasticity. For all tests, significance level = 0.05. All statistical analyses were performed in R (R Core Team, 2019).

3. Results

3.1 Ploidy

From flow cytometry, most samples from triploid crosses were verified triploid, and all samples from diploid crosses were verified diploid. In April of 2015, all oysters from triploid crosses were verified triploid (n=25) except VVM, where 24 of 25 were triploid and the other was diploid. In February/March 2016, all triploids were verified triploid (n=15) except for LLM at PG and LLM at RR, where 14 of 15 were triploid and the other was diploid.

3.2 Mortality

Mean cumulative mortality for crosses at all sites ranged from 6% to 76% by the end of the field trial (November 2016) (Figure 3). At all sites, MM had the highest and VV had the lowest cumulative

mortality. A large increase in cumulative mortality was observed in triploid crosses at ND between April 14 and June 7. During this time, cumulative mortality increased from 0% in all triploid crosses to 27% in VVV, 22% in VVM, and 17% in LLV (Figure 3). Increase in mortality for LLM was only 8%.

A significant interaction existed between site and cross during the window "triploid mortality" was expected (Table 1). At RR, PG, and OC, all crosses had a low probability of mortality (< 0.15) (Figure 4). At ND, the probability of mortality was 0.01 for VV, 0.29 for VVV, 0.27 for VVM, 0.20 for LLV, and 0.14 for LLM. The probability of mortality was significantly different between VV and the triploid crosses and was statistically indistinguishable among the triploid crosses (Figure 4).

There was a significant interaction between site and cross among the Virginia and Maine diploids (Table 1). Estimates of probability of mortality in Virginia and Maine diploids shared a similar pattern among sites (Figure 5). The VV cross always had the lowest probability of mortality (< 0.1) and was significantly different from other crosses within a site, with the only exception that VV and VM were statistically indistinguishable at OC. The hybrids (VM and MV) had a statistically indistinguishable probability of mortality within each site, and the probabilities for the hybrids ranged from 0.13 to 0.31. Maine diploids (MM) had the highest probability of mortality estimates, ranging from 0.46 to 0.76, which were significantly different from the other crosses within each site (Figure 5).

The Virginia-Maine hybrids exhibited mid-parent heterosis. In terms of cumulative mortality, heterosis accounted for 16% lower mortality at RR, 11% at ND, 5% at PG, and 20% at OC. Heterosis in probability of mortality accounted for 0.16 lower probability at RR, 0.11 at ND, 0.05 at PG, and 0.21 at OC.

3.3 Pathology

All triploids sampled on May 24 at ND were processed for histological analysis because of the spike in mortality in VVV, VVM, and LLV in late spring. Infection prevalence was low for *H. nelsoni* (≤

309 10%) in all three crosses and infection intensity in all but one oyster was light, with one light-to-
310 moderate from an LLM individual (Table 2). No infections of *P. marinus* or *Haplosporidium costale* were
311 detected, and no evidence of bacterial infections was observed in the cross section. Prevalence of
312 chlamydia ranged from 5% to 29% among crosses. Prevalence of hemocytosis was low (≤ 10%) with light
313 intensity (Table 2).

314 Samples of MM from ND and RR were processed for histological analysis because of high
315 mortality. MM individuals were selected from August, a period immediately preceding the largest
316 increase in cumulative mortality at these sites. No infections of *H. nelson* or *H. costale* were detected,
317 however, infections of *P. marinus* were common, with incidences of 66% from RR and 60% from ND. For
318 RR, intensities ranged from rare (90% of infections) to moderate-heavy (10% of infections). For ND,
319 intensities ranged from rare to heavy, with moderate infections most common (44%).

320 **3.4 Live vs. Dead Triploids at ND: Shell Height**

321 Data for shell height of live and dead triploids collected on June 7, 2016 at ND were normally
322 distributed and met the assumptions of homogeneity of variance among factors. Residuals from the
323 ANOVA were normally distributed and homoscedastic. The number of dead oysters in a bag ranged
324 from 3 to 26 (Table 3). The interaction of status (live or dead) and cross was significant ($p < 0.05$). For
325 only the LLV cross, median shell length of dead oysters (91 mm) differed by more than 5 mm than that in
326 live oysters (83 mm) (Figure 6).

327 **3.5 Shell Height**

328 Mean shell height of each cross ranged from 22 mm to 27 mm prior to field deployment in June
329 2015. In February/March at the start of the experiment, mean shell height of crosses at all sites ranged
330 from 44 mm to 82 mm (Figure 7). VVV had the greatest mean shell height at RR with 82 mm ± 2
331 (standard error) and ND with 79 mm ± 2, LLV had the greatest at PG with 63 mm ± 3, and LLM had the

greatest at OC with 74 mm ± 4. At all sites in February/March 2016, MM had the lowest mean shell height (RR: 60 mm ± 2), (ND: 57 mm ± 2), (PG: 44 mm ± 1), (OC: 58 mm ± 2).

In August of 2016, mean shell height among crosses at each site ranged from 61 mm to 91 mm, with LLM at ND having the greatest mean shell height and MM at PG having the lowest mean shell height (Figure 7). Shell height data for August 2016 was normally distributed and met the assumption of homogeneity of variance. The ANOVA revealed cross ($F=16.9$, $p< 0.05$) and site ($F=41.3$, $p< 0.05$) were significant, but the interaction was not significant ($F=1.5$, $p=0.07$) (Table 4). Least square means for shell height of each cross and each site were determined from the ANOVA (Table 4). Overall, LLM had the greatest mean shell height (84 mm), and MM had the smallest mean shell height (68 mm). For sites, oysters at RR had the greatest mean shell height (84 mm) and oysters at PG had the smallest mean shell height (71 mm).

Results from Tukey's HSD test for comparisons for RR minus other sites were 2 mm, 13 mm, and 6 mm for ND, PG, and OC, respectively. The 95% confidence intervals that did not contain zero were for RR vs. PG and RR vs. OC (Table 4).

3.6 Meat Weight

Overall, mean meat weight ranged from 1.1 g (MM, PG, March) to 12.4 g (LLM, ND, June) throughout the experiment (Figure 8). Variation in the range of meat weight was about the same and highest at RR (9.1 g) and ND (9.5 g), followed by OC (7.2 g), and lowest at PG (4.9 g).

In general, the difference in mean meat weight between diploids and triploids was greater in summer than spring (Figure 8). In early May, mean meat weight of VVV was greater than VV by 27% at RR, 15% at ND, 20% at OC, and 6% at PG. Mean meat weight substantially decreased in most of the diploid crosses from early May to August. Among all diploid crosses, mean meat weight decreased by 29% at RR, 36% at ND, 37% at OC, and 33% at PG. During the same time, mean meat weight among all triploid crosses at RR, ND, OC, and PG changed by +8%, -12%, +1%, and +27%, respectively. By August,

356 the mean meat weight of VVV was greater than of VV by 51% at RR, 80% at ND, 53% at OC and 24% at
357 PG.

358 **3.7 Environmental Conditions**

359 Average daily temperatures ranged from -1.7 °C during winter to 33.4 °C in the summer. No
360 major differences were observed in average daily temperature among the experimental sites from the
361 available data (Figure 9). Some comparisons among sites were not possible due to gaps in the data
362 (Figure 9). During the period of the spike in mortality at ND (May 10-June 27), average daily
363 temperatures were within 1°C at all sites (Table 5).

364 The mean of average daily salinities was within 1 ppt at ND, PG, and OC (Figure 10). Average
365 daily salinities ranged from 16.7 ppt to 19.5 ppt at ND, 16.3 ppt to 19.7 ppt at PG, and 16.4 ppt to 21.3
366 ppt at OC. Average daily salinity was lower for RR, ranging from 11.4 ppt to 16.3 ppt. A large decrease
367 in salinity from 16.3 ppt to 12.6 ppt was measured July 15 to July 16, caused by a rainfall event.

368 Dissolved oxygen concentration, pH, chlorophyll *a*, and turbidity monitored from May 10 to July
369 12 at ND and PG are reported in Appendix Figures A.1, A.2, A.3, and A.4. ND had the lowest average
370 daily concentration of dissolved oxygen in May but was never lower than 6 mg/L. Average daily pH
371 ranged from 7.7 to 8.3 at both sites and was lowest at ND on May 31 (7.7) and lowest at PG on July 4
372 (7.7). The range of average daily concentration (mg/L) of chlorophyll *a* was higher at ND (2.3 to 8.0)
373 than PG (1.0 to 3.2). Turbidity, in Nephelometric Turbidity Units, was higher on average at ND (5.8) than
374 PG (1.1) for the time both had measurements (May 10 to June 27), although the maximum turbidity was
375 greater at PG (115.2) than ND (75.3).

376 **4. Discussion**

377 **4.1 Triploid Mortality**

378 The primary objective in this study was to investigate the cause of the recurring mortality events
379 of triploid *C. virginica*, or triploid mortality, in the lower Chesapeake Bay. Triploid mortality observed in

this project was expected to match reports from farmers in previous years (K. Hudson, pers. comm., Guévélou et al., 2019). Most reports indicated mortalities would occur in near market sized oysters in late spring (May-June), many (>20%) would die within a matter of weeks, and then relatively little mortality would occur throughout the summer.

A mortality event fitting the description of triploid mortality was observed only at Nandua Creek (ND). Between March and the end of June at ND, mortality ranged from 15 to 29% in the triploid crosses. There was a small increase in cumulative mortality in these crosses for the subsequent four months of the trial (< 10%). This mortality event was mild compared to previous reports from some farmers in 2014 (Guévélou et al., 2019) and 2019 (K. Hudson, pers. comm.), however the timing and brief period of the event suggest it is a signature example of triploid mortality.

The onset of the mortality event at ND occurred between May 10 to May 24, when the mean of the average daily temperature was approximately 20°C, and ended between June 7 and June 27, when the mean of the average daily temperature was approximately 27°C. Temperature and salinity, however, were similar between ND and the other nearby Eastern Shore sites throughout the field trial. DO concentration and pH were similar between ND and PG, but triploid mortality obtained only at ND. As Guévélou et al. (2019) suggested, environmental conditions implicated in summer mortality of *C. gigas,* such as quantity of food available and stressors from the sediment (Samain and McCombie, 2008), are likely worthy candidates of investigation for future study into the etiology of triploid mortality.

The results from this trial demonstrate that triploid mortality in the Chesapeake Bay involves more than a specific cross or brood stock source. The severity of triploid mortality was measured as the probability of mortality between February/March to the end of June. Despite their heterogeneous genetic lineage, the severity of triploid mortality among VVV, VVM, LLV, and LLM was statistically indistinguishable at the only site with substantial mortality in the spring (>20%). Guévélou et al. (2019)

404 also measured mortality among different triploid crosses in late spring in the Chesapeake Bay, however
405 the triploids differed only by the source of diploid brood stock. Guévélou et al. (2019) reported triploids
406 made from a Maine diploid, or "Northern cross," tended to have the lowest mortality across sites and
407 triploids made from DEBY tended to have the highest. The test of genetic lineage in Guévélou et al.
408 (2019), however, was not ideal because the crosses were not controlled, the seed were obtained from
409 multiple commercial hatcheries, and the seed varied in age. Through simultaneous, controlled crosses,
410 the present study found that neither a genetically distinct source of diploid brood stock (V or M) nor
411 genetically distinct source of tetraploid brood stock (VV or LL) significantly affected the severity of
412 triploid mortality.

413 Like Guévélou et al. (2019), the present study found no support for the hypothesis that genes
414 passed by the "Northern diploid" are a cause of triploid mortality. Some of the other findings of
415 Guévélou et al. (2019) are similar to this study: observed mortality on commercial sites on the bay-side
416 of the Eastern Shore that matched previous reports of triploid mortality, found no relationship between
417 mortalities and infections from *H. nelsoni* or *P. marinus*, and found, within crosses, the largest oysters
418 were not especially susceptible to mortality in late spring.

419 The current study controlled for many of the factors that confounded the "rapid institutional
420 response" reported by Guévélou et al. (2019). Still, the study presented here had its own design
421 limitations. Each cross in the current study was produced by a single mass spawn. Replication of these
422 spawns with multiple independent crosses would have provided more precise estimation of a brood
423 stock effect (e.g. Dégremont et al. 2012). Regarding genetic effects, future research on triploid mortality
424 should shift the focus from mass spawn studies to estimating variance in groups of siblings, or families,
425 in order to possibly uncover a genetic basis for susceptibility to triploid mortality. High heritability for
426 survival related to summer mortality has been observed in *C. gigas* (Dégremont et al., 2007).

Is “triploid mortality” truly *triploid* mortality? There has been scant data for mortality rates in diploids during a triploid mortality event. Results from Guévélou et al. (2019) were ambiguous: between the two sites with the signature triploid mortality pattern, Nassawadox Creek, diploids and triploids had similar mortality at Nassawadox Creek, while diploids had much lower mortality than triploids at Pungoteague Creek. In this study, where only one site had the signature triploid mortality, only the triploids were affected. The VV cross had less than 2% mortality during the triploid mortality event at ND. Clearly, the conditions causing the triploid mortality event at ND did not cause a similar mortality event in the diploid control group. From these results, we hypothesize that conditions inducing triploid mortality events in the Chesapeake Bay do not cause mass mortality in diploids and suggest classifying the events as “triploid mortality” is justified. Additional data from controlled field tests involving a greater variety of sites and diploids, and in which triploid mortality events occur, are required to further evaluate this hypothesis.

High mortality in triploid oysters on farms in the Southeastern United States have invited comparisons with triploid mortality in the Chesapeake Bay. In 2016, oyster farmers in Alabama and Louisiana observed unexpectedly high mortality in triploid stocks (Wadsworth et al., 2019). The mortality was investigated in a follow-up study by Wadsworth et al. (2019) in which the survival of diploids and triploids was tracked at commercial sites. Wadsworth et al. (2019) found high mortality at several of the sites and higher cumulative mortality in the triploids than diploids at most sites.

It is not clear if mortalities reported in Wadsworth et al. (2019) represent triploid mortality as it has been defined in the Chesapeake Bay. A major difference is that Wadsworth et al. (2019) recorded well-known stressors around the time of mortality events. Sudden drops in salinity, periods of very low salinity (< 5 ppt) and high temperature (> 30°C), and strong disease pressure from *P. marinus* were associated with mortality in Wadsworth et al. (2019a) , all of which have been absent in the recent investigations in triploid mortality in the lower Chesapeake Bay. Instead of classic “triploid mortality” as

451 defined in the Chesapeake Bay, differential mortality in Wadsworth et al. (2019) could have been due to
452 the diploids having higher resistance to stress from low salinity and *P. marinus*.
453 Susceptibility to triploid mortality, like susceptibility to mortality owing to pathogens such as
454 OsHV-1 µvar (Azéma et al., 2016; Petton et al., 2015), *Vibrio aestuarianus* (Azéma et al., 2016), and
455 *Roseovarius crassostreae* (Bricelj et al., 1992; Ford and Borrero, 2001), likely varies based on ontogeny.
456 Reports from farmers have suggested that while market or near-market oysters die during triploid
457 mortality events, juvenile oysters or seed have low mortality. For this reason, only oysters older than
458 one year were used during the field trials in Guévélou et al. (2019) and the current study. The factors
459 involved in this ontogeny-based susceptibility are not clear, however one explanation is that
460 susceptibility is positively related with reproductive effort. Reproductive effort, which has been linked
461 to vulnerability to bacterial infections (De Decker et al., 2011; Wendling and Wegner, 2013) and
462 "summer mortality" in *C. gigas* (e.g. Koganezawa, 1975; Samain and McCombie, 2008), is expected to
463 increase with age in oysters (Bayne, 2017). Although triploid oysters are considered reproductively
464 sterile, they may expend considerable reproductive effort during gametogenesis (Allen and Downing,
465 1990; Jouaux et al., 2010).
466 The closest analogy for triploid mortality in the Chesapeake Bay may be "summer mortality"
467 observed in diploid *C. gigas*. Summer mortality episodes have regularly occurred in Japan (Koganezawa,
468 1975; Takeuchi et al., 1960; Ventilla, 1984), Western Europe (Cotter et al., 2010; Goulletquer et al.,
469 1998; Parache, 1989; Samain and McCombie, 2008; Watermann et al., 2008), and the west coast of the
470 US (Cheney, 2000; Glude, 1975; Perdue et al., 1981), often resulting in high crop losses (> 50%) of adult
471 diploid *C. gigas* during the summer months. Like triploid mortality, many cases occur within a period of
472 several weeks (e.g. Cheney 2000, Ropert et al. 2008, Cotter et al. 2010), and are not typically explained
473 by extreme environmental conditions or intense disease pressure (Cheney, 2000; Glude, 1975;
474 Koganezawa, 1975; Ropert et al., 2008).

The important overlap between summer mortality and triploid mortality may be in their generalized etiology. Summer mortality was originally suspected to be due to a "physiological disorder" that arose when warm temperatures and eutrophic conditions led to extensive gonad formation (Koganezawa, 1975), and additional research has repeatedly associated physiological processes of gametogenesis and spawning with summer mortality (Cotter et al., 2010; Perdue et al., 1981; Samain et al., 2007; Samain and McCombie, 2008). Physiological dysfunction related to gametogenesis and spawning may also be causing triploid mortality. Triploid mortality consistently occurs in late spring, when the physiology of oysters in the Chesapeake Bay is expected to be strongly influenced by gametogenesis and spawning. In oysters, it has been well documented that gametogenesis and spawning are also strongly influenced by environmental conditions (Delaporte et al., 2006; Dutertre et al., 2010, 2009; Liu et al., 2010) that can explain the strong site and inter-annual effects of summer mortality (Glude, 1975; Ropert et al., 2008) and triploid mortality.

A connection between gametogenesis and mortality in triploid *C. virginica* has been recently examined. Guévélou et al. (2019) raised the question of whether "the degree of triploid gametogenesis and reproduction" effects mortality, and Wadsworth et al. (2019) examined gonad development as a potential factor to explain mortality in triploids. Both found no evidence for an association between gonad development in triploids and mortality, however they both primarily classified gonad development using methods developed for diploid oysters. Triploid oysters have much different gonad development than diploids (Allen and Downing, 1990; Barber and Mann, 1991; Guévélou et al., 2019; Jouaux et al., 2010; Lee, 1988), and thus precision to detect differences may be lacking in a classification system developed for diploids. Further study in triploid mortality would benefit from a more specific classification system for gonad development in triploid *C. virginica*, which may reach the precision needed to detect a relationship between gametogenesis and susceptibility.

4.2 Mortality in Virginia and Maine Diploids

The control for triploid mortality in this study was the diploid DEBY (VV) cross. Diploids with a Maine genetic origin (VM, MV, and MM) were produced as an experiment. The VV cross exhibited low mortality while the Virginia-Maine hybrids and Maine diploids had significantly higher mortality. MM had especially high mortality and the most cumulative mortality at all sites by a wide margin (>20%).

In general, mortality in diploids with a Maine genetic origin was gradual and most of the mortality occurred in late summer. Gradual mortality over the summer is not unusual for oysters in the Chesapeake Bay, in part because disease pressure from *P. marinus* is expected to increase over summer and near a maximum by late summer (Burreson and Ragone Calvo, 1996). Diploids with Maine genetic origin were expected to be susceptible to *P. marinus* and have general unsuitability to warm estuarine conditions of the Chesapeake Bay. The Maine brood stock used in this study had been selected in a region with higher salinity, lower temperatures, and less *P. marinus* pressure than the sites in this study (e.g. Proestou et al., 2016). Many of the MM diploids (60 to 66%) were infected with *P. marinus* when examined in August, suggesting *P. marinus* may have been responsible for at least some of the mortality in the diploids with Maine genetic origin.

For mortality among the diploid constructs, Virginia-Maine reciprocal hybrids were statistically indistinguishable within sites. Differences between reciprocal hybrids have been observed in shellfish in the larval stage and may often be attributable to variance in egg quality (Cruz and Ibarra, 1997; Deng et al., 2005). Some studies, however, have also observed reciprocal effects in adults or juveniles, such as yield in *C. gigas* (Hedgecock and Davis, 2007), growth in the disk abalone *Haliotis discus hannai* (Deng et al., 2010), and growth and survival in the Atlantic deep-sea scallop *Placopecten magellanicus* (Wang and Côté, 2012).

The hybrids exhibited heterosis for mortality at all sites. Heterosis was defined by the deviation of the mean of the reciprocals from the mean of their parental populations (Falconer, 1981), often

523 referred to as mid-parent heterosis. Heterosis was for higher survival, in the direction of the selectively
524 bred VV line, or DEBY line. DEBY has been selected for resistance to the major diseases in the
525 Chesapeake Bay since about 1987, or about 14 generations (Ragone Calvo et al., 2003). Our hypothesis
526 is that disease resistance from the DEBY contributes more than just an additive effect, perhaps by
527 improving the overall health of the hybrid beyond fending off disease, per se. Callam et al. (2013)
528 showed that triploids made from disease resistant tetraploids resulted in nearly completely resistant
529 progeny no matter the source of the diploid parent, be it wild or selectively bred, also suggesting effects
530 other than the additivity of disease resistance.
531
532 **4.2 Growth & Condition**
533 Shell height was a minor source of variation among crosses in this field trial and was similar in
534 diploids and triploids. The variation can be attributed largely to the MM cross, the slowest growing cross
535 by a wide margin. The most direct comparison between triploid and diploids was afforded by VVV
536 versus VV crosses because of the similarity of pedigree between the tetraploid and diploid parents that
537 comprised the triploid: mean shell height of VVV was only 3 mm greater than that of VV by August 2016
538 (18 months post-spawn).
539 Triploid *Crassostrea* spp. generally seem to grow substantially faster than their diploid
540 counterparts based on shell height (Dégremont et al., 2012; Matthiessen and Davis, 1992; Nell and
541 Perkins, 2005; Qin et al., 2019), however, several studies, including the current one, observed only a
542 modest difference (Barber and Mann, 1991; Callam et al., 2016; Hand et al., 1998; Stone et al., 2013).
543 Some authors have suggested the growth advantage in triploid oysters depends on the favorability of
544 the growing conditions (Davis, 1994; Guo et al., 2009). Callam et al. (2016) suggested stress from low
545 salinity inhibited triploids from growing faster than diploids at a low salinity site and drew a parallel with
546 results from Davis (1994), which found *C. gigas* triploids only performed better than diploids at more

547 productive sites. This explanation, however, does not account for the small difference in shell height
548 between triploids and diploids from selectively bred lines at the more mesohaline site in Callam et al.
549 (2016), and contrasts with findings from Ibarra et al. (2017). Ibarra et al. (2017) found mated triploid *C.*
550 *gigas* grew substantially faster than diploids at two tropical sites, characterized by low productivity and
551 high temperatures, but not at a more productive temperate site. In this study, although PG seemed to
552 be a less favorable growing site, there was only a small difference (> 10 mm) in mean shell height
553 between VVV and VV at all sites by August.

554 Meat weight provided a clearer distinction between diploids and triploids in this field trial. Meat
555 weight fluctuates during the year due to physiological changes in the oyster, such as, gametogenesis,
556 spawning, and rebuilding of biochemical reserves (Loosanoff, 1942; Thompson et al., 1996). Mean meat
557 weight decreased sharply in the diploids between May and August, which is likely attributable to
558 spawning, which in *C. virginica* generally occurs between May and September in the Chesapeake Bay
559 (Kennedy and Krantz, 1982). For triploids, mean meat weight was stable during this time, which
560 demonstrates triploid *C. virginica* maintain high meat quality during the spawning season similar to
561 previous findings in *C. gigas* (Allen and Downing 1986, Allen, 1988).

562 Despite the maintenance of body weight in triploids, spawning activity cannot be ruled out.
563 Significant variation in fecundity of triploids has been observed (Allen and Downing, 1990; Jouaux et al.,
564 2010), with some triploids producing significant amounts of gametes and spawning concurrently with
565 diploids, while other triploids are virtually sterile. Allen and Downing (1990, 1986) found triploid *C.*
566 *gigas* to have stable mean meat weight during the spawning interval for diploids, despite histological
567 evidence that some triploids spawned. Normand et al. (2008) also inferred that triploid *C. gigas*
568 spawned within the same time interval as diploids based on histological evidence. In their study, dry
569 meat weight decreased substantially in triploids in parallel with diploids. Histological assessment is
570 required to more accurately estimate the gonad development and spawning activity in the triploids.

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Table 1. Results from Type III Wald tests from the mixed effects logistic regression models to compare "triploid mortality" and mortality among diploids made from Virginia and Maine brood stock. df: degrees of freedom, Chisq: Chi-squared test statistic, p: p value at α=0.05.

Model	Factor	Chisq	df	p
Triploid Mortality	Cross	15.5	4	< 0.05
	Site	9.1	3	< 0.05
	Cross x Site	33.9	12	< 0.05
Virginia and Maine Diploids	Cross	174.6	3	< 0.05
	Site	2.6	3	0.45
	Cross x Site	35.3	9	< 0.05

Table 2. Prevalence (P %) and weighted prevalence (WP) of infections of *Haplosporidium nelsoni*, *Perkinsus marinus*, *Haplosporidium costale,* nematopsis, chlamydia, and hemocytosis determined via histology for *Crassostrea virginica* sampled on May 24 and August 10 at Nandua Creek (ND) and August 8 at Rappahannock River (RR). Only prevalence was measured for nematopsisand chlamydia. n: number of observations. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

				H. nelsoni		*P. marinus*		*H. costale*	Nematopsis	Chlamydia	Hemoctyosis	
Date	Site	Cross	n	P (%)	WP	P (%)	WP	P (%)	P (%)	P (%)	P (%)	WP
May	ND	VVV	21	0	0	0	0	0	5	14	5	0.1
	ND	VVM	21	5	0.05	0	0	0	0	5	10	0.2
	ND	LLV	21	5	0.05	0	0	0	0	29	5	0.1
	ND	LLM	21	10	0.1	0	0	0	0	24	10	0.2
Aug	RR	MM	15	0	0	66	0.9	0	0	0	13	0.3
	ND	MM	15	0	0	60	1.5	0	7	13	20	0.4

Table 3. Number of dead oysters per bag from each cross and results from the ANOVA for shell height (mm) of live and dead triploid *Crassostrea virginica* sampled from Nandua Creek on June 7, 2016. Seven live oysters were sampled from each bag. df: degrees of freedom, SS: sums of squares, MS: mean sums of squares. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

Dead Oysters					
Cross	Bag 1	Bag 2	Bag 3		
VVV	17	23	26		
VVM	24	14	9		
LLV	8	7	13		
LLM	8	7	3		
ANOVA					
Factor	df	SS	MS	F	p
Status	1	34	34	0.31	0.6
Cross	3	2625	875	7.85	< 0.05
Bag_{Cross}	2	166	83	0.75	0.5
Status x Cross	3	1120	373	3.35	< 0.05
Residuals	233	25977	111		

Table 4. Results from the ANOVA for shell height (mm) in August of 2016 for *Crassostrea virginica* crosses and sites, least square means from the ANOVA, and Tukey's Honest Significant Difference (HSD) test for VVV and VV among all other crosses and RR among all other sites. Results from Tukey's HSD test are reported with 95% family-wise confidence intervals in parentheses. df: degrees of freedom, SS: sums of squares, MS: mean sum of squares, $\bar{x}$: sample mean, ci: 95% confidence interval. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia. Sites are RR=Rappahannock River, ND=Nandua Creek, PG=Pungoteague, and OC=Occahannock Creek.

Shell Height: August 2016

ANOVA

Factor	df	SS	MS	F	p
Cross	7	11100	1586	16.9	< 0.05
Site	3	11610	3870	41.3	< 0.05
Cross x Site	21	2981	142	1.5	0.07
Residuals	449	42069	94		

Least Square Means: (Cross)

	$\bar{x}$	ci
LLM	84	82, 87
VVV	82	80, 85
LLV	82	79, 84
VVM	80	78, 83
VV	79	76, 81
MV	77	74, 79
VM	75	72, 77
MM	68	66, 71

Multiple Comparisons of Means: Tukey Contrasts (Cross)

	VVV	VVM	LLV	LLM	VV	VM	MV	MM
VVV	--	2 (-3, 7)	0 (-5, 5)	-2 (-7, 3)	4 (-2, 9)	7 (2, 13)	5 (0, 10)	14 (9, 20)
VV	--	-2(-7, 3)	-3 (-9, 2)	-6 (-11, 0)	--	4 (-2, 9)	2 (-4, 7)	10 (5, 16)

Least Square Means: (Site)

	$\bar{x}$	ci
RR	84	82, 85
ND	82	80, 84
OC	77	76, 79
PG	71	69, 73

Multiple Comparisons of Means: Tukey Contrasts (Site)

	RR	ND	PG	OC
RR	--	2 (-1, 5)	13 (10, 16)	6 (3, 10)

Table 5. Mean of average daily temperature values (°C) at all sites during the mortality event at ND (May 10-June 27). Means are reported within sampling visits to ND: from May 10 to May 24, from May 24 to June 7, and from June 7 to June 27. *No data were available between May 24 and June 5 for RR. Sites are Rappahannock River (RR), Nandua Creek (ND), Pungoteague (PG), and Occohannock Creek (OC).

	May 10-May 24	May 24-June 7	June 7-June 27
RR	19.9*	--*	26.8
ND	19.6*	25.4*	26.5
PG	19.0*	25.4*	26.0
OC	19.3*	25.1*	26.3

Figure 1. The spawning design for *Crassostrea virginica* produced by crossing diploid oysters (2N) from Virginia (V) and Maine (M) in a 2x2 matrix, resulting in four diploid crosses (VV, VM, MV, and MM), as well as crossing the same female diploid oysters to male tetraploid oysters (4N) with Virginia (VV) and partial Louisiana (LL) origin to create four triploid crosses (VVV, VVM, LLV, and LLM).

♀ \ ♂		2N		4N	
		V	M	V	L
2N	V	VV	MV	VVV	LLV
	M	VM	MM	VVM	LLM

Figure 2. Map of commercial farms where the eight crosses of *Crassostrea virginica* were reared from June 2015 to November 2016. Map produced using ArcGIS® software by Esri. 1: Rappahannock River (RR); 2: Occohannock Creek (OC); 3: Nandua Creek (ND); 4: Pungoteague (PG).

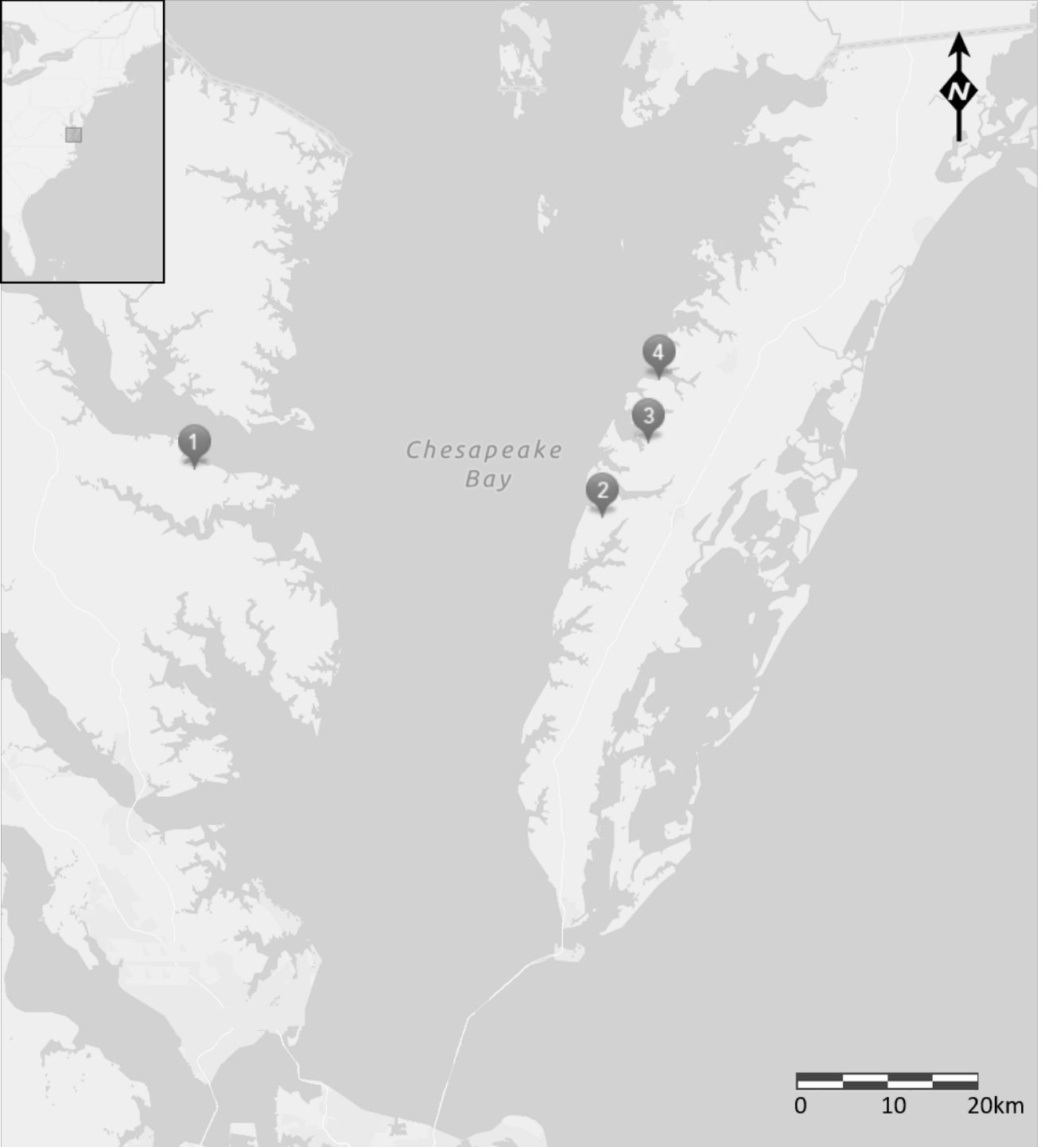

1
2
3
4
Chesapeake
Bay
N
0
10
20km

Figure 3. Mean cumulative mortality (%) in four triploid crosses (black) and four diploid crosses (white) of *Crassostrea virginica* at four sites in the Chesapeake Bay from April to November of 2016. Figure insets are closer views of cumulative mortality from early May to early June. Error bars represent ± standard error. RR: Rappahannock River; ND: Nandua Creek; PG: Pungoteague; OC: Occohannock Creek. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

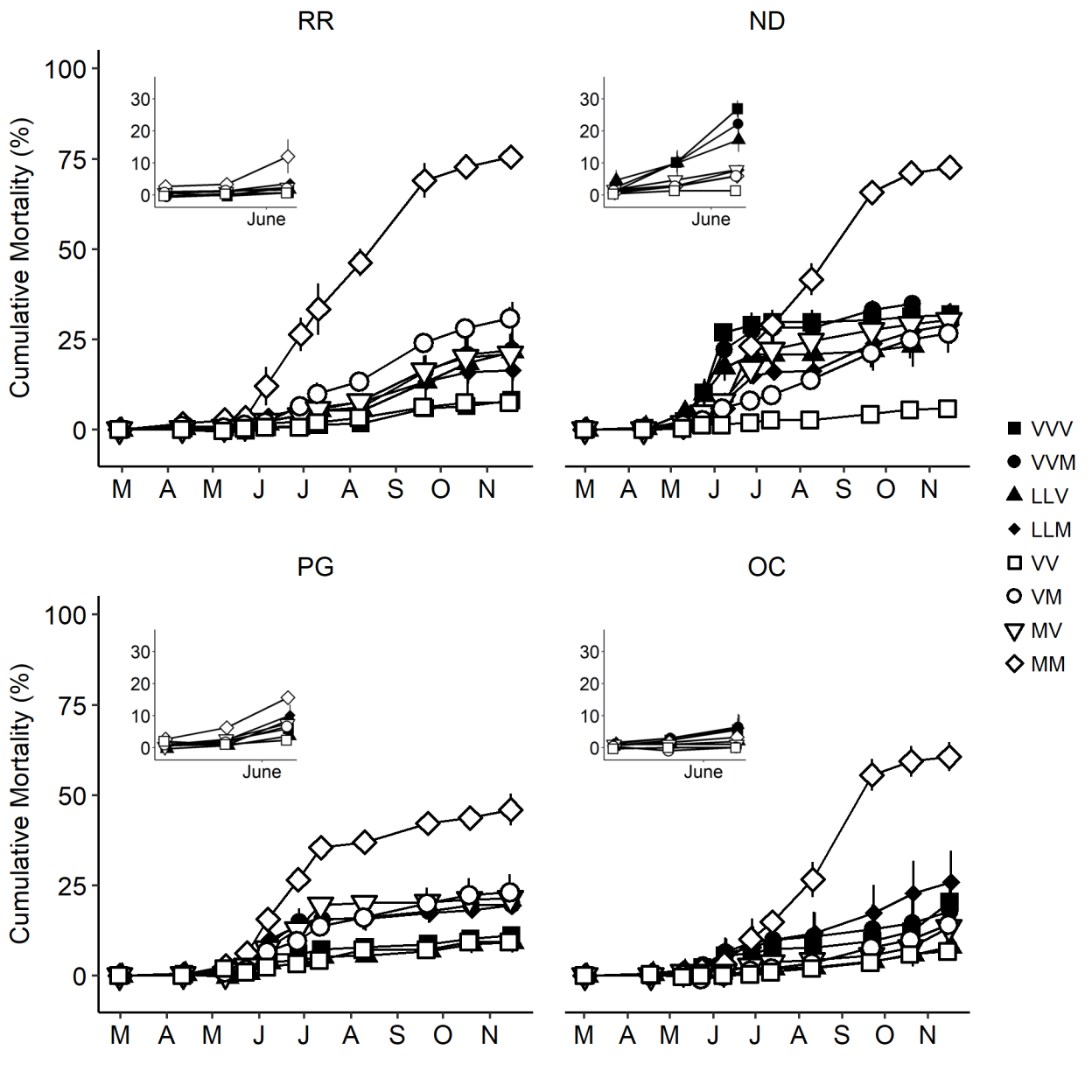

RR
ND
PG
OC
Cumulative Mortality (%)
100
75
50
25
0
30
20
10
0
June
M A M J J A S O N
VVV
VVM
LLV
LLM
VV
VM
MV
MM

Figure 4: Probability of mortality estimate, 95% confidence intervals, and pair-wise comparisons using Tukey's Honest Significant Differences test from the mixed effect logistic regression for mortality of a diploid cross (white) and triploid crosses (black) of *Crassostrea virginica* at four sites in the Chesapeake Bay from February through June of 2016. Sites are Rappahannock River (RR), Nandua Creek (ND), Pungoteague (PG), and Occohannock Creek (OC). Pair-wise comparisons were only made within sites. Crosses that share a letter are not significantly different at $\alpha = 0.05$. Abberviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

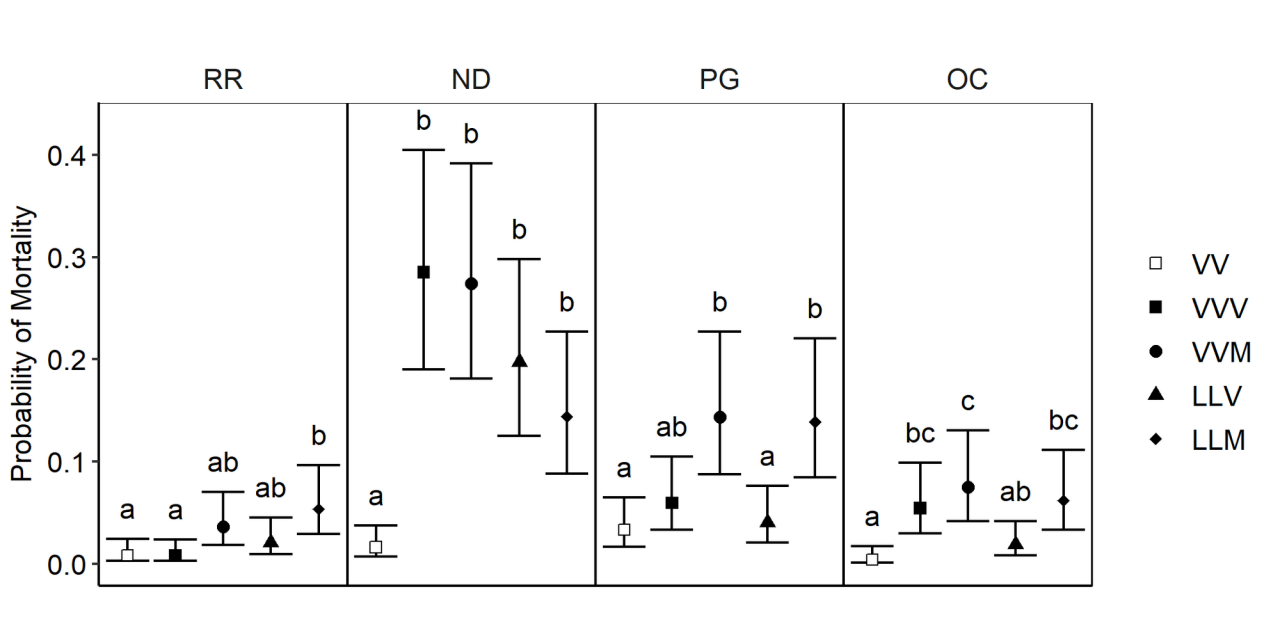

RR
ND
PG
OC
Probability of Mortality
0.0
0.1
0.2
0.3
0.4
a
a
ab
ab
b
a
b
b
b
b
a
ab
b
a
b
a
bc
c
ab
bc
VV
VVV
VVM
LLV
LLM

Figure 5: Probability of mortality estimates, 95% confidence intervals, and pair-wise comparisons using Tukey's Honest Significant Differences test from the mixed effect logistic regression for mortality of diploid crosses of *Crassostrea virginica* at four sites in the Chesapeake Bay from February to November of 2016. Sites are Rappahannock River (RR), Nandua Creek (ND), Pungoteague (PG), and Occohannock Creek (OC). Pair-wise comparisons were only made within sites. Crosses that share a letter are not significantly different at $\alpha = 0.05$. Abberviations for crosses are found in Figure 1: M=Maine, V=Virginia.

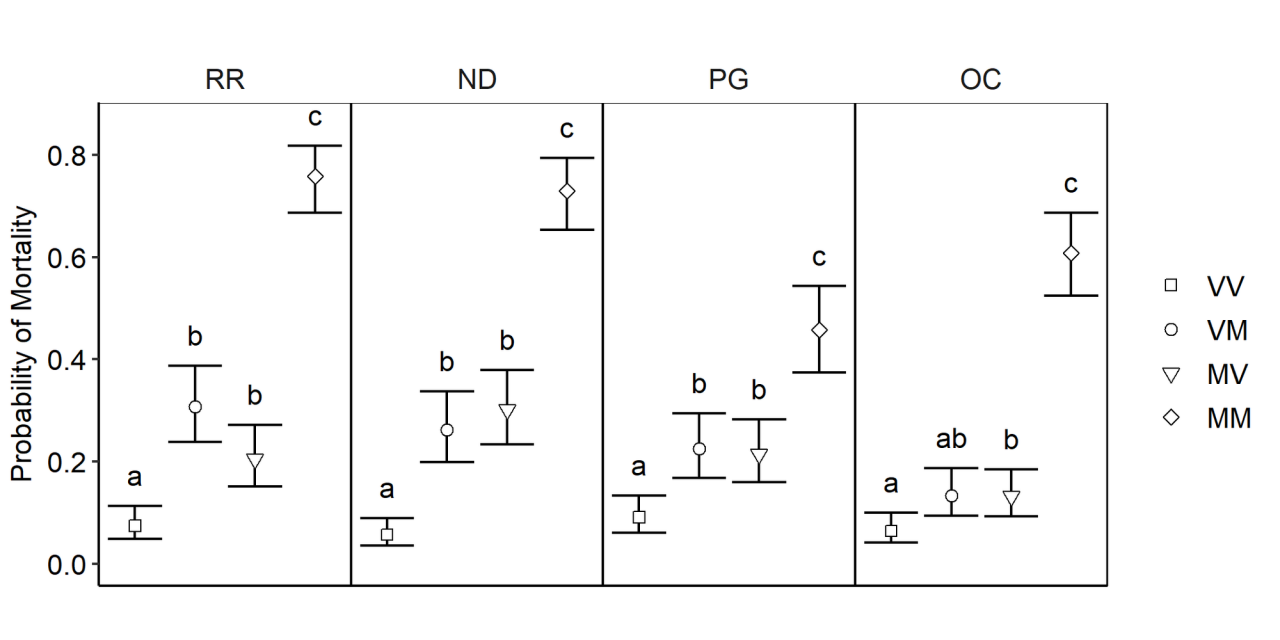

RR
ND
PG
OC
Probability of Mortality
0.0
0.2
0.4
0.6
0.8
a
b
b
c
a
b
b
c
a
b
b
c
a
ab
b
c
VV
VM
MV
MM

Figure 6. Shell length (mm) of live and dead *Crassostrea virginica* of four triploid crosses (VVV, VVM, LLV, LLM) sampled from Nandua Creek on June 7, 2016. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

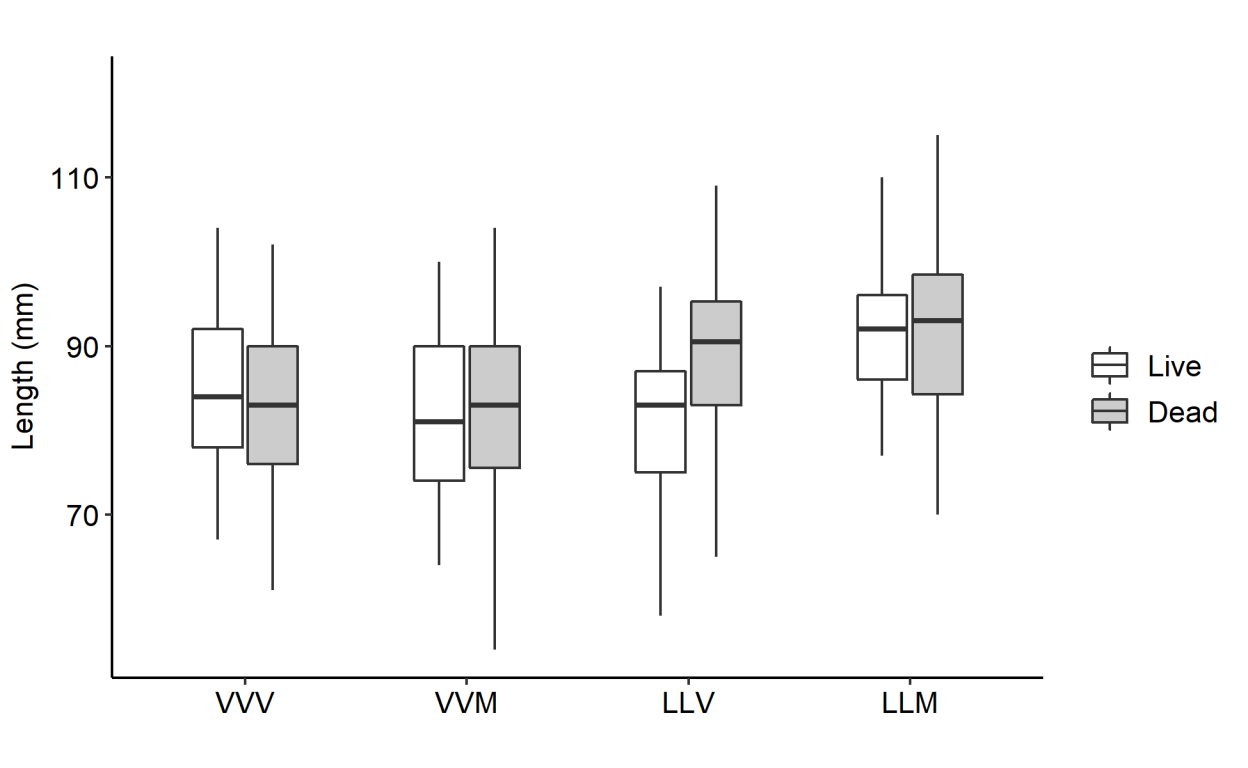

Length (mm)
110
90
70
VVV
VVM
LLV
LLM
Live
Dead

Figure 7. Mean shell height (mm) for four triploid crosses (black) and four diploid crosses (white) of *Crassostrea virginica* at each site at the start of the experiment in February/March 2016 and at the end of the experiment in August 2016. RR: Rappahannock River; ND: Nandua Creek; PG: Pungoteague; OC: Occohannock Creek. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

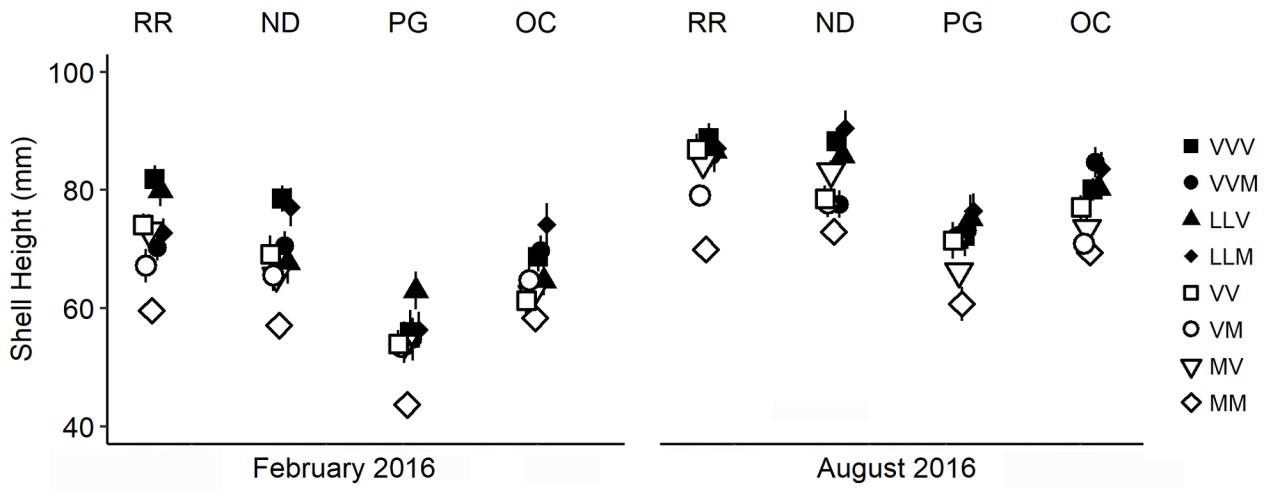

RR
ND
PG
OC
RR
ND
PG
OC
100
80
60
40
Shell Height (mm)
February 2016
August 2016
VVV
VVM
LLV
LLM
VV
VM
MV
MM

Figure 8. Mean meat weight (g) for four triploid (black) and four diploid (white) crosses of *Crassostrea virginica* reared at four sites from February-August of 2016. Error bars represent ± standard error. RR: Rappahannock River; ND: Nandua Creek; PG: Pungoteague; OC: Occohannock Creek. Abbreviations for crosses are found in Figure 1: L=Louisiana, M=Maine, V=Virginia.

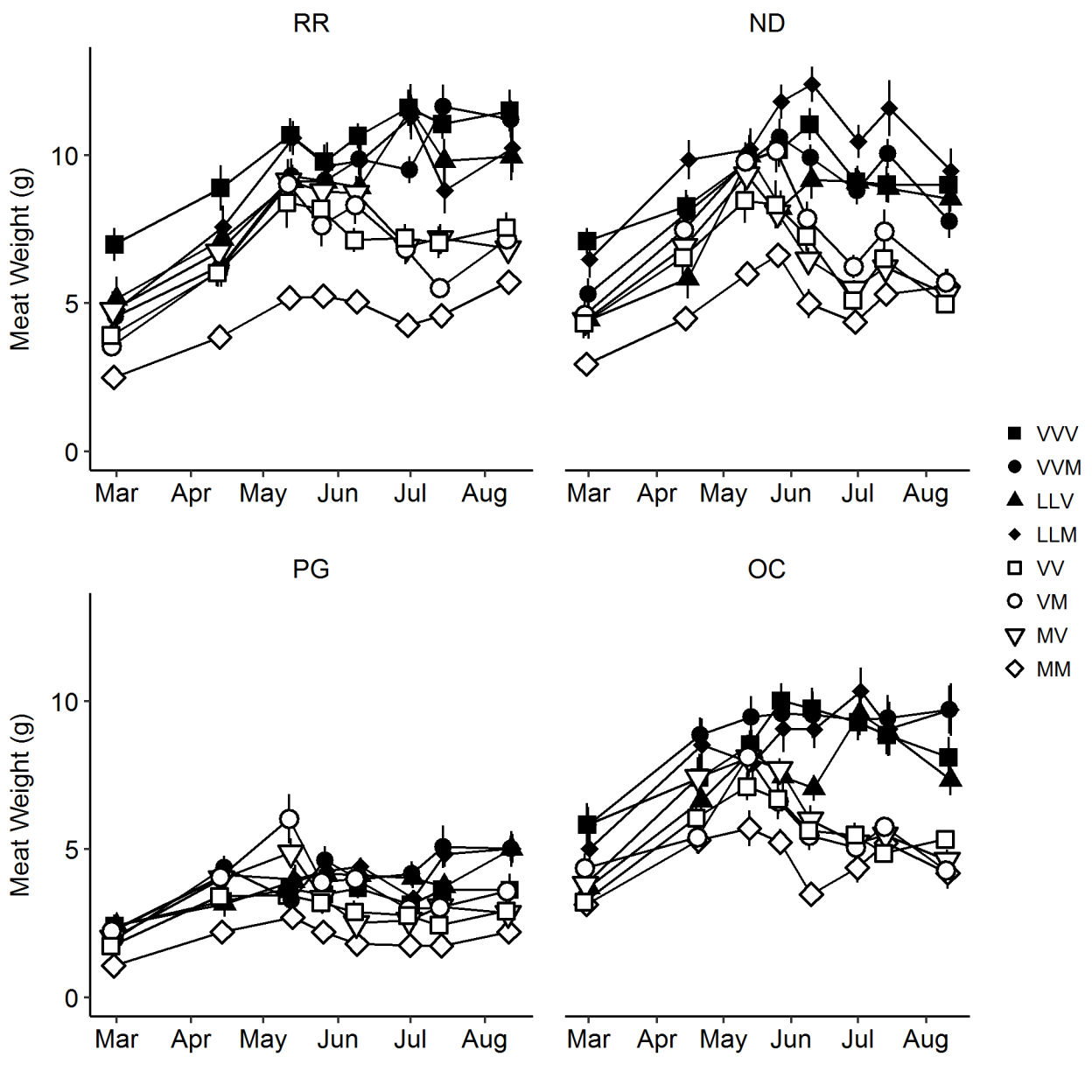

RR
ND
PG
OC
Meat Weight (g)
0
5
10
Mar
Apr
May
Jun
Jul
Aug
VVV
VVM
LLV
LLM
VV
VM
MV
MM

Figure 9. Average daily temperature (°C) from January 1 to August 10 of 2016 at each of the four sites where crosses of *Crassostrea virginica* were reared from June 2015 to August 2016. RR: Rappahannock River; ND: Nandua Creek; PG: Pungoteague; OC: Occohannock Creek.

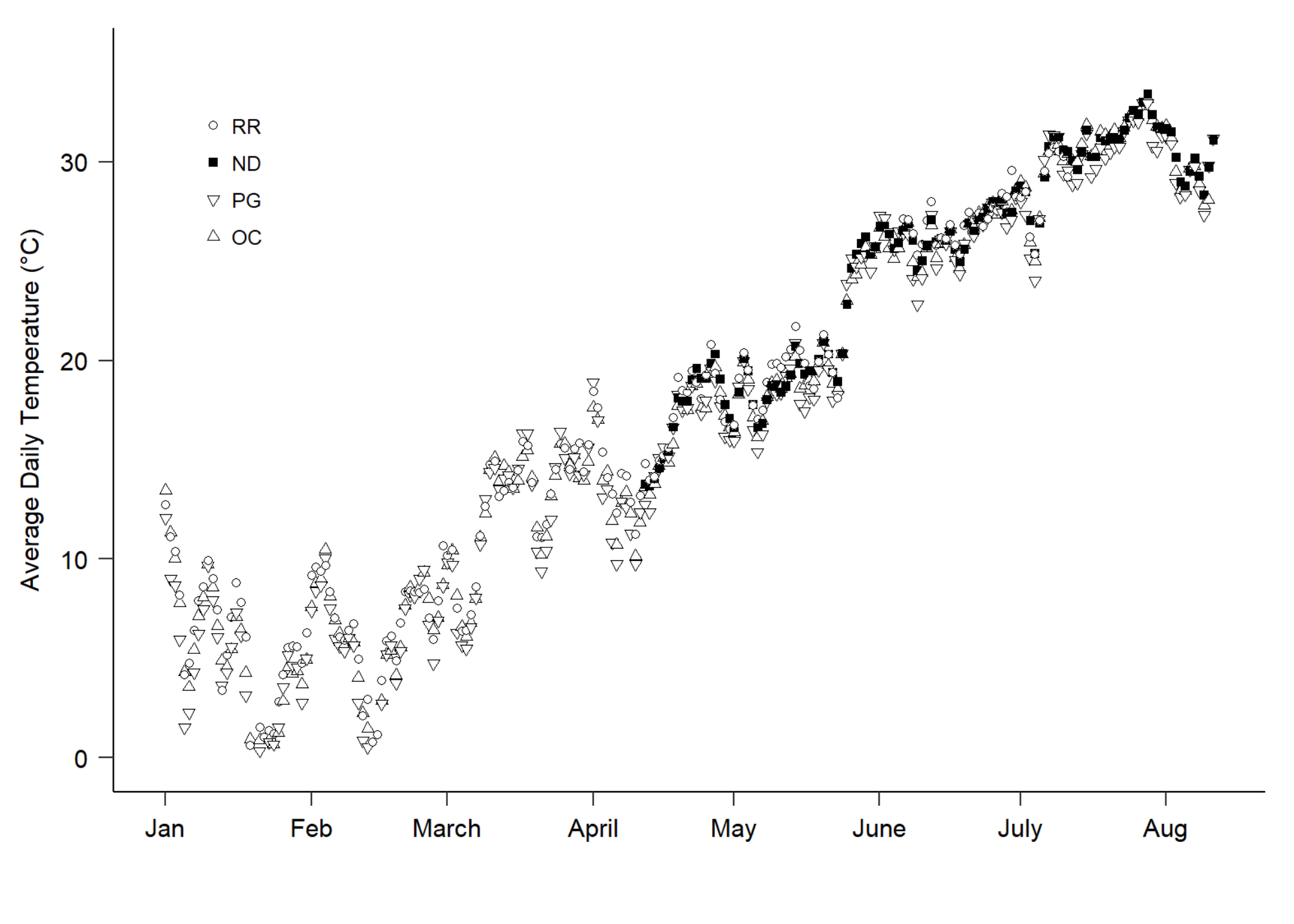
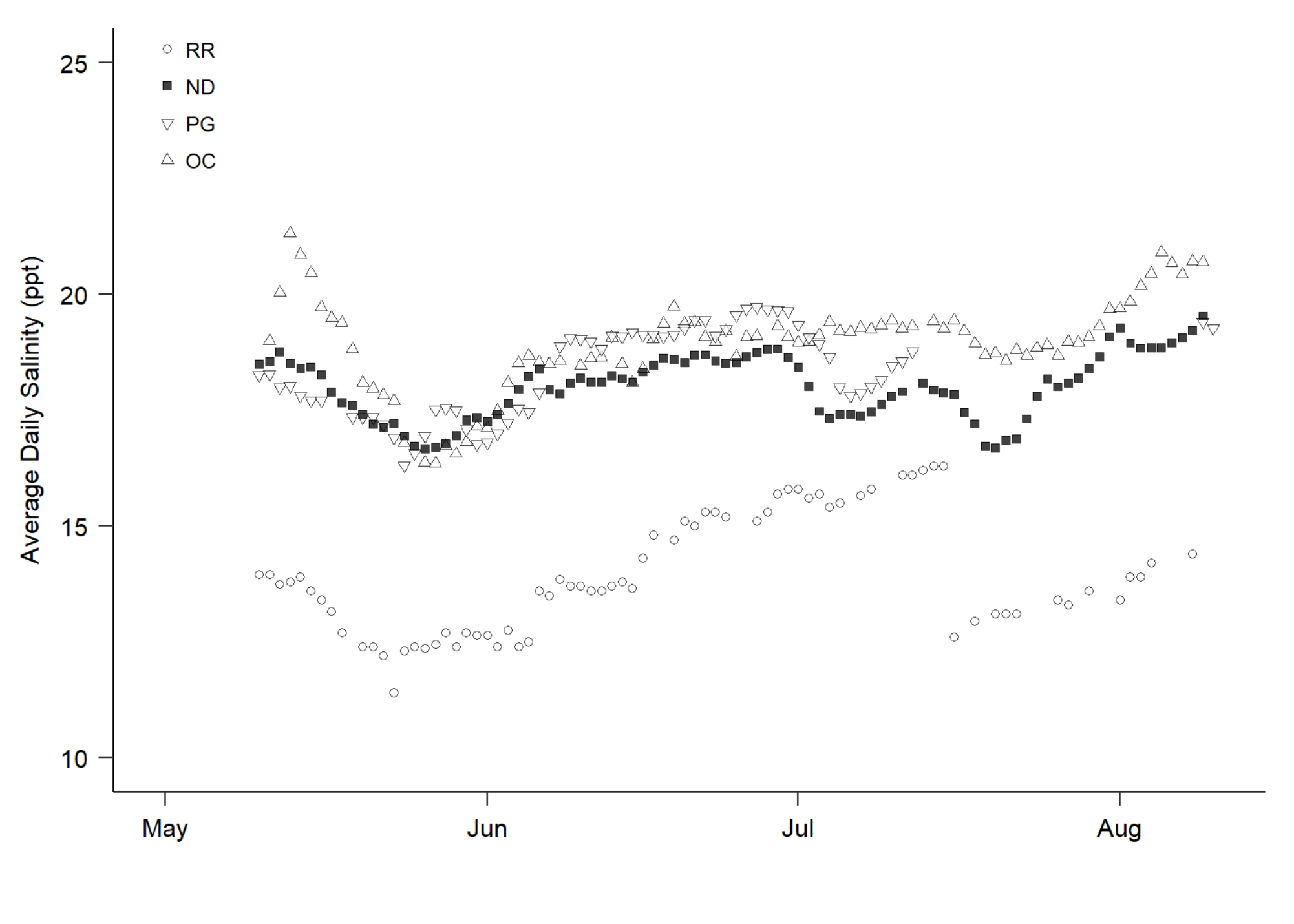

RR
ND
PG
OC
Average Daily Temperature (°C)
0
10
20
30
Jan
Feb
March
April
May
June
July
Aug

Figure 10. Average daily salinity (ppt) from May 10 to August 10,2016 at each of the four sites where crosses of *Crassostrea virginica* were reared from June 2015-August 2016. RR: Rappahannock River; ND: Nandua Creek; PG: Pungoteague; OC: Occohannock Creek.


RR
ND
PG
OC
25
20
15
10
Average Daily Salinity (ppt)
May
Jun
Jul
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