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## BREEDING AND DOMESTICATION OF EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) LINES FOR CULTURE IN THE MID-ATLANTIC, USA: LINE DEVELOPMENT AND MASS SELECTION FOR DISEASE RESISTANCE

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**ABSTRACT** A selective breeding program for *Crassostrea virginica* was established in 1997 as part of an initiative in Virginia to address declining oyster harvests caused by the two oyster pathogens *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo). Housed in the Aquaculture Genetics and Breeding Technology Center (ABC), the objective of the program was to develop and disseminate disease-resistant lines that would enable an oyster culture industry. Today, culture of disease-resistant cultivars accounts for more than 90% of oyster production in the state, where 28.1 million half-shell oysters and 2 billion eyed larvae were sold in 2012. Results of our line development program as of 2006 are reported. Eight ABC lines from 3 genetic groups—East Coast (EC), Louisiana (LA), and hybrids between the 2 (HY)—and 1 wild control line, were produced and tested. These 9 groups were deployed in 4 replicates across 4 Virginia sites characterized by low (Kinsale (KIN)), medium (York River (YRK) and Lynnhaven (LYN)), and high (Wachapreague (WAC)) salinity regimes. Groups were sampled routinely for survival, growth, and disease diagnosis between November 2004 and December 2006. At KIN, where salinity was low and below the threshold for MSX and Dermo, survival was 41%–46% greater than survival at the other 3 sites by the end of the trial. Where the diseases were present (LYN, YRK, and WAC), ABC lines in general had greater survival than the control, but this varied by genetic group. The EC groups had 52%–82% greater survival, the HY groups had as much as 40% greater survival, and the LA groups performed worse than the control. Poor performance of the LA groups was a result of their susceptibility to MSX, and the majority of them died before the end of the study. The genetic effects varied with site, and the rank of the lines was inconsistent, such that the best line in one site was, in some cases, one of the worst in another. Genotype-by-environment interaction is clearly driven by disease and salinity. Growth was also influenced by site, genetic group, and an interaction between them. Compared with the wild control, ABC lines were 31%, 20%, 42%, and 24% heavier at the end of the trial in the KIN, YRK, LYN, and WAC sites, respectively. However, unlike survival, the best performers were those from the LA and HY groups. Again, line rankings changed across sites. For this reason, a salinity-specific breeding strategy to develop lines that perform optimally within a salinity range has been adopted.

**KEY WORDS:** *Crassostrea virginica*, oysters, selective breeding, disease resistance, Chesapeake Bay, growth, genetic improvement, MSX, Dermo

### INTRODUCTION

#### Fostering Breeding Technologies

In 1997, the Virginia General Assembly passed an initiative that gave rise to the Aquaculture Genetics and Breeding Technology Center (ABC) with the following legislative language: “To enhance our capabilities of fostering aquaculture in Virginia, we propose the establishment of an Aquaculture Genetics and Breeding Technology Center through the auspices of VIMS and the Commonwealth of Virginia. The mission of the Center will be to foster the development of breeding technologies to promote increased yields, improve disease resistance, and to make available genetically-improved broodstocks for the aquaculture industry.” The goal was later paraphrased to be “do something about the oyster problem.” The problem was the decline of commercial production on public and private grounds. Initially, ABC participated in a number of activities, including strategies for the use of selectively bred lines for restoration (Allen & Hilbish 2001, Allen et al. 2003, Carlsson et al. 2008), clam breeding (Camara et al. 2006), testing the nonnative *Crassostrea ariakensis* (Allen 2005), developing remote setting techniques (Congrove et al. 2009), and evaluating heritability of traits

(Dégremond et al. 2007). But, the bedrock of activity at ABC from the outset was breeding for disease resistance. This article chronicles the initial steps to develop commercial lines of *Crassostrea virginica* before the actual advent of an industry to use them.

The story of the demise of landings in the Chesapeake Bay has been told (Mackenzie 2007), with disease, if not the major culprit, certainly the contemporary one in 1997 (Ragone Calvo et al. 2003, National Academies of Sciences 2004). To allow aquaculture to thrive, disease-resistant oysters seemed essential, not just as an experimental breeding program, but as a broodstock distribution center. Aquaculture would also require a paradigm shift for the oyster industry—a change from the capture of oysters to cultivation. For Virginia, aquaculture had consisted of transplanting wild harvested seed to leased growing grounds (Hargis & Haven 1988). In 1997, intensive husbandry of hatchery-reared product was artisanal but, more important, was deemed impossible on a commercial scale because of disease-induced mortalities. The role of selective breeding in oyster aquaculture there would, therefore, become unique and pivotal. Selection would not only precede the establishment of an industry, but also enable it. Using a combination of selective breeding and triploidy, aquaculture of oysters has become a reality. In 2012, 28.1 million cultured oysters were sold in Virginia, a 35-fold increase since 2005 (Murray & Hudson 2012), plus sales of 2 billion eyed larvae contributing to spat-on-shell production that, in 2012, produced more than 40,000 bushels from private grounds (Dr. J. Wesson, Virginia Marine

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Resources Commission, pers. comm.). It is not hard to envision the day when oyster aquaculture routinely surpasses fisheries in the Bay.

#### *Disease Resistance in the Mid-Atlantic*

It has been clear for decades that resistance to infections of MSX disease from *Haplosporidium nelsoni* was heritable, and therefore selection for resistance works (Haskin & Ford 1979). MSX-resistant populations were developed by breeding survivors in disease-endemic areas after epizootic events (Haskin & Ford 1979, Ford & Haskin 1987, Burreson 1991). Survivors were then bred over successive generations with reduced disease-induced mortalities in each successive generation (Haskin & Ford 1979, Ford & Haskin 1987, Burreson 1991) compared with wild oysters. Histopathological data revealed they had lighter and more localized infections (Ford & Haskin 1987). In addition, disease-resistant oysters exhibited physiological superiority over wild animals, such as greater clearance rates and more energy reserves in months when MSX disease pressure was greatest (Barber et al. 1991).

Less clear is whether resistance to *Perkinsus marinus*, the causative agent of Dermo disease, is heritable. Successive drought years between 1985 and 1988 caused intensification and spread of *P. marinus* and, since 1985, has gradually replaced *Haplosporidium nelsoni* as the primary oyster pathogen in Chesapeake Bay (Burreson 1991, Carnegie & Burreson 2011). Burreson (1991) compared the survival of 6 populations—2 MSX resistant and 4 wild populations—in a natural disease challenge to both MSX disease and Dermo disease. Although the selected lines showed less susceptibility to MSX disease, all 6 lines were highly susceptible to *P. marinus*. The selected MSX-resistant groups actually demonstrated greater infection prevalence and intensity for Dermo disease than the unselected populations. Burreson (1991) concluded that MSX resistance was not a generalized response to pathogens, but was specific to MSX and seemed to increase susceptibility to Dermo disease. Ragone Calvo et al. (2003) reported that after four generations of selection in the York River, Virginia, an environment where both *H. nelsoni* and *P. marinus* are endemic, oysters developed resistance to both Dermo and MSX diseases, creating a line of oysters with dual resistance to both MSX and Dermo diseases. Resistance to Dermo disease was also reported by Guo et al. (2003). Although there seems to be a response to selection for Dermo disease, a definitive study eliminating MSX resistance (i.e., enhanced robustness) as a factor in better survival from Dermo disease is lacking.

In 1997, it was clear that progress could be made in domesticating oysters by selecting for disease resistance. The task, however, would be to change the focus from running experiments that verify tolerance/resistance to running a breeding program—in other words, moving from line testing to production and dissemination of genetically improved animals. The progress made with mass selection since the last publication by Ragone Calvo et al. (2003) is presented, including the new lines produced, strategies used, and results of our in-house testing.

## MATERIALS AND METHODS

### *Founding Populations and Genetic Material*

A systematic introduction of new genetic material into our lines had been ongoing since 1998 and was completed by 2004.

The primary motivation was to reduce the risk of inbreeding in the two original disease resistance lines at the time: DBY, derived at the Virginia Institute of Marine Science (VIMS), and the regionally-derived line, CROSSBreed (XB). They had been subject to intense selection from disease pressure. DBY was in its sixth generation of selection by 2004, with several years of unknown bottlenecks in the population as a result of hatchery failures. XB, although only in its fourth generation, was a synthetic line (DeBrosse & Allen 1996) comprised of several severely inbred lines (Haskin & Ford 1979, Ford & Haskin 1987) held for many generations at the Haskin Shellfish Research Laboratory.

Material from Louisiana and wild natives of Chesapeake Bay were crossed into these lines. Louisiana, and probably Gulf populations in general, have a long history of exposure to *Perkinsus marinus* and have developed innate resistance to Dermo disease (Bushek & Allen 1996, Ragone Calvo et al. 2003, Encomio et al. 2005). Wild oysters from the Louisiana Grant Terre (LGT) and Caminada Bay, as well as a selected line from Louisiana State University (LSU), the so-called OBOY (Oyster Bayou) line (Leonhardt 2010) were obtained. Last, wild oysters from Mobjack Bay, Virginia, where both Dermo and MSX diseases are endemic (Burreson 1991), were obtained both for hybridizing and as a control population. Comparing our selected lines with wild oysters from this site tests the wild type against artificial selection (*cf.* Carnegie & Burreson 2011) and serves as a yardstick for breeding progress, or lack thereof. Using these various imports, a number of new and hybrid lines were created. By 2004 there were 8 hatchery-reared lines under test (Table 1).

### *Hatchery and Nursery*

The 8 hatchery lines and the control, hereafter named lines, were produced at the VIMS, ABC hatchery in Gloucester Point, Virginia, in June 2004, following the then-standard ABC hatchery protocols. All broodstock were allowed to ripen naturally in the York River and were brought into the hatchery for spawning. Individual oysters were shucked, and sex was determined using a microscope. Gametes were stripped spawned by making lacerations on the gonad tissue and the gametes gently teased out using pressure and the flushing of filtered seawater. After stripping, the gametes from each individual were checked again to confirm sex assignment and, for females, to rule out hermaphrodites. Gametes were then assessed for quality. Sperm from males were assessed for motility. Eggs were filtered through a 43- $\mu$ m screen onto a 20- $\mu$ m screen, assessed, and counted. Equal numbers from each female were pooled. The pool of eggs was then divided into equal aliquots among the total number of viable males, and each aliquot was fertilized with sperm from an individual male. This process was repeated for each line.

The goal was to reach an effective breeding number ( $N_e$ ) of at least 50 for each spawn, determined by the equation:

$$N_e = \frac{4N_mN_f}{N_m + N_f},$$

where  $N_m$  is the number of males and  $N_f$  is the number of females.

The effective breeding numbers for the lines are shown in Table 1. After fertilization, pooled zygotes were stocked into

TABLE 1.  
ABC line names and their genetic and geographical origins in the 2004 cohort.

Line	Genetic origin	Geographical origin	F <sub>0</sub>	Gen	N <sub>e</sub>
DBY	Delaware Bay, selected in Virginia	East Coast (EC)	1987	6	54
XB	CROSBreed line begun at HSRL, NJ	East Coast (EC)	1992	4	35
DMO	DBY × wild Mobjack Bay hybrid	East Coast (EC)	2000	3	53
DXB	DBY × XB hybrid	East Coast (EC)	2002	2	24
DBLA	LGT × DBY hybrid	Hybrid EC × Louisiana (HY)	2004	1	57
CAMX	XB × wild Caminada Bay, LA	Hybrid EC × Louisiana (HY)	2004	0	57
LGT	Line started from wild Grand Terre, LA	Louisiana (LA)	2002	2	55
OBOY	LSU line originating from Oyster Bayou, LA	Louisiana (LA)	2002	2	NA
MBC	F <sub>0</sub> of wild oysters from Mobjack Bay, VA	East Coast control (C)	Various	—	30

F<sub>0</sub>, first year the line was spawned; Gen, generation for the 2004 year-class; NA, data not available; N<sub>e</sub>, effective population size for 2004 spawn.

and reared in aerated 2- $\mu$ m filtered, UV-treated York River water in 200-L tanks. Water was changed every other day and larvae retained on mesh screens. Daily feeding for larvae began on day 1 with a minimum ration of *Pavlova* sp. at 20,000 cells/mL. Larval feeding increased by 5,000 cells/mL every few days depending on the health, size, and density of each culture. On about day 4, 5% *Chaetoceros neogracile* was introduced and was increased 5% daily. On about day 8, or when most larvae were between 120–150  $\mu$ m, 5% of their ration included *Tetraselmis* sp.<sub>2</sub> and by day 12, the larval diet was 45% *Pavlova*, 45% *Chaetoceros*, and 10% *Tetraselmis*, continuing through setting on microcultch in downwellers. When spat were large enough to be retained on a 500- $\mu$ m screen (>700  $\mu$ m), they were transferred to the land nursery system of upwellers where they fed on raw water from the York River. They were held in the nursery until they attained a size of 10 mm, after which they were held in the York River until they were deployed in the field.

### Field Testing

#### Sites

Four sites throughout the Virginia portion of the Chesapeake Bay were used in the study (Fig. 1). Sites were characterized by different salinity regimes. The high-salinity site (20–30 ‰) was located on the sea side of the eastern shore of Virginia in Wachapreague. Two sites at medium salinity (15–23 ‰), where disease pressure from both diseases is high, were located in the York River on the VIMS beach at Gloucester Point and in Broad Bay on the Lynnhaven River. Last, the Yeocomico River at Kinsale was the low-salinity testing site (9–15 ‰), where disease pressure from *Perkinsus marinus* (or Dermo) is low and for *Haplosporidium nelsoni* (or MSX) is absent.

Lines were deployed in all sites for 2 y starting in November 2004 and ending in December 2006. The grow-out method used was rack and bag culture. All racks were placed in the intertidal

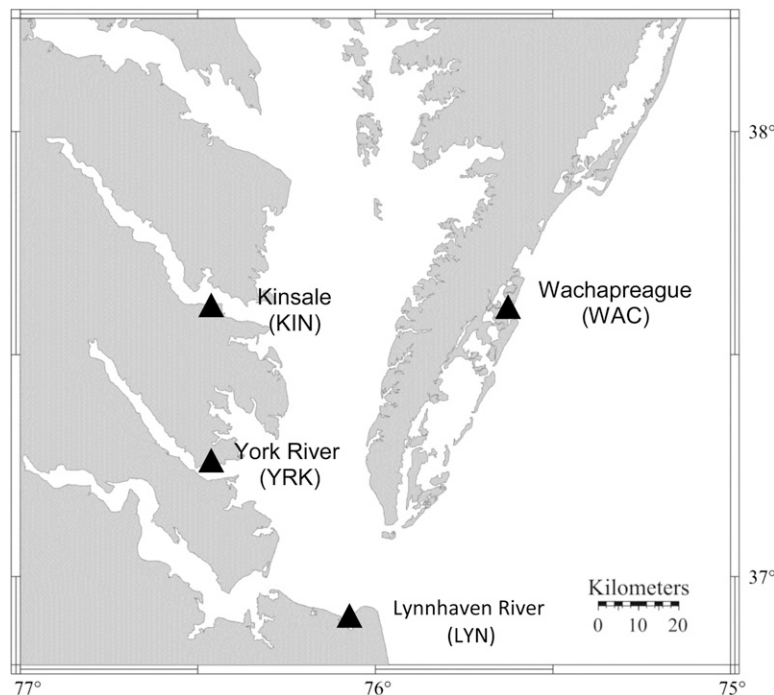


Figure 1. Location of test sites for selectively bred lines of *Crassostrea virginica* in Chesapeake Bay.

zone from March to November and were exposed daily at low tide. From December to March, racks were put in the subtidal zone to prevent freezing during the winter months. Each line was deployed in 8 replicates of 200 oysters. Four of these bags were used in a survival trial and the other 4 bags were used for growth and disease sampling.

#### Survival Data

All live and dead oysters within each of the 4 survival bags were counted every 4–8 wk from March to December for the 2-y test period. Cumulative mortality was calculated as the total number of oysters that died up to each interval divided by the number of live oysters at the beginning.

#### Disease Sampling

Twenty-five oysters from each line within each site were collected during the spring and fall in each of the 2 years of the experiment to assess disease status. Disease diagnosis was conducted by the VIMS Shellfish Pathology Laboratory. Briefly, *Perkinsus marinus* was diagnosed using Ray's fluid thioglycollate medium (RFTM) assays (Ray 1952) on combined mantle, gill, and rectal tissue. Infection intensity was rated based on Mackin (1962) and Ray (1954). Histological examination was performed for the diagnosis of *Haplosporidium nelsoni* and other parasites, pathogens, and abnormalities. Shucked oyster meats were preserved in Davidson's AFA and processed using standard histological techniques. Tissue sections were cut at 6  $\mu\text{m}$  and stained with Harris' hematoxylin–eosin (Burreson et al. 1988). The presence of parasites was detected by light microscopy. Infection intensities of *H. nelsoni* were rated as light, moderate, or heavy, as described by Burreson et al. (1988). For both *P. marinus* and *H. nelsoni* infections, the proportion of seriously infected oysters was determined from the intensity data. Following the procedures of Haskin and Ford (1982) and Carnegie and Burreson (2011), this proportion was calculated as the number of oysters characterized by moderate or greater intensity (for *P. marinus*, these were all the oysters at 3–5 on the Mackin scale; for *H. nelsoni*, all the oysters in the moderate and heavy categories) divided by the number of oysters sampled for disease diagnosis.

#### Growth data

One of the 4 growth bags was sampled randomly for 30 oysters per line every time a mortality count was conducted. At each sampling, length and whole live body weight were measured. Length is defined as the longest dimension from umbo to growing edge, and was measured with calipers to the nearest millimeter. Using these data, the percentage of oysters that reached market size (76 mm) at the end of the test period was obtained. Whole live weight was measured to the nearest 0.1 g using electronic scales.

#### Environmental Parameters

Temperature and salinity were recorded monthly using a YSI probe (Yellow Springs Instruments, Yellow Springs, OH) and, additionally, temperature was recorded every 3 h using 2 iBCod temperature loggers (Maxim Integrated Products, Sunnyvale, CA) in each site.

#### Statistical Analysis

All statistical analyses were performed using the GenStat for Windows software package, 15<sup>th</sup> edition. Cumulative survival

data at the end of the test period were arcsine-transformed before analysis. Survival and growth data were analyzed using the REML procedure (Payne et al. 2012) with the model

$$Y_{ijk} = \mu + \text{line}_i + \text{site}_j + (\text{line} \times \text{site})_{ij} + \text{rep}_{k(ij)} + e_{ijk},$$

where  $Y_{ijk}$  is the dependant variable (survival, length, or weight);  $\mu$  is the overall mean; "line" is the fixed effect of line; "site" is the fixed effect of site; "line  $\times$  site" is the interaction between line and site; "rep" is the random effect of replicate, nested within site and line (for survival only); and  $e_{ijk}$  is the residual error. To test whether replicate was significant, a test statistic for the likelihood ratio was calculated by subtracting the deviance obtained when rep was included in the model from that obtained when it was excluded in the model. The significance of the statistic was tested by comparing with critical values of chi square at the 95% significance level. For pathology, data for the proportion of serious infections (medium and higher) were tested. A chi square analysis ( $\alpha = 0.05$ ) was performed to compare the observed proportions of serious *Perkinsus marinus* infections with expected proportions. Analyses were done among sites to test for site effects, and within each site to test the differences between lines at each site. The line OBOY was excluded from growth and disease analysis because all animals from this line died in all but the KIN site.

## RESULTS

#### Temperature and Salinity

Mean monthly temperatures at each site are presented in Figure 2. All 4 sites showed clear and similar seasonal patterns throughout the 2 y of the study, with water temperatures varying little from site to site. The estimated cumulative day-degrees were 5,944 for Wachapreague, 7,010 for Lynnhaven, 6,710 for York River, and 6,526 for Kinsale. Mean temperatures of the sites throughout the experiment were Wachapreague, 16.1°C; Lynnhaven, 19.8°C; York, 17.3°C; and Kinsale, 17.1°C. Salinity varied from consistently higher than 20 ‰ at Wachapreague on the sea side of the eastern shore of Virginia to always lower than 15 ‰ at Kinsale (Fig. 3). The Lynnhaven and York River sites had similar salinity ranges from 14–22 ‰.

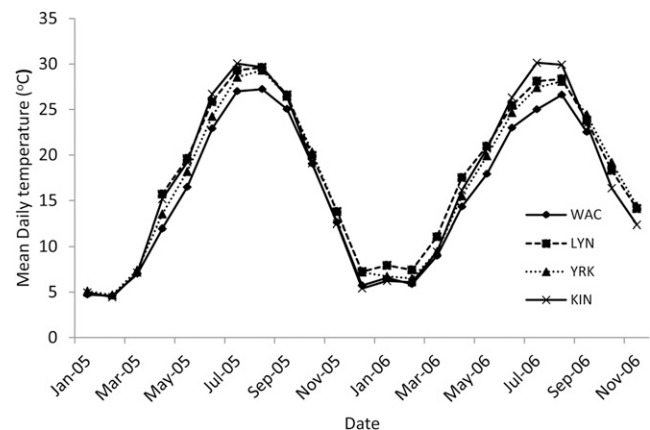


Figure 2. Mean water temperatures recorded by the iBCod temperature logger from January 2005 until December 2006 at all 4 test sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

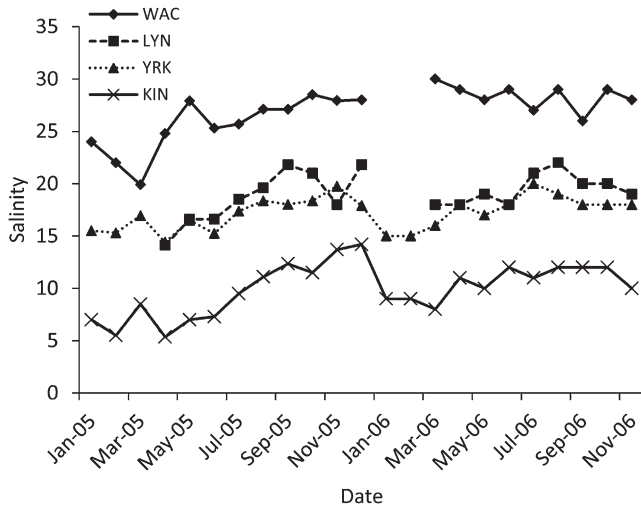


Figure 3. Monthly salinity at each of the test sites—WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale—recorded using a YSI probe on monthly sampling visits.

#### Survival

The results from the REML analysis showed a significant effect of site ( $P < 0.001$ ), line ( $P < 0.001$ ), and site  $\times$  line interaction ( $P < 0.001$ ; Table 2). Replicate was not significant ( $P > 0.05$ ), meaning there was little variation within site for each line among bags. The proportion of live oysters (i.e., overall mean survival of all lines) deployed in each of the 4 sites at each sampling time is shown in Figure 4, and illustrates the site differences in mean survival. At the end of the 24-mo testing period, survival was the lowest at the Lynnhaven and York River sites (22%–23%), intermediate at the Wachapreague site (28%), and the greatest at Kinsale (69%). The mortality patterns from the Lynnhaven and York River sites were similar, with accelerated mortalities occurring between summer and fall of both years. In Wachapreague, the majority of the mortality

TABLE 2.

Results of REML analyses showing effect of site, line, and site-and-line interactions on survival, shell length, and whole body weight for lines of selectively bred *Crassostrea virginica*.

	Wald statistic	df	F	F prob
Survival				
Site	2,268.58	3	762.05	<0.001
Line	1,343.61	8	167.95	<0.001
Site $\times$ line	691.92	24	28.83	<0.001
Shell length				
Site	35.27	3	11.76	<0.001
Line	148.29	7	21.18	<0.001
Site $\times$ line	63.03	21	3	<0.001
Whole body weight				
Site	29.73	3	9.91	<0.001
Line	313.16	7	44.74	<0.001
Site $\times$ line	58.53	21	2.79	<0.001

Analyses were done at the last sampling point (fall 2006).

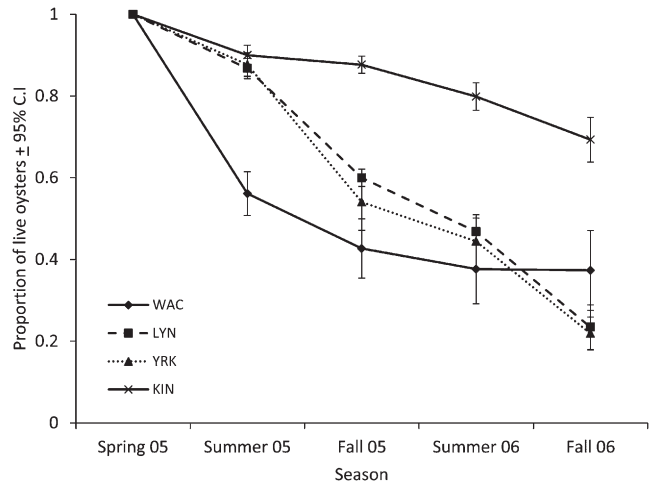


Figure 4. Proportion of live oysters ( $\pm 95\%$  CI) from selectively bred lines of *Crassostrea virginica* and the control at each sampling site from spring 2005 to fall 2006. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

occurred during the first year of the study, between deployment and the first summer. Mortality at Kinsale was highest between the summer and fall of the second year of the study.

Some of the source of variation in survival among lines can be explained by grouping lines by genetic origin (i.e., East Coast (EC), hybrids between the EC and Louisiana lines (HY), Louisiana (LA), and the wild control (C)). At Kinsale, survival was similar among genetic origins (Fig. 5). At all other sites, survival was consistently the greatest for the EC lines, intermediate for the HY lines, and lowest for the LA lines, for which more than 70% mortality occurred by the fall of the first year (Fig. 5).

By the end of the trial, at all 4 sites, the survival of the selected lines was, on average, greater than that of the wild control (Fig. 6). However, not every line outperformed the control (Fig. 6, Table 3), and in all sites, some selected lines had greater survival than the control and some had lower, demonstrating the nature of the line  $\times$  site interaction. The rankings among lines were only similar between Lynnhaven and the York River. Where salinity and disease pressure was high (i.e., Wachapreague, Lynnhaven, and the York River), established EC lines (DBY, XB) had greater survival than the wild control, demonstrating genetic improvements for survival. Hybrids between established EC lines (DBY  $\times$  XB and DBY  $\times$  wild Mobjack Bay) also did better than controls, except DMO in Wachapreague. The effect of introducing Gulf genes into DBY and XB lines—yielding HY crosses DBLA (DBY  $\times$  LGT) and CAMX (XB  $\times$  Caminada Bay)—was mixed. Where salinity and likely disease influence was high, DBLA underperformed and CAMX outperformed the wild control, whereas it was the opposite at Kinsale (Fig. 6, Table 3). There were also differences between the two Louisiana lines (LA), with greater survival for the LGT than the OBOY (Table 3), the latter having nearly complete mortality in all three high-salinity sites.

#### Disease

#### Dermo

The proportion of serious infections for *Perkinsus marinus* was tested by chi square analysis on the last sampling in fall

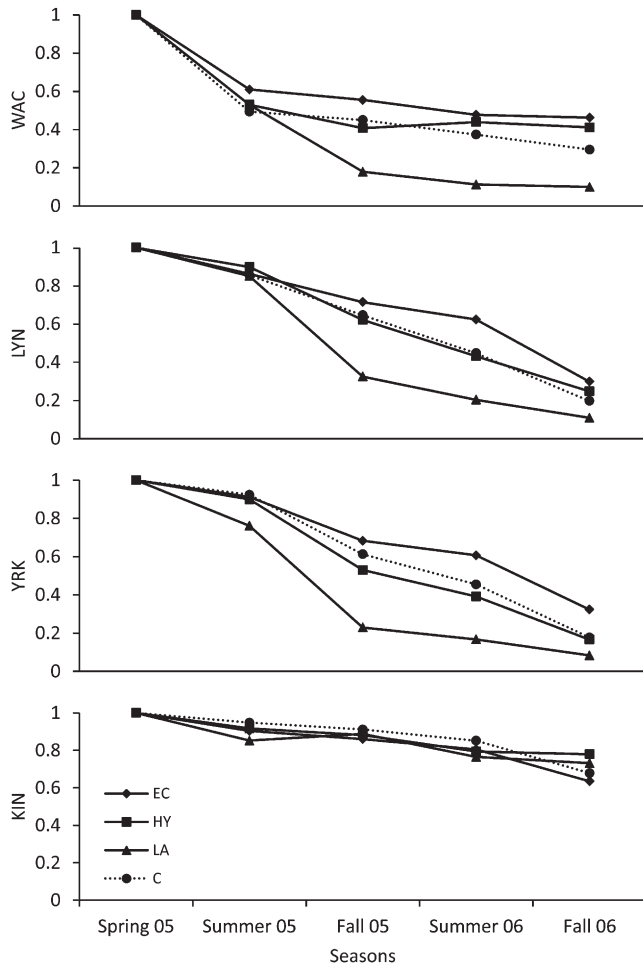


Figure 5. Proportion of live *Crassostrea virginica* at each sampling site averaged across genetic origins: EC, East Coast; HY, hybrid between EC and Louisiana; LA, Louisiana; plus the wild control (C) from spring 2005 to fall 2006. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

2006. There were significant site effects (chi square = 83.74,  $P < 0.001$ ). Within all sites, there were no significant differences among lines apart from in Wachapreague (chi square, = 28.80,  $P < 0.001$ ). Overall, the levels of infection (prevalence), intensity of *P. marinus*, and percentage of serious infections increased during the 24 mo of the trial. In Wachapreague, Dermo disease intensity, as indicated by the Mackin rating for *P. marinus*, peaked at 1.7 by fall 2006, so although the disease was present, serious infections remained low until fall 2006. Dermo disease intensity (Mackin) was the greatest and peaked at 2.7 and 2.9 in Lynnhaven and the York River, respectively. By fall 2006, 75% of all oysters sampled at Lynnhaven site had serious infections. In the York River, this number was 63%, whereas at Kinsale the intensity reached 1.6 by the end of the study and only 1.7% of oysters sampled had serious infections (Table 4), accounting for the significant differences between the sites.

The percentage of serious infections at the end of the trial is shown in Figure 7, by line and by site, and, as with mortality, the ranking of lines was not consistent across sites. At Wachapreague, the EC lines had greater serious infections than the HY and Louisiana line LGT. At Lynnhaven and the York River,

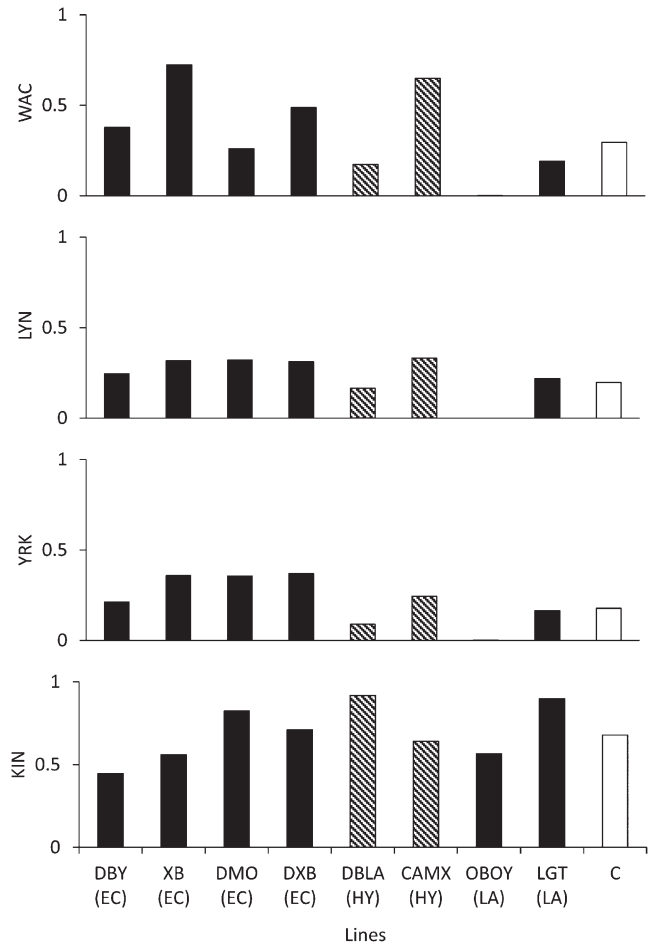


Figure 6. Proportion of live oysters from selectively bred lines of *Crassostrea virginica* and control at the end of the trial in fall 2006 within each line at each site. East Coast (EC; solid), hybrid between EC and Louisiana (HY; striped), and Louisiana (LA; solid) refer to geographical origin of lines: East Coast, hybrid, or Louisiana. C, control (white). Line names are listed in Table 1. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

where infections were greatest by fall 2006, the 3 genetic origins showed greater percentages of serious infections, with no general trend among lines. At Kinsale, serious infections were similar between the EC lines, slightly greater than the HY, and LA had the least serious infections (Fig. 7).

MSX

Prevalence, percentage of serious infections, and Mackin index for *Haplosporidium nelsoni* infections in all sites are presented in Table 4. Infection by *H. nelsoni* was not detected at all at Kinsale. At Wachapreague, it appeared that infections declined rapidly after the first year and were undetectable by fall 2006, whereas at Lynnhaven and the York River, there was only a gradual decline in prevalence and intensity. Serious infections peaked in fall 2005.

Prevalence and percentage of serious infections for *Haplosporidium nelsoni* infections at Wachapreague, Lynnhaven, and York River for all 4 sampling events are shown in Table 5. During the first two sampling periods, when MSX disease incidence was greatest, infection and intensity reflected genetic

TABLE 3.

Difference (%) in survival and whole weight over wild controls for selectively bred lines and control of *Crassostrea virginica* at the end of the trial in fall 2006.

Line	Origin	Survival					Weight				
		Wach	Lynn	York	Kin	Mean	Wach	Lynn	York	Kin	Mean
DBY	EC	128	125	119	66	109.5	100	127	116	103	111.5
XB	EC	245	161	201	82	172.2	105	104	92	82	95.8
DMO	EC	88	163	201	121	143.2	100	123	91	99	103.2
DXB	EC	165	158	208	105	159.0	108	115	107	98.5	107.1
Mean		156.5	151.8	182.2	93.5		103.2	117.5	101.5	95.6	
DBLA	HY	59	84	50	135	82.0	134	143	101	141	129.8
CAMX	HY	220	168	138	94	155.0	134	163	122	143	140.5
Mean		139.5	126.0	94.0	114.5		134.0	153.0	111.5	142.0	
OBOY	LA	0.4	0	0.7	83	21.0	NA	NA	NA	153	153
LGT	LA	64	111	93	132	100.0	135	157	146	156	148.5
Mean		32.2	55.5	46.8	107.5		135.0	157	146	154.5	
MBC	C	100	100	100	100		100	100	100	100	

Percent difference is calculated as  $\left(\frac{\text{No. of oysters remaining in a line}}{\text{No. of oysters remaining in control}}\right) \times 100$  for survival and  $\left(\frac{\text{Mean length of oysters in a line}}{\text{Mean length of control oysters}}\right) \times 100$ . Values less than 100% performed worse than the control. Kin, Kinsale; Lynn, Lynnhaven; NA, No data available mortality precluded measurements; C, control. York, York River; Wach, Wachapreague.

origin across all 3 sites. Over all three high-salinity sites in 2005 (Wachapreague, Lynnhaven, and York River), the average prevalence and percentage of serious infections was the lowest for EC, intermediate for HY and control, and the greatest for LA.

#### Growth

Based on REML analysis of data collected in fall 2006, there were, again, significant effects of site ( $P < 0.001$ ) and line ( $P < 0.001$ ), and significant site  $\times$  line ( $P < 0.001$ ) interactions for both length and weight (Table 2). For length, growth at Wachapreague and Kinsale seemed retarded initially, followed by a period of compensatory growth at both sites (Fig. 8). The slow start can be attributed to acclimation of the mid-salinity lines to the high- or low-salinity sites. Basically, nearly all the oysters at all sites achieved market size (75 mm) by fall 2006, including the control.

For whole body weight, growth at all sites was similar until winter 2005 and 2006, when growth at Lynnhaven and York River continued, but did not at Wachapreague or Kinsale (Fig. 9). By fall 2006, the average body weight at the Kinsale site was about 10% less than the other sites. Figure 10 depicts the final whole body weight of all lines deployed to all sites, and comparisons are drawn to the control in Table 3. In general, all lines with LA genes exceeded those of EC origin and the control at all sites (Fig. 10).

#### DISCUSSION

For commercial oyster aquaculture, survival to harvest is paramount. In the waters of the Chesapeake Bay, where *Haplosporidium nelsoni* and *Perkinsus marinus* persist, disease resistance is the most important trait. During the past three decades, genetic resistance or tolerance to these diseases has been the focus of numerous studies (Haskin & Ford 1979, Ford & Haskin 1987, Barber et al. 1991, Burreson 1991, Bushek & Allen 1996, Ragone Calvo et al. 2003, Encomio et al. 2005). The purpose of our work at ABC is the implementation of these

results—as well as improving other traits—for the advancement of oyster aquaculture—specifically, incorporating and improving lines for commercial use as broodstock. As with previous authors (Burreson 1991, Ragone Calvo et al. 2003, Encomio et al. 2005, Nell & Perkins 2006), our data indicate that mass selection for disease resistance is successful and improvements can be realized against the wild type. Performance, though, depends on three factors: location (site), background genetics (line), and a site-by-line interaction.

In the current study, differences in performance across sites for the same line are environmental effects, and the interaction between the site and the line is a genotype-by-environment interaction ( $G \times E$ ). Genotype-by-environment interactions occur when different genotypes, in this case lines, do not respond the same way to different environments. Thus, a specific difference in the environment (site) may have a greater effect on some genotypes than others. Alternatively, there may be a change in the rank of genotypes when measured under different environmental conditions (Falconer & Mackay 1996). Both scenarios are true in this study. Thus, selection decisions made under 1 set of conditions will correlate poorly with genetic gains when progeny are reared under disparate environments (Kvingedal et al. 2008).

#### Survival and Disease Testing

Statistically, each site can be viewed as a different replicate of the same experiment because a random assemblage of the same genes were deployed at each of the sites. Within each site, temperature and salinity were measured. Temperature profiles across sites showed little variation, but the salinity profiles varied markedly (Figs. 2 and 3). Thus, salinity drives survival differences. The greatest mortalities were observed at the York River and Lynnhaven sites. These 2 sites had salinity and temperature conditions most favorable for the pathogenic proliferation of *Haplosporidium nelsoni* ( $>20^{\circ}\text{C}$ ,  $>15\text{‰}$ ) and *Perkinsus marinus* ( $>25^{\circ}\text{C}$ ,  $>15\text{‰}$ ). This was corroborated by histopathological results; the infection intensity, percentage of



TABLE 4.

Mean percent infection (prevalence), percentage of serious (moderate to high) infections, and Mackin indices for infections of *Perkinsus marinus* and *Haplosporidium nelsoni* across all lines at each test site throughout the course of the trial of selectively bred lines and control of *Crassostrea virginica*.

	Wachapreague	Lynnhaven	York River	Kinsale
<i>P. marinus</i>				
Prevalence (%)				
Summer 2005	6.2	36.4	22.0	1.8
Fall 2005	33.3	86.8	95.5	22.2
Summer 2006	23.8	87.5	80.4	13.3
Fall 2006	81.0	98.0	100	77.1
Percentage of serious infections				
Summer 2005	0	15.6	6.2	0
Fall 2005	10.2	50.3	55.8	0.3
Summer 2006	6.1	59.5	39.0	0.4
Fall 2006	36.5	75.0	63.2	1.7
Mackin index				
Summer 2005	0.1	0.7	0.3	0.01
Fall 2005	0.5	2.0	2.3	0.3
Summer 2006	0.3	2.3	1.7	0.3
Fall 2006	1.7	2.9	2.7	1.6
<i>H. nelsoni</i>				
Prevalence (%)				
Summer 2005	28.0	24.4	27.6	0
Fall 2005	5.8	17.2	22.7	0
Summer 2006	1.6	11.9	19.1	0
Fall 2006	0	13.0	14.2	0
Percentage of serious infections				
Summer 2005	21.3	3.6	11.1	0
Fall 2005	3.1	13.7	15.0	0
Summer 2006	0.5	6.4	11.5	0
Fall 2006	0	5.5	7.0	0
Mackin index				
Summer 2005	1.1	0.3	0.6	0
Fall 2005	0.2	0.7	0.7	0
Summer 2006	0.03	0.3	0.6	0
Fall 2006	0	0.3	0.3	0

serious infections, and prevalence was greatest at those sites for both pathogens (Table 4). Salinity was too low for MSX disease at Kinsale; hence, there were no detectable levels of *H. nelsoni*. Levels of infection for *P. marinus* were also low, and it should not be the cause of the mortality. At Wachapreague, the survival rate was low. With the exception of a high percentage of serious *H. nelsoni* infections in summer 2005, the cause of the mortality throughout the course of the trial cannot be attributed easily to either *H. nelsoni* or *P. marinus* (Table 4). Disease screening was accomplished in April 2005, with prevalence of both diseases less than 7%. It is evident that there was another cause for mortalities on the sea side than in the Bay.

To try to clarify the cause of the line effects, survival of the genetic groups within each site was plotted in Figure 5. The progenitors of the EC group were domesticated animals from

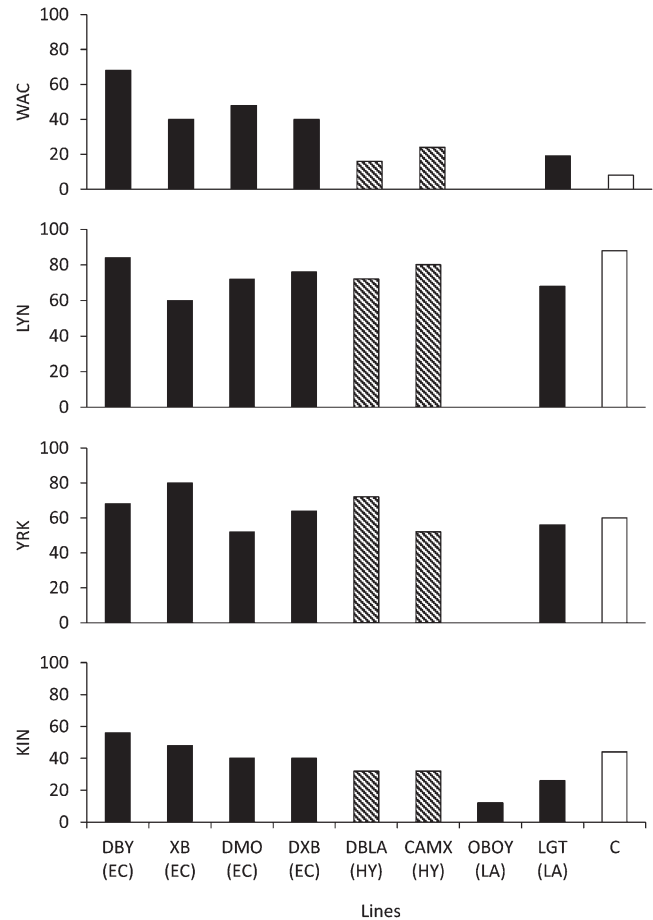


Figure 7. Percentage of serious (moderate to high) infections of *Perkinsus marinus* for each line of selectively bred *Crassostrea virginica* and the control at each site at the end of the trial in fall 2006 sites. East Coast (EC; solid), hybrid between EC and Louisiana (HY; striped), and Louisiana (LA; solid) refer to geographical origin of lines: East Coast, hybrid, or Louisiana. C, control (white). Line names are listed in Table 1. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

trials conducted by Ford and Haskin (1987) (XB) and Burreson (1991) (DBY), and as such had been selected for resistance to *Haplosporidium nelsoni* over several to many generations. Consequently, their survival during the first year of the trial, when MSX-induced mortality is usually highest (Burreson 1991), was relatively high compared with the other groups and the control. Conversely, the survival of the LA groups was low at high-salinity sites, with about 20% by fall 2005 (Fig. 5). Pathology data for the trial period (Table 5) at Lynnhaven and York River show that this is likely MSX disease mortality. Comparing the intensity and prevalence of *H. nelsoni* among all 4 groups, it is clear that LA groups were more susceptible to the parasite. In addition, the EC groups had lower prevalence, thereby demonstrating a level of resistance. Furthermore, the survival of the HY groups were intermediate, which shows the nature of the additive effect for MSX resistance. Another clear indication of the heritability for MSX disease resistance can be seen in survival and severity between LGT and OBOY. The LGT line was imported to Virginia before OBOY and had been subjected to MSX disease for two generations. The LGT line had clearly developed considerable

TABLE 5.

Mean percent infection (prevalence) and percent serious infections (moderate to high) of *Haplosporidium nelsoni* in selectively bred oyster lines and control of *Crassostrea virginica* grown in Wachapreague, Lynnhaven, and York River.

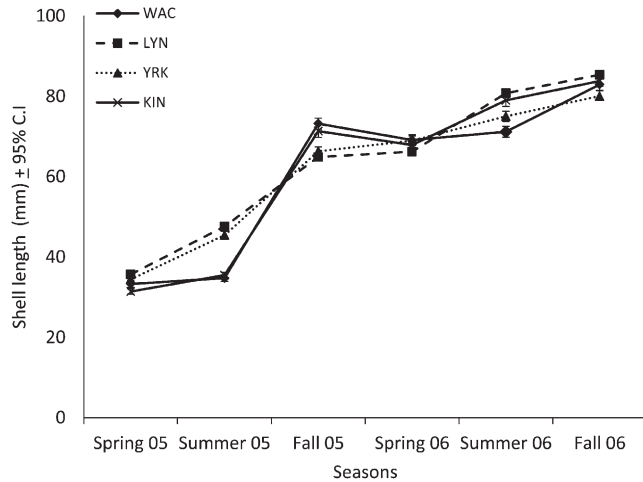
Line	Origin	Summer 05		Fall 05		Summer 06		Fall 06	
		Prevalence (%)	Serious (%)	Prevalence (%)	Serious (%)	Prevalence (%)	Serious (%)	Prevalence (%)	Serious (%)
Wachapreague									
DBY	EC	8	8	4	4	4	4	0	0
XB	EC	0	0	4	0	0	0	0	0
DMO	EC	12	8	4	4	0	0	0	0
DXB	EC	4	0	0	0	0	0	0	0
Mean		6.0	4.0	3.0	2.0	1.0	1.0	0	0
DBLA	HY	32	28	0	0	4	0	0	0
CAMX	HY	32	8	8	0	0	0	0	0
Mean		32.0	18.0	4.0	0	2.0	0	0	0
OBOY	LA	100	100	0	0	NA	NA	NA	NA
LGT	LA	52	40	12	4	0	0	0	0
Mean		76.0	70.0	6.0	2.0	0	0	0	0
MBC	C	12	0	20	16	4	0	0	0
Lynnhaven									
DBY	EC	4	0	4	0	8	4	0	0
XB	EC	4	0	4	4	12	0	0	0
DMO	EC	8	4	0	0	8	4	0	0
DXB	EC	4	0	8	0	4	4	4	0
Mean		5.0	1.0	4.0	1.0	8.0	3.0	1.0	0
DBLA	HY	24	8	16	8	23	15	32	24
CAMX	HY	28	0	4	0	20	16	16	0
Mean		26.0	4.0	10.0	4.0	21.5	15.5	24.0	12.0
OBOY	LA	80	12	79	79	NA	NA	NA	NA
LGT	LA	48	8	36	28	8	4	28	8
Mean		64.0	10.0	57.5	53.5	8.0	4.0	28	8
MBC	C	20	0	4	4	12	4	24	12
York River									
DBY	EC	12	4	4	4	8	4	12	0
XB	EC	4	4	4	0	8	8	4	4
DMO	EC	0	0	4	0	16	4	8	4
DXB	EC	16	0	4	4	0	0	8	0
Mean		8.0	2.0	4.0	2.0	8.0	4.0	8.0	2.0
DBLA	HY	8	4	0	0	32	20	40	20
CAMX	HY	44	12	36	4	32	20	24	12
Mean		26.0	8.0	18.0	1.0	32.0	20.0	32.0	16.0
OBOY	LA	96	60	91	87	NA	NA	NA	NA
LGT	LA	24	12	40	24	20	16	8	4
Mean		60.0	36.0	65.5	55.5	20.0	16.0	8.0	4.0
MBC	C	40	4	28	12	40	20	20	12

“Line” refers to crosses detailed in Table 1. EC, East Coast; HY, hybrid between EC and LA; LA, Louisiana origin; NA, mortality precluded measurements; C, control.

disease resistance compared with the naive OBOY line, which had also been selected for 2 generations, but in Louisiana for Dermo disease resistance only (Leonhardt 2010). Altogether, the significant  $G \times E$  interaction for survival in this study can be defined more specifically as a salinity-by-genotype interaction.

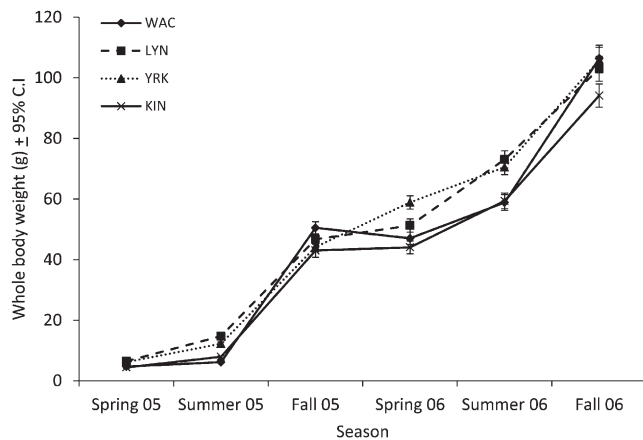
There appears to be a nongeneralized response for susceptibility to the two parasites *Haplosporidium nelsoni* and *Perkinsus marinus*—that is, resistance to one parasite does not confer resistance to the other. A nongeneralized response was also observed by Burreson (1991), although the reverse seemed to be true in his study; *H. nelsoni*-resistant oysters were more susceptible to *P. marinus*. In a similar experiment to the current study,

Burreson (1991) compared the performance of 6 groups, 3 of which had been selected previously for MSX resistance in Delaware Bay and 3 wild lines from Virginia. He looked at susceptibility to *H. nelsoni* and *P. marinus* by assessing mortality and sampling oysters periodically to look for the presence and intensity of both pathogens. The MSX-resistant lines showed lower susceptibility to *H. nelsoni*, but all 6 lines were highly susceptible to *P. marinus*. Throughout the 3 y of the study, mortality of the MSX disease-resistant lines was significantly greater than wild groups, which died of Dermo disease. He suggested that MSX resistance was not a generalized response to pathogens, but is specific to MSX.

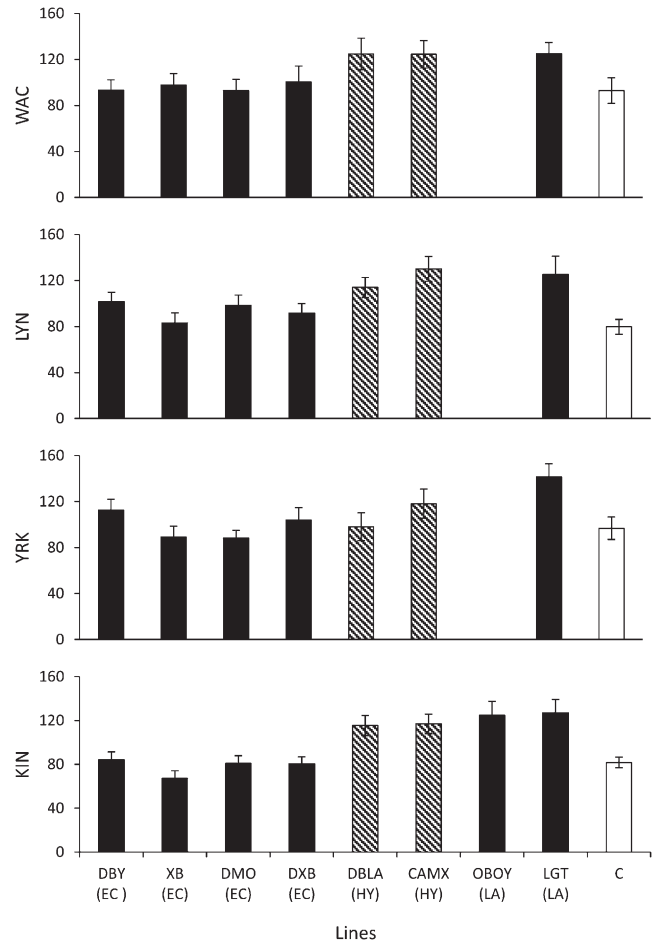


**Figure 8.** Mean shell length ( $\pm 95\%$  CI) of oysters sampled from selectively bred *Crassostrea virginica* and the control by site from spring 2005 to fall 2006. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

In contrast, Ragone Calvo et al. (2003) reported that after 2 generations of selection in the presence of *Perkinsus marinus*, one of their lines developed dual resistance to both parasites. In this case, the resistant lines compared were of different origin than the ones used by Burreson (1991). They compared the VIMS line (DBY) with the same wild control line used by us (Mobjack Bay), as well as a wild Louisiana population and another Virginia wild population, all deployed to 3 sites. Two of these sites were identical to the current study (York River and Wachapreague; the third site was the Wicomico River). They conducted the experiment over 2 generations ( $F_3$  and  $F_4$ ), and in both generations they found similar results as observed here (i.e., significant site, line, and site-by-line interactions). The Louisiana group showed greater susceptibility to *Haplosporidium nelsoni* and the VIMS line survived the best. In their trial, however, the largest source of variation was not site, but line. In our study, site was the largest source of variation. This minor discrepancy can be explained by the designs of the studies. Both



**Figure 9.** Mean whole body weight ( $\pm 95\%$  CI) of oysters sampled from selectively bred *Crassostrea virginica* and the control by site spring 2005 to fall 2006. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.



**Figure 10.** Mean whole body weight ( $\pm 95\%$  CI) of oysters sampled from selectively bred *Crassostrea virginica* and the control at each site at the end of the trial in fall 2006. East Coast (EC; solid), hybrid between EC and Louisiana (HY; striped), and Louisiana (LA; solid) refer to geographical origin of lines: East Coast, hybrid, or Louisiana. C, control (white). Line names are listed in Table 1. Sites: WAC, Wachapreague; LYN, Broad Bay, Lynnhaven; YRK, York River; and KIN, Yeocomico River, Kinsale.

used a similar range of salinities (Ragone Calvo et al. (2003), Wicomico to Wachapreague; ours, Kinsale to Wachapreague). However, the study by Ragone Calvo et al. (2003) used 3 wild populations and a domesticated 1, whereas 8 domesticated lines and 1 wild strain were used in the current study. Intuitively, variance attributed to “line” would seem smaller in the latter case.

It is possible to make direct comparisons with this study and that of Ragone Calvo et al. (2003) because one of the lines (DBY), the wild control (Mobjack), and two test sites (York River and Wachapreague) are the same. It is also plausible to compare the effects of selection. In our study, the DBY line had been under selection for two additional generations ( $F_6$ ). Ragone Calvo et al. (2003) reported that, in the York River, 47% of DBY oysters were alive at the end of the trial, whereas in the current study, 21% remained. Similarly, survival of the line at Wachapreague was also greater—64% for  $F_4$  compared with 38% for  $F_6$ .

Comparing the histological results between the two trials is also possible because they were both done by the VIMS

Shellfish Pathology Laboratory. Prevalence and intensity of heavy infections in the current study were greater. This observation highlights one of the largest complications with breeding oysters. Oysters are grown under conditions that cannot be controlled, and that change temporally and spatially. For selection for disease resistance, environmental conditions, such as temperature and salinity, determine the activity and virulence of both *Haplosporidium nelsoni* and *Perkinsus marinus* within the host (Haskin & Ford 1982, Villalba et al. 2004). For breeding, this means that repeatable disease challenges are almost impossible because disease pressure could change from one generation to the next, possibly making cumulative gains for disease resistance difficult. For MSX resistance, lack of pressure has not been problematic at some sites, such as the York River, where disease pressure is constant (Burrenson 1991). The same selection program at, for example, Lynnhaven would be much less successful. Selection for Dermo disease is more problematic because constant disease pressure is much less predictable, and highly dependent on environment and contagion (Villalba et al. 2004), even in the York River. But perhaps more important, the effects of Dermo disease are progressive. Little effect is seen in younger populations (Burrenson 1991), with increasingly pathogenic effects in subsequent years. Lack of predictable disease challenge also complicates the process of evaluating genetic gain because, as observed in this instance, comparing across generations can be futile if the challenge is different.

It is clear from other studies that heritability for resistance to MSX disease is high. It is not as clear that this is true for Dermo disease. For MSX disease, high heritability was demonstrated decades ago by Haskin and Ford (1979, 1987), who compared the survival of wild oysters with hatchery-reared groups that had survived a natural challenge over 5 generations. After just one generation of selection under high disease pressure, they observed a 4-fold increase in survival of the hatchery line compared with the wild control. By the third generation, all the realized gain had been achieved, with the largest generational gain between the  $F_0$  and  $F_1$ . The pattern of selection response observed by Haskin and Ford (1979, Ford & Haskin, 1987) suggests two things. First, heritability for resistance to MSX disease is high, perhaps controlled by only a few genes; second, selection was intense. When heritability is high and selection pressure is also high, there is an increased risk of inbreeding, followed by diminished performance as a result of inbreeding depression, including lower response to selection. Thus, careful attention has to be given to the management of genetic variation in domesticated lines under these circumstances.

### Growth

The literature suggests that there is a relationship between the presence of disease and other measures of vitality. Oysters infected with *Perkinsus marinus* have severely retarded growth (Burrenson 1991, Paynter & Burrenson 1991), poorer condition, and reduced reproductive activity (Dittman et al. 2001). These negative effects of the infections on growth and condition are likely a consequence of a decrease in the available energy in infected hosts (Villalba et al. 2004). Barber et al. (1988) showed that oysters infected with MSX had poorer condition, as determined by condition index, and reduced fecundity. In this study, no relationship between illness and size were found, at

least judged by the relationship between survival and body weight. There is a lack of correlation between improved survival over the wild control and final meat weight (Table 3). For example the EC line in the York River survived 182% greater than the control and grew just about as well as the control; the LA lines in the York River had half the survival of the control and were about 150% heavier by the end of the study. In addition, even though disease pressure was high at Lynnhaven, oyster growth performance at that site was comparable with Kinsale, where disease pressure was low. Of course, those groups with greater mortality end up with lower density in grow-out containers, but this explanation is rejected as the general explanation for the lack of relationship between survival and final body weight; from the start, oysters were stocked at densities that were not density limited.

### Genotype-by-Environment Interaction and Implications

Notably, our breeding program encompasses a wide array of estuarine environments. That there is significant  $G \times E$  with which to contend is not surprising, but it is problematic for breeding. Observations of significant  $G \times E$  interactions are in agreement with previously published work in other shellfish studies. Dégremont et al. (2012) detected significant site-by-cohort (line) effects for mortality of diploid lines tested at three of the same sites used in the current study (Kinsale, York River, and Lynnhaven). Based on a statistical test, they showed that these interactions were caused by salinity differences influencing disease pressure. Kvingedal et al. (2008) reported significant  $G \times E$  for growth in silver-lip pearl oysters (*Pinctada maxima*) grown in two different environments: a hatchery and the ocean. They also found significant family reranking among five families grown under different conditions of food quality and food availability. However, there were no  $G \times E$  effects observed for salinity effects under experimental conditions in the hatchery, indicating that multiple factors likely contribute to  $G \times E$  effects. In *Crassostrea gigas*, Evans and Langdon (2006) found significant  $G \times E$  for growth and survival in a study using 25 full sib families across four environments on the Pacific Coast of the United States. Dégremont et al. (2005) tested 44 full sib families at 3 test sites in France. They observed significant  $G \times E$  for survival and yield, but not growth. Swan et al. (2007) also observed no significant reranking of families for growth across five Australian farms; however, there were significant scaling effects, with two farms showing greater growth performance than the other 3. In *Crassostrea virginica*, Mallet and Haley (1983) showed that  $G \times E$  influenced growth rates, body weight, and length using 6 lines grown at 2 sites in New Brunswick, Canada. As with the current study, performance was site dependant, and significant reranking of lines between 2 sites was observed.

Oysters are grown in environments that cannot be controlled or predicted easily. Thus, without growing the animals away from disease-endemic waters, selective breeding is the only viable option for disease management. Furthermore, this poor degree of control over the rearing environment increases the environmental variation among sites and the probability that  $G \times E$  will be significant and problematic (Evans & Langdon 2006). An understanding of the potential impact of  $G \times E$  is essential to ensure maximum genetic gains are achieved before

target breeding begins. In general, it is our opinion that this is poorly appreciated in other breeding programs.

For selective breeding, the presence of this sort of  $G \times E$  interaction has significant consequences. First, the best genotype in one environment is not the best in another. Second, it is clear that the advantage of years of selection for improved survival might be lost in the environment where the trait is not required (Table 5). Breeding animals from a single-selection environment to improve performance in dissimilar environments is pointless. For the Chesapeake, and probably the mid-Atlantic in general (if not the entire East Coast), a *Crassostrea virginica* breeding program that incorporates environment specific breeding is apt. Where disease is not an issue (i.e., in the low-salinity zones), new production traits can be explored that influence the profitability of production, traits such as growth rate and harvest traits (e.g., meat weight), which

influence production profits. On the other hand, in areas where disease is an issue, the focus can be shifted to fast growth in addition to disease resistance.

#### ACKNOWLEDGMENTS

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