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PERFORMANCE OF "NATURAL DERMO-RESISTANT" OYSTER STOCKS—SURVIVAL, DISEASE, GROWTH, CONDITION AND ENERGY RESERVES

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ABSTRACT   To determine if natural populations of the eastern oyster possess resistance to *Perkinsus marinus*, progeny representing several oyster stocks from the Chesapeake Bay and the Gulf of Mexico were deployed at two sites within the Chesapeake Bay. Mortality, *P. marinus* infection (prevalence and intensity), shell height, condition index, and energy reserves (glycogen, protein, and lipid) were compared between these stocks. Oyster stocks from the Chesapeake Bay had higher intensities of Dermo infection than Louisiana stocks, with differences among individual stocks. Throughout the 2-y study, a natural Dermo-resistant stock from Tangier Sound (CTS), was identified. Despite infection intensities approaching those of a susceptible Rappahannock River stock (CRB) and higher than a Gulf of Mexico stock (LOB), CTS consistently had lower mortality for the 2-y grow out, and was comparable to a hatchery disease-resistant strain (XB). At a site (Port Kinsale) where the significant parasite was *P. marinus*, the LOB stock grew to the largest shell heights and had significantly lower intensities of infection. However, the performance of the LOB stock was comparatively poorer at the other deployment site (Regent Point) where MSX was present. Shell heights were highest overall in the CRB stock at Regent Point, despite high susceptibility to disease. Condition index varied between stocks, although not necessarily along trends of disease resistance since condition was highest in the CRB and XB stocks. Variations in energy reserves were strongly influenced by season, but not disease, or stock origin. The present study shows that differences between stocks contain an underlying genetic component. Differences seen between deployed stocks in mortality, growth, and condition have strong implications for development of selective criteria for an aquaculture-based industry.

KEY WORDS: Crassostrea virginica, Perkinsus marinus, Dermo, disease resistance, condition index, energy reserves, oyster

INTRODUCTION

Diseases caused by 2 protozoan pathogens, *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) have devastated oyster populations along the east coast of the United States, particularly the Chesapeake Bay region. Extensive annual mortalities have hampered efforts to enhance oyster populations for commercial and restoration purposes. Efforts to circumvent the effects of disease have mostly focused on selective breeding to produce more disease-resistant strains, achieving the most success with MSX resistance (Haskin & Ford 1979, Ford & Haskin 1987, Ford 1988). Selection for resistance to Dermo, however, has been more difficult to achieve (Ford & Tripp 1996).

Dermo disease is chronic and disease-associated mortality does not typically occur until the second year of growth or when oysters are at or near market size (75mm) (Andrews & Hewatt 1957, Andrews & Ray 1988). Individuals that grow beyond market size and have survived more than two seasons of exposure to *P. marinus,* are believed to be resistant to Dermo. However, these resistant individuals are often continually removed from most populations during commercial harvests, thus preventing long term establishment of native resistant populations (Andrews & Ray 1988, Ray 1954, Kennedy 1996.). Some native populations, however, are suspected to possess inherent resistance or increased tolerance to *P. marinus* (Andrews 1954, Mackin & Sparks 1962).

Past field studies have noted differences in resistance of translocated oyster stocks (Andrews & Hewatt 1957). Lab experiments indicated varying resistance among eastern oyster populations from different regions supportive of natural selection for Dermo resistance (Bushek & Allen 1996). Native populations naturally selected for Dermo resistance could provide the basis for development of more resistant strains. Until recently very few of these

populations have been identified in the Chesapeake Bay and none had been reported from the Gulf of Mexico (Ford & Tripp 1996).

In 1996, Virginia oystermen discovered substantial numbers of large eastern oysters (lengths >110 mm) in Tangier Sound of the Chesapeake Bay (Blankenship 1997). Similarly, populations from the Gulf of Mexico in Louisiana (Grande Terre and Oyster Bayou) were found that were characterized by predominantly large-sized individuals in areas of high Dermo prevalence (Dr. J. Supan, Louisiana State University, pers. comm.). Because these populations possessed large and presumably long-lived ($\geq$2–3 years) individuals in an enzootic area for Dermo, it was presumed that these populations may possess some natural Dermo resistance (Dr. J. Wesson, Virginia Marine Resources Commission, pers. comm.).

The Dermo resistance of a particular oyster strain may be a function of its ability to withstand the pathogenic effects of *P. marinus*. Secretion of proteases by *P. marinus* may be a virulence factor in causing mortality (La Peyre et al. 1995, Oliver et al. 1999). Mortality associated with disease is due to depletion of the energetic reserves of the oyster (Choi et al. 1989), resulting in impaired physiologic function and degraded condition. One of the systemic effects of diseases such as Dermo and MSX is a reduction in soft tissue growth, resulting in a decreased condition index and changes to the biochemical composition of the host (Stein & Mackin 1957, Crosby & Roberts 1990, Gauthier et al. 1990, Paynter & Burreson 1991, Barber et al 1988a, Barber et al. 1988b, Newell 1985, Ford et al 1988). Changes in condition and biochemical composition may indicate differences among individuals or groups that exhibit variable response to disease stress. Few studies have examined the effects of infection by *P. marinus* on biochemical composition, particularly in relation to the progression of disease. Moreover, no study has compared condition and energy reserves among disease-resistant and susceptible oyster strains.

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To determine if there are native Dermo resistant oyster populations we collected oysters from several Chesapeake Bay and Gulf of Mexico populations. We compared disease resistance in F_0 progeny from presumptive "natural Dermo-resistant" (NDR) and non-resistant populations deployed in Chesapeake Bay. We determined whether some stocks showed natural resistance to disease and whether alterations in static physiological indices reflect response to disease. To achieve these objectives, mortality, disease, growth, condition, and energy reserves among several presumptive Dermo-resistant oyster stocks were compared in a common-garden experiment at two Chesapeake Bay sites. Oysters were naturally exposed to *P. marinus* from September 1999 to December 2001 at these sites. Dermo resistance was defined in this study as either low prevalence or intensity of *P. marinus*, and/or lower disease-associated mortality (Valiulis 1973, Bushek & Allen 1996). Resistant populations may maintain lower prevalences and intensities of infections, in addition to improved survival. (Gaffney & Bushek 1996).

MATERIALS AND METHODS

Stock and Site Selection

Oysters from representative populations from the Chesapeake Bay (Tangier Sound—CTS, lower Rappahannock River—CRB, Choptank River—CCR), the Gulf of Mexico (Louisiana region) (Grande Terre—LGT, Oyster Bayou—LOB, Hackberry Bay—LHB) (Fig. 1A, 1B), and one disease-resistant hatchery strain (CROSBreed—XB, bred for MSX resistance 9 generations, Dermo resistance 4 generations) were spawned in the Virginia Institute of Marine Science (VIMS) oyster hatchery and reared from larval stage to settlement. Natural oyster stocks were selected for testing based on the preponderance of large-sized individuals within those populations and designated NDR, or "natural Dermo resistant" stocks (CRB, CTS, LGT, and LOB). Parents from these stocks were greater than market size (75-mm shell height), and many were larger than 100 mm. For spawning, only individuals >100 mm were collected. Susceptible stocks (CCR and LHB) were characterized by a history of low exposure to *P. marinus*. The progenies from these seven stocks were transferred to mesh bags at 3–5 mm shell height and grown in Taylor floats (Luckenbach et al. 1997). Oyster stocks were placed in separate floats containing 800 individuals per float. Two replicate floats per stock were deployed at 2 sites (Regent Point Marina–Rappahannock River and Port Kinsale–Yeocomico River, see Fig. 1A) within the Chesapeake Bay from September 1999 to December 2001. Salinity ranges for both sites were 10–16 ppt (Fig. 2). Sites were selected primarily because salinity ranges would be favorable for *P. marinus* exposure but not *H. nelsoni*. Initial surveys of wild oysters at these sites showed that MSX was not present at either site. Ease of access and availability of space were also factors in the choice of these sites.

Sampling

Sampling was conducted every 4–5 wk during each of 2 growing seasons from spring to late fall. These seasons coincide with the peak disease periods. Temperature and salinity were measured on each sampling day by thermometer and refractometer. Oysters from each float were counted to assess mortalities. Oysters from each float were randomly sampled and returned to the lab to measure shell heights, dry weights, energy reserves, and infection levels of *P. marinus*. Ten oysters were sampled for shell heights, dry
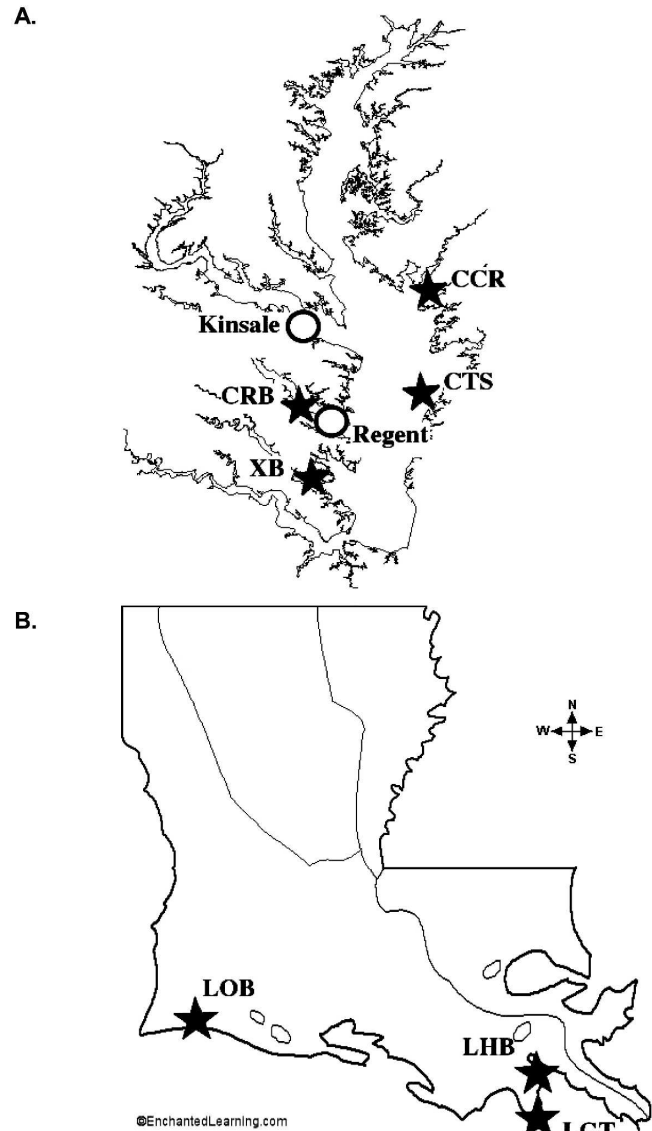

Figure 1. A. Chesapeake Bay and B. Louisiana maps showing locations of natural oyster stocks. Location of oyster stocks are indicated by a star (★). Stock acronyms are CCR = Chesapeake Bay Choptank River, CRB = Chesapeake Bay Rappahannock River, CTS = Chesapeake Bay Tangier Sound, LGT = Louisiana Grande Terre, LHB = Louisiana Hackberry Bay, LOB = Louisiana Oyster Bayou. Open circles (○) indicate sites where oyster stocks were grown (Port Kinsale, Yeocomico River; Regent Point, Rappahannock River).

weights, and *P. marinus* infections. Five to 10 oysters/float were used for individual protein and glycogen measurements and 3 oysters/float were sampled for lipid analysis.

Analyses were performed on 4 stocks (CRB, CTS, LOB, and XB). This was based on variation in survival and Dermo infection of these stocks after 1 y of growth. As the study progressed, the CRB stock exhibited high mortalities and high *P. marinus* infection intensities at Regent Point in the first year (2000). We reclassified the CRB stock as disease-susceptible, despite it matching our initial criteria for an NDR stock. All Gulf oysters exhibited a similar pattern of mortality at Regent Point; therefore one stock (LOB) was selected to represent the Gulf of Mexico populations. Thus, the remaining stocks represented the range of characteristics
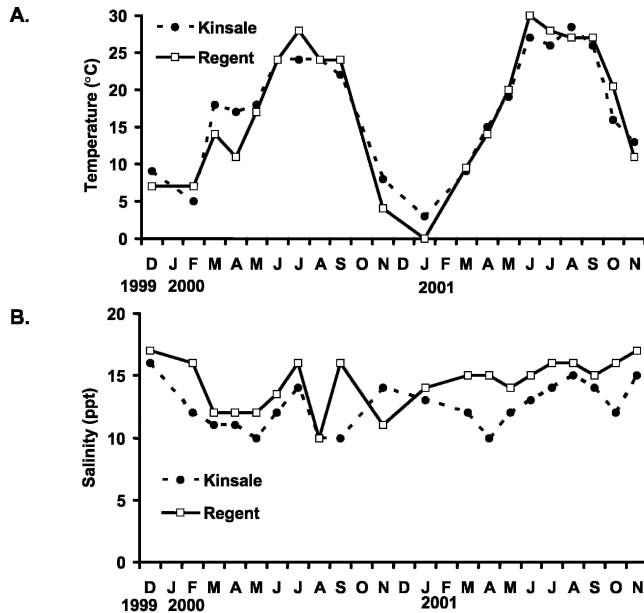
Figure 2. A. Water temperatures (°C) and B. salinities (parts per thousand, or ppt) at 2 sites in the Chesapeake Bay: Port Kinsale (Yeocomico River) and Regent Point (Rappahannock River) from 1999–2001.

(CRB disease susceptible, Chesapeake NDR—CTS, Gulf NDR—LOB, XB) pertinent to our general hypothesis that natural resistance to Dermo exists in distinct oyster populations.

Mortality

Live and dead oysters were counted at each sampling. Data was expressed as percent cumulative mortality [# dead oysters at time t (current period sampled) + # total dead over previous samplings ÷ total # live oysters at t = 0 − # removed for sampling). Cumulative mortalities were compared between each group at each site.

Shell Height

Shell height was measured from the anterior (shell hinge) to posterior (edge at the highest point) using vernier calipers. Shell heights were reported in mm.

Tissue Sampling

After shell height measurement, oysters were shucked, and tissues removed. Total wet weight of oyster tissue was recorded and whole tissue was divided in two fractions. To ensure equal organ representation between tissue fractions, tissues were sectioned in half along the left and right valve axes. One fraction was used to determine the intensity of infection by *P. marinus*, and the other was used for analysis of energy reserves.

Diagnosis of **P. marinus (Dermo)** *Infection*

Prevalence and intensity of *P. marinus* infection in experimental oysters were determined using total body burden assessment (Choi et al. 1989, Nickens et al. 2002). Oyster tissues were removed from the shell. The tissue fraction used for *P. marinus* diagnosis was homogenized in 10-mL of 0.2 M phosphate buffer and weighed. One milliliter of tissue slurry was added to 10 mL alternative fluid thioglycollate medium (AFTM, Sigma Biochemicals) containing the antibiotic chloramphenicol and the anti-fungal

agent nystatin. The homogenate was reweighed after tissue removal to estimate the wet weight of tissue used to assess counts of *P. marinus*. The tissue aliquot added to AFTM was incubated in the dark at room temperature for 5–7 days. Tissues were then pelleted by centrifugation, resuspended in 2 M NaOH and incubated overnight at 60°C. Tissue pellets were washed two to three times with distilled water and resuspended in 0.1 M phosphate buffer containing 0.2% sodium azide. Tissue suspensions were diluted if necessary, to facilitate counting and 100 μl-aliquots were added to a 96 well plate. One to two drops of Lugol solution (1:9 dilution) was added to each well, and *P. marinus* cells were counted using an inverted microscope. *Perkinsus* infection intensity was expressed as number (#) of *P. marinus* cells/g wet tissue weight (ww). Presence or absence of *P. marinus* was used to calculate prevalence data and expressed as percentage (%) of infected oysters.

Diagnosis of **H. nelsoni (MSX)** *Infection*

Periodically, oysters were examined histologically for *H. nelsoni* infection (Burreson et al. 1988). A transverse tissue section across gill, stomach, intestine, and digestive diverticula was removed from each oyster. Tissues were fixed in Davidson solution, dehydrated, cleared, and embedded in paraffin. Sections 5-μm thick, were cut, mounted on glass slides, and stained with iron hematoxylin and eosin. Intensities of infection were categorized as high, medium, or low

Dry Weight and Condition Index

Tissue fractions, except the 1.0-mL aliquot of tissue used to diagnose *P. marinus*, were freeze dried for 48 h and weighed. The relationship between wet weight and dry weight of the *P. marinus* tissue fraction was calculated so as to estimate dry weight in tissue slurries added to AFTM. Total dry weights of soft tissues were measured by adding all aforementioned dry weight fractions together. Shells were dried in an oven at 60°C for 48 hours. Total dry weights and dry shell weights were used to calculate condition index (tissue dry weight / dry shell weight × 100) (Walne & Mann 1975, Lucas & Beninger 1985). This method is comparable to, but varies from the shell capacity method recommended by Crosby and Gale (1990) and is more amenable to rapid processing of numerous samples (Rainer & Mann 1992).

Energy Reserve Measurements

Glycogen Analysis

Freeze-dried tissues were analyzed for glycogen content using the anthrone reagent method (Van Handel 1965). Tissues were homogenized in phosphate buffer (50 mM sodium phosphate, 1 mM EDTA and 0.5 mM PMSF, pH 7.2), digested in boiling 30% potassium hydroxide and precipitated with 95% ethanol and saturated sodium sulfate. The glycogen precipitate was dried overnight at 60°C. Anthrone reagent (0.15% in 72% sulfuric acid) was added to the precipitate and incubated at 90°C for 20 min and then cooled in ice. Positive reaction with the anthrone reagent produced a blue color, directly proportional to the amount of glycogen contained within the sample. Samples were added to a 96-well microplate and read on a microplate spectrophotometer at 620 nm. Commercially purified oyster glycogen (Sigma Biochemicals) was used to generate a standard curve for each set of microplates. Glycogen content was expressed as mg glycogen/g dry weight of tissue.

Total Protein Analysis

Total protein concentration was determined by a modified Lowry protocol (DC BioRad assay, Lowry et al. 1951). An aliquot of the homogenate prepared for glycogen analysis was removed and diluted 1:3 in 1.0 M NaOH. Diluted samples were boiled in water for 5 min centrifuged and supernatant assayed for protein content on 96-well microplates at 690 nm. Protein amounts were expressed in mg/g dry weight.

Lipid Analysis

Total lipids were extracted from freeze-dried oyster tissue (~50 mg dry weight) with chloroform: methanol: water (2:2:1) according to the procedure of Bligh and Dyer (1959). The extracted lipids were evaporated under N_2 at 40°C and resuspended in chloroform to 10–12 mg/mL total lipid, capped under N_2, and stored at −20°C until analysis. Lipid contents and lipid class composition were analyzed by thin layer chromatography and flame ionization detection (TLC/FID) using an Iatroscan TH-10, MK-III analyzer (Iatron Laboratories, Japan) (Chu & Ozkizilcik 1995). Briefly, silica coated glass rods (S-III chromarods, Iatron Laboratories Inc., Japan) were activated and cleaned by flame ionization on the Iatroscan. One micro liter of the lipid extract was then spotted on to each chromarod using a Hamilton syringe. Neutral (steryl esters, triacylglycerol, free fatty acids, and cholesterol) and polar (total phospholipid content only) lipid classes were separated on the chromarods after development in hexane:diethyl ether:formic acid (85:15:0.04). Following development, lipid contents and lipid class composition were quantified by FID on the Iatroscan. Operating conditions were 2,000 mL/min air flow, 0.73 kg/cm^3 hydrogen pressure, and a scan speed of 3.1 mm/sec. Peak area integrations were performed using PeakSimple software (SRI Inc.). Peak areas corresponded to the amount of ionized lipid in each separated component. Lipid class concentrations were determined by comparison to a standard curve for each lipid class component. Lipid class standards were cholesteryl palmitate (steryl ester), triolein (triacylglycerol), oleic acid (free fatty acid), cholesterol, and phosphatidylcholine (phospholipid). Units were expressed in mg lipid/g dry tissue weight.

Statistics

Cumulative mortalities were compared by contingency table analysis (Zar 1996), using stock as row variables and live and dead numbers as column variables. Comparisons were controlled for site. Prevalence of *P. marinus* was also compared by contingency table analysis with stock as row variables and infected and uninfected as column variables. Prevalence comparisons were controlled for by date and by site. For both contingency table analyses, stock replicates were pooled.

For infection intensities, shell height, condition index, and energy reserves, oyster stocks were compared by an unreplicated repeated-measures ANOVA (Underwood 1997) with stock and sampling period as main effects. Replicate floats were nested within stock as a within subjects random factor. Analyses were separated by year (2000 and 2001) and by site. Stock differences and monthly trends were mainly uniform across both sites. Cases where there were interactions between stock and site were described qualitatively. In several comparisons, month x rep (stock) interactions were significant, making interpretation difficult and precluding the application of this statistical design (Underwood 1997). In those cases, variables were compared separately by month with alpha (probability of Type I error) levels adjusted by Bonferonni correction (alpha (α)/# samplings: Year 2000 α = 0.05/6, Year 2001 α = 0.05/5) (Underwood 1997). Application of this procedure also allowed comparisons of stocks in months where the CRB stock died, resulting in an unbalanced design. Shell height, dry weight, condition index, and energy reserves (glycogen, lipid, and protein) were analyzed separately. All variables were transformed to meet assumptions of normality and homogeneity of variance when necessary. Pair-wise comparisons from ANOVA analyses were determined by Student Newman Keul procedure. Statistical analyses were conducted using Statistical Analysis System, Version 8 (SAS Institute Inc., 1999).

RESULTS

Temperature and Salinity

Temperatures were similar at both Port Kinsale and Regent Point, and salinities were generally, although slightly, higher at Regent Point (Fig. 2A, 2B). Seasonal variation in temperature was apparent with maximum temperatures in the summer months (28°C to 30°C) of both years and near freezing-to-freezing temperatures in January 2001 (3°C at Port Kinsale and 0°C at Regent Point). Salinities ranged from 10–17 ppt. Differences in salinities between the sites were most apparent in September 2000 and April 2001. During these months salinity at Regent Point was 5–6 ppt higher than Port Kinsale. Mean salinity was ~2 ppt higher at Regent Point (14.4 ppt) than at Port Kinsale (12.5 ppt).

Mortality

Significant differences in cumulative mortalities were found among stocks at each site ($P < 0.0001$). High mortalities were seen in the CRB (53.8 ± 3.1%, n = 2) and LOB (45.1 ± 1.0%, n = 2) stocks by November 2000 (Fig. 3). Mortality was highest in the CRB group with 100% mortality at Regent Point by July 2001 and 82.8 ± 1.4% at Kinsale at the end of the experiment. The CTS and XB stocks showed lower (average of both sites: 46.6% and 52.6%, respectively) cumulative mortalities. Mortalities were higher at Regent Point (CTS—72.4%, XB—75.0%) than Port Kinsale (CTS—20.2%, XB—31.0%). Cumulative mortalities for the LOB stock (47.4%) were intermediate between the CRB and XB/CTS groups at Port Kinsale. High mortalities in the LOB stock at Port Kinsale, however, were observed in the March, 2001 sampling. Mortality of LOB oysters reached 100% at Regent Point, by the end of the study.

Perkinsus marinus *Infections*

Prevalence

Perkinsus marinus infections were detected at Regent Point in June of 2000 and at Port Kinsale in August, 2000 (Fig. 4A). Infections progressed more rapidly at Regent Point, resulting in higher prevalences compared to Port Kinsale. Prevalence at Kinsale was lower (8.8 ± 3.0%) in May 2001 compared to Regent Point (70.0 ± 10.0%), suggesting that infections may have cleared in oysters at Port Kinsale during the overwintering period. The prevalence remained high at Regent Point during May 2001, indicating that oysters were exposed to *P. marinus* earlier and at higher levels than at Port Kinsale. Significant variation in prevalence between stocks occurred from 2000 to June 2001 (Fig. 4B, 4C). Within each month, prevalence fluctuated between stocks.
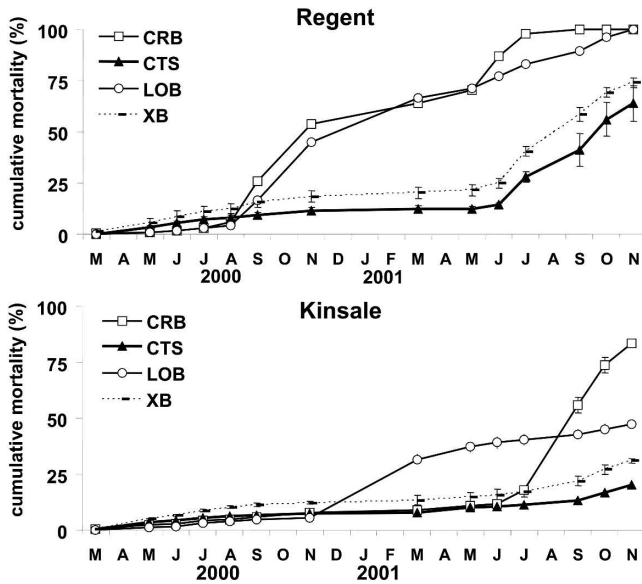
Figure 3. Cumulative mortality (%) data of three native oyster stocks and one hatchery disease-resistant strain at A. Port Kinsale and B. Regent Point. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain. Data was collected every 4–5 wk in 2 seasonal periods (May to November 2000 and May to November 2001). Sampling is not continuous between November 2000 and May 2001. Data are presented as mean percentage (%) mortality ± standard error of the mean (SEM) n = 2 replicate floats per stock.

Figure 4. Prevalences of *Perkinsus marinus* at Port Kinsale and Regent Point from 2000 to 2001. Prevalence is percentage (%) of infected oysters. Data presented in the top figure (A.) are mean prevalence ± SEM. (n = 4 oyster stocks [20 oysters/stock] for each site). Prevalences of individual stocks (CRB, CTS, LOB, and XB) at each site (B. and C.) are shown separately. Prevalences of each stock are collapsed across two replicate floats/stock. At Regent Point, prevalence data for the CRB stock from September to October 2001 is absent due to 100% mortality in that stock. Note that data are presented for each month sampled. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

Despite inter-stock variation in prevalence, across months and sites there remained a significant effect of stock on prevalence (P = 0.0002). Prevalence was lower in LOB and CTS stocks (70.0% and 74.8%), whereas prevalences in CRB and XB stocks were higher (88.8% and 81.9%, respectively). By July 2001, prevalences were at or near 100% for all stocks through the remaining sampling periods.

Infection Intensities

Stocks were compared for differences in intensities of infection (# of *P. marinus* cells/g ww) by month for each site. In 2000, *Perkinsus* infections varied significantly by month (P < 0.0001) at both sites. At Port Kinsale, intensities showed no significant effects between stocks. Differences between stocks were most apparent at Regent Point. Mean intensities for all stocks were 8.4 × 10^6 ± 8.3 × 10^6 cells/g ww at Regent Point and 1.2 × 10^3 ± 1.0 × 10^3 cells/g ww at Port Kinsale. Because of significant interactions between replicates and months at Regent Point, data were analyzed separately by month. Nevertheless, differences in infection between stocks were apparent and significant starting in June 2000 (P < 0.01), as overall mean infection levels were low (87.0 ± 0.6 cells/g ww) and fluctuated from June to August 2000. By November 2000, infections at Regent Point were highest in the CRB stock (1.8 × 10^7 ± 2.1 cells/g ww) (P < 0.01). The CTS and LOB stocks had intermediate mean intensities and the XB oysters had the lowest intensities of *P. marinus* (Fig. 5). At both sites and across all stocks, *P. marinus* infections increased over time and were highest at Regent Point. Intensity decreased from Nov 2000 to May 2001.

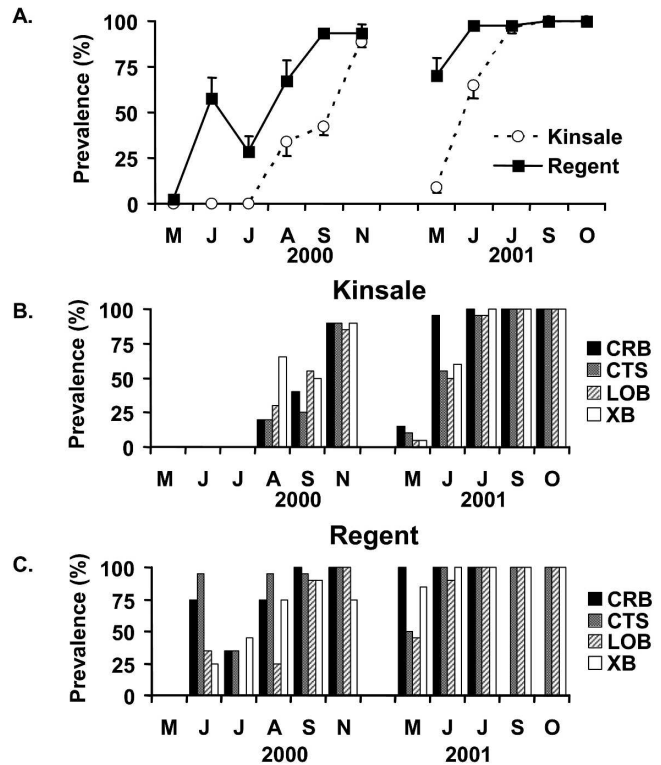From May to October 2001, intensities increased (Fig. 5) coincident with increased mortalities (Fig. 3). Significant month by stock interactions reflected high variability in infections among replicate floats at both Kinsale and Regent. Despite this, there were significant differences in infection levels among stocks. Consistent with the previous year, the CRB stock had the highest infections and was significantly higher than the other stocks at Regent Point, before reaching 100% mortality by May to July 2001 (Fig. 3). At Port Kinsale, LOB infections remained below 1.0 × 10^6 cells/g ww throughout October, with maximum infections reaching 5.0 × 10^5 cells/g ww. Infection levels in the LOB stock were significantly lower at both Kinsale and Regent. Overall, infections at Regent were higher than at Kinsale, and mortality increased as infections reached or exceeded 1.0 × 10^6 cells/g ww at both sites. By October 2001 all oysters at Regent Point had infections above 1.0 × 10^7 cells/g ww.

MSX Infections

At Regent Point, oysters examined between July and November 2000 showed presence of *Haplosporidium nelsoni*. At Regent Point in 2001, MSX was detected in 48/124 animals examined in May, July, and October. No significant differences between stocks were observed, but MSX was not detected in any XB oysters
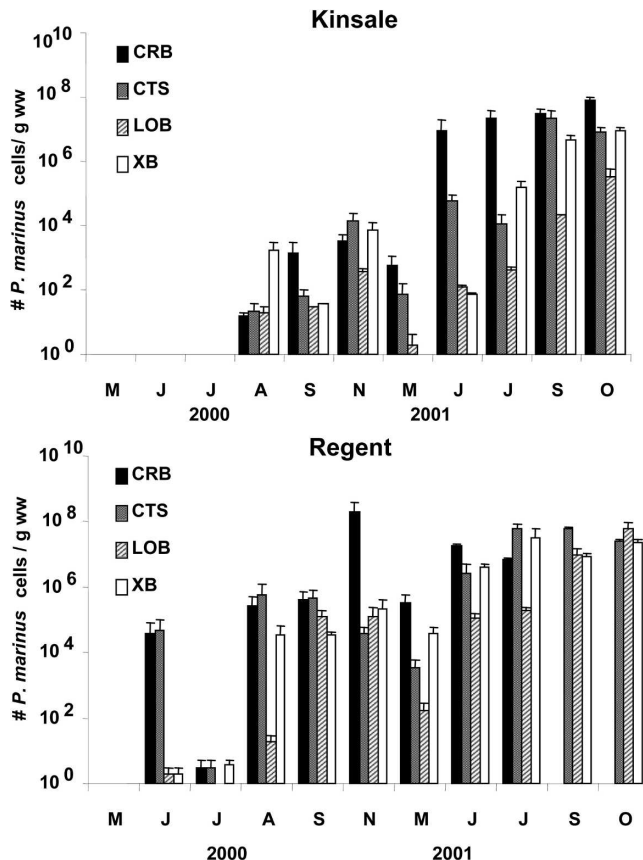
Figure 5. Infection intensities of CRB, CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point from 2000 to 2001. Data are presented as mean number (#) of *P. marinus* cells (enlarged hypnospores)/g wet weight (ww) ± SEM. (*n* = 2 replicates of 10 animals for each stock). Numbers of *P. marinus* cells/g ww are graphed on a logarithmic scale. Note break in sampling periods between November 2000 and May 2001. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.
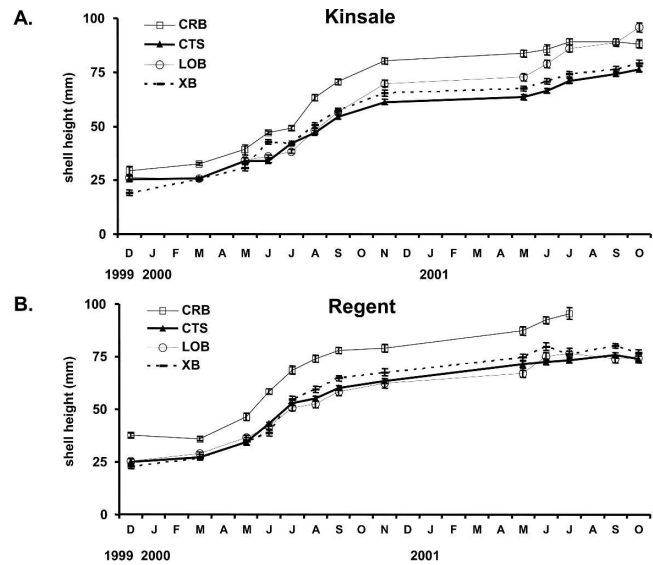

Figure 6. Comparisons of shell heights among CRB, CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point during periods (shown as months) sampled in 2000 to 2001. Data are mean shell heights (mm) ± SEM (*n* = 2 replicates of 10 oysters for each stock). CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROS-Breed strain.

examined. *Haplosporidium nelsoni* was not detected at Port Kinsale in 129 animals examined.

Shell Heights

In 2000, shell heights increased over time and were significantly different between stocks ($P < 0.0001$). Comparisons between stocks showed that CRB grew significantly more than CTS, LOB, and XB stocks at both sites (Fig. 6). At Port Kinsale, the CTS stocks had significantly lower shell heights than the other three stocks, whereas the LOB and XB stocks were not significantly different. Shell heights were larger at Regent Point than at Port Kinsale. Interactive effects were significant as well, but as with *P. marinus* infections, overall trends in shell height across stock, site, and month were consistent.

In 2001, increases in shell height over time and among stocks were significant at Port Kinsale ($P < 0.0001$). At Port Kinsale, the CRB and LOB stocks grew to the largest average shell heights. Final shell heights measured in October 2001 were CRB: 88.1 ± 0.2 mm, LOB: 95.8 ± 1.4 mm (mean ± standard error of the mean, or SEM, *n* = 20 oysters from each stock). The CTS and XB shell heights were lower (76.4 ± 0.5 mm and 79.3 ± 0.7 mm). From May

to July, shell heights at Regent Point were significantly different between stocks ($P < 0.0001$). At Regent Point the CRB stock grew to the largest sizes but reached 100% mortality by July 2001. Shell heights at Regent Point were similar among the CTS, LOB, and XB stocks (73.9 ± 0.8, 74.3 ± 1.3, 76.8 ± 2.7 mm, respectively) from May to July. During September to October, XB oysters grew to significantly greater sizes ($P = 0.0053$ for main effects of stock only) than the LOB group. The LOB shell heights were the lowest at Regent Point, but highest at Port Kinsale, indicating an interactive effect of site on this stock. Within the other stocks, shell heights were greater at Regent Point than at Port Kinsale.

Dry Weights

Dry weights varied significantly among stocks and months at both sites ($P < 0.0001$) in 2000. Dry weight differences among stocks reflected differences in shell height (CRB > LOB, XB > CTS). Increases in dry weight were highest from September to November sampling dates, particularly in the CTS, LOB, and XB stocks (Fig. 7). Differences in dry weight among stocks showed consistent trends from month to month, although stock x month effects were significant ($P < 0.0001$). At Regent Point, the CTS stock grew rapidly from September to November and had the highest dry weights compared to the other stocks. At both sites, growth was greatest during the fall with dry weights increasing from September to November by 1.5 and 2.0 times at Regent Point and Port Kinsale.

Tissue dry weights in 2001 were characterized by a sharp decrease in June from peak dry weights in May (Fig. 7) and no longer reflected changes in shell height. Significant differences between stocks were seen at both sites. At Port Kinsale, dry weights were highest in the LOB stock. At Regent Point, differences among stocks were similar to 2000, with the CRB stock having the highest dry weights from May to July, although the CRB stock did not
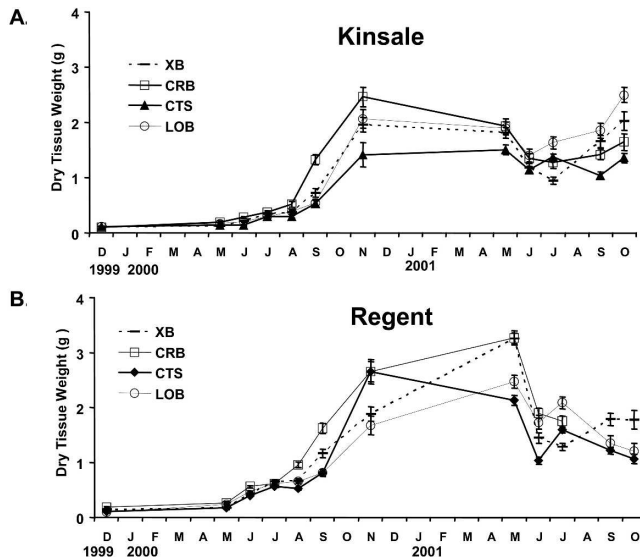
Figure 7. Dry tissue weight measurements of CRB,CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point during periods (shown as months) sampled in 2000 to 2001. Data are mean dry weights (g) ± SEM (*n* = 2 replicates, 10 oysters/replicate/stock). CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

differ significantly from the LOB and XB stocks. The CTS stocks had the lowest dry weights in the fall. As in the previous year, dry weights were higher at Regent Point than at Port Kinsale. This was uniform across all stocks except for the LOB stock, which had lower dry weights at Regent Point than at Port Kinsale.

Condition Index

In 2000, condition index (CI) varied significantly between stocks and months ($P < 0.0001$), and reflected changes in dry weight. Condition index decreased from May to July and increased in all stocks at both sites from September to November (Fig. 8). Condition index increased from the fall to winter, coinciding with an accumulation of glycogen and triacylglycerol (TAG) (see following sections).

In 2001 the highest condition index was during the May sampling with decreases in the subsequent month. Variation among stocks in condition index was consistent with the previous year, the XB strain maintained the highest condition over the other stocks at both sites ($P < 0.0001$). At Regent Point, changes in LOB condition reflected changes in shell height and dry weight. Prior to 100% mortality, CRB oysters also had a significantly higher condition index than CTS and LOB stocks at both sites.

Glycogen

Glycogen content varied seasonally, although not significantly, with maximum values seen during the winter–spring periods (Fig. 9). Decreases in glycogen occurred during the summer months. Patterns in glycogen content were consistent across both sites. No significant differences, however were noted due to month, stock, or site.

Protein

Protein content did not vary by stock but seasonal variation was seen with minimum levels observed in the fall (Fig. 10). Changes
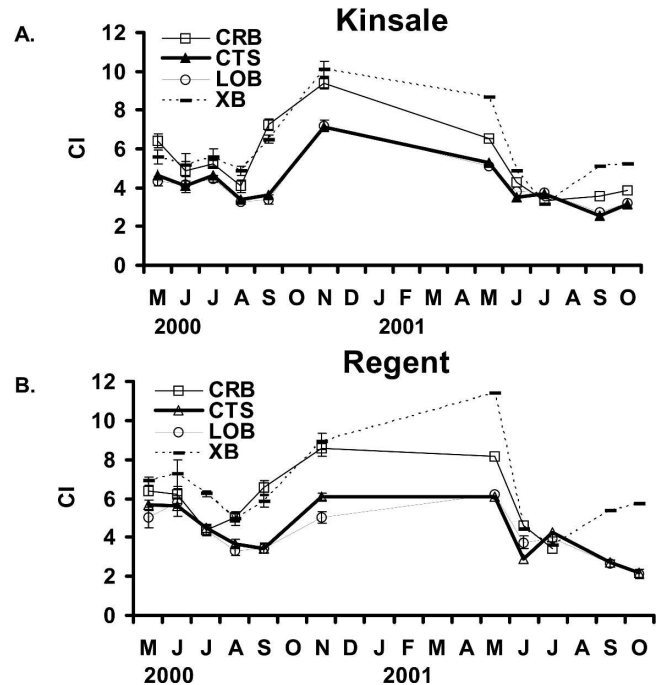

Figure 8. Condition index (CI) values of CRB, CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point during periods (shown as months) sampled in 2000 to 2001. Condition index was measured according to Lucas and Beninger (1985) as (dry tissue weight/dry shell weight) × 100. Data are presented as mean CI ± SEM (*n* = 2 replicates of 10 oysters/rep/stock). CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

in weight-specific protein amounts (mg/g dry weight) decreased during periods of increased glycogen.

Lipid

Lipid class composition (steryl esters, triacylglycerol, free fatty acids, cholesterol, and phospholipids) was compared among oyster stocks in August and November 2000 and May to July and September-October, 2001 (Fig. 11). Significant differences were found in triacylglycerol (TAG) content due to month and stock. Interaction between month and stock were significant at Port Kinsale but not at Regent Point. Seasonal variation in TAG was most apparent at Kinsale, with an increase in TAG in all stocks except the CRB stock, between November 2000 and May 2001 (Fig. 11A). At both sites TAG decreased in June and July 2001 (Fig. 11A and 11B). TAG levels were highest in LOB and CRB stocks at both sites.

DISCUSSION

Natural Dermo Resistance

Our results showed that one native stock (CTS) possesses resistance to Dermo disease, comparable to that of a hatchery strain (XB) selected for resistance to Dermo and MSX. Performance of this stock supported the assumption that individuals from this population was Dermo-resistant and that resistance may be genetically based. Furthermore, we have tested the F_1 progeny of the F_0 CTS stock. The performance (growth, condition, *P. marinus* infection, and survival of the F1 CTS oysters was similar to the F0
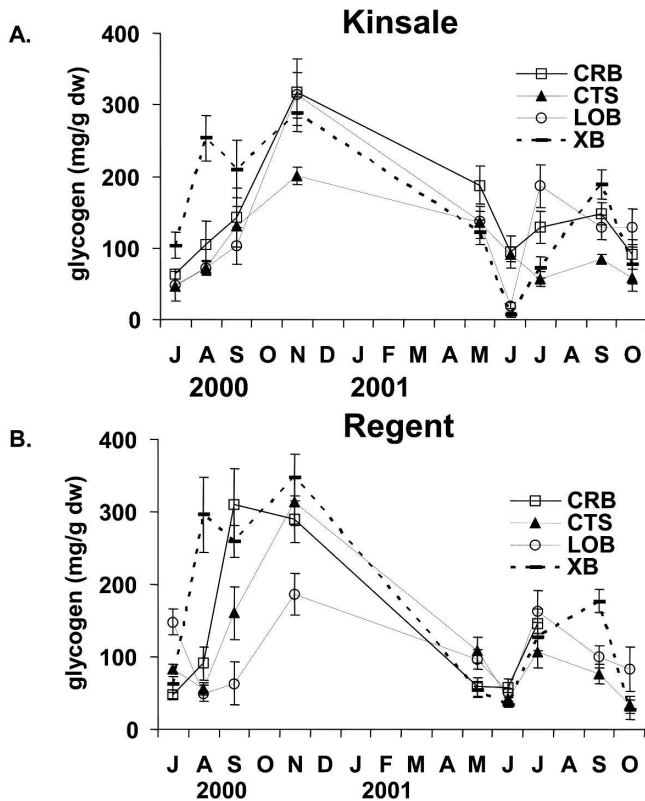
Figure 9. Glycogen contents of CRB, CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point during periods (shown as months) sampled in 2000 to 2001. Data presented are mean glycogen (mg/g dry weight, or DW) ± SEM n = 2 replicates of 6–10 oysters per replicate per stock. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.
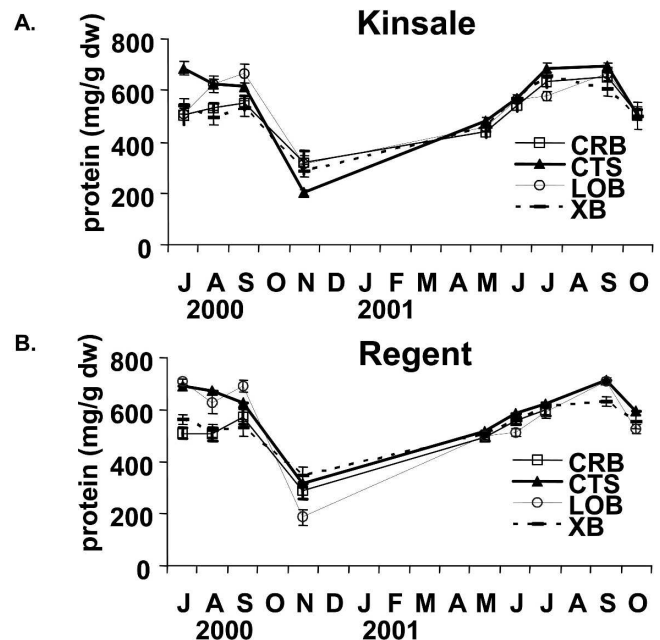

Figure 10. Comparisons of protein contents among CRB, CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point during periods (shown as months) sampled in 2000 to 2001. Data presented are mean protein (mg/g dry weight, or DW) ± SEM, n = 2 replicates of 6–10 oysters each. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

stock tested in the present study (Encomio 2004, Encomio & Chu 2005).

Dermo disease is generally characterized as causing mortality in oysters in their second year of growth, as they approach or attain market size and accumulate *P. marinus* to a critical infection level (Burreson 1991, Paynter & Burreson 1991), generally at 10^6 cells/g ww (Choi et al. 1989, Bushek et al. 1994). The CTS and XB oysters were at or near market size (75-mm shell height) and had infections exceeding critical infection levels of 1.0×10^6 cells/g ww, when mortalities began to increase. Cumulative mortalities in the CTS and XB groups, however, remained lower than the CRB stock, despite comparable infection levels. Because mortalities did increase rapidly in those stocks at 1.0×10^6 cells/g ww, mortality was delayed, implying an ability to resist infections for longer periods. Delayed mortality in disease-resistant oysters was also demonstrated in strains of *O. edulis* exposed to *Bonamia ostreae*, a protistan parasite that causes mortality after chronic infection, a similar characteristic of Dermo disease (Naciri-Graven et al. 1998). Stock differences in survival, growth, and condition were consistent between grow-out sites, further suggesting a genetic basis for their differences.

It is not certain whether the LOB stock was Dermo-resistant. Cumulative mortalities of the LOB stock were higher than the XB and CTS groups at both sites, particularly at Regent Point. However, although LOB cumulative mortality was higher than the CTS or XB, at Kinsale most of that mortality was attributed to deaths

that occurred just prior to the March, 2001 sampling. This mortality event was believed to have been caused by exposure to freezing temperatures during extreme low tides that occurred during the weeks before sampling. Mortality only occurred in the exposed floats that contained Louisiana oysters. Chesapeake and XB floats that were similarly exposed did not contain noticeable mortalities. After this mortality event, monthly mortality rates were low in the LOB stock and lower than all other groups. At Regent Point, where LOB mortalities were very high, MSX disease may have been a contributing factor to those high mortalities (see following section). Additionally, at Port Kinsale, LOB oysters grew beyond market size (>100 mm), had lower levels of infection, and lower mortality rates during the period of *P. marinus* exposure. At Port Kinsale, mean intensities of *P. marinus* infection in the LOB stock remained below critical infection levels of 10^6 cells/g ww for the entire study. At Regent Point, infection intensities of the LOB stock remained at sublethal levels until September 2001. Furthermore, LOB oysters from the same cohort as described in this study, had higher survival, grew to greater sizes, and had lower intensities of *P. marinus* than the CRB, CTS, and XB groups in grow-out comparisons conducted concurrently in Louisiana (Stickler 2004). These observations, particularly the performance of the LOB oysters at Port Kinsale, suggest that the LOB stock could be a potential candidate for Dermo resistance. However, further tests of Louisiana stocks must be performed to determine whether Dermo resistance in these stocks is geographically broad, as demonstrated in Gulf stocks by Bushek and Allen (1996), and not site-specific.

Previous studies have shown that disease resistance among eastern oysters contains a genetic component (Haskin & Ford 1979, Burreson 1991, Bushek & Allen 1996). Bushek and Allen
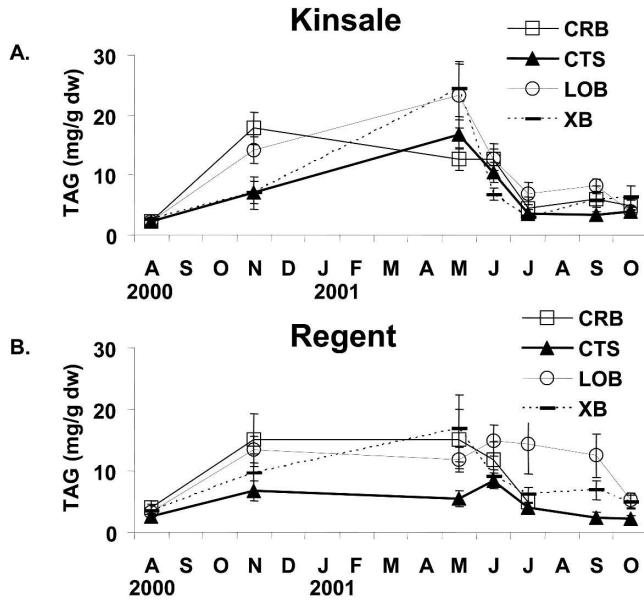
Figure 11. Triacylglycerol (TAG) contents in CRB, CTS, LOB, and XB oysters at A. Port Kinsale and B. Regent Point during periods (shown as months) sampled in 2000 to 2001. Mean TAG (mg/g dry weight, or DW) ± SEM $n = 2$ replicates of 3–8 oysters/replicate per stock are presented. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

(1996) found that Gulf of Mexico oysters were uniformly resistant to a range of geographic strains of *P. marinus*, but these relationships were not tested in the field. Field comparisons have shown variation in survival between groups of oysters (Andrews & Hewatt 1957, Burreson 1991, Ragone-Calvo et al. 2003) exposed to Dermo. Those studies differed from the present study in several ways. Andrews and Hewatt (1957) compared mortalities in translocated oysters, rather than stocks spawned and reared under identical conditions. Burreson (1991) examined differences between native stocks similarly to our study but primarily focused on comparisons with MSX resistant strains and response to Dermo and MSX. Further, stocks were deployed in disease enzootic sites at different ages, making stock comparisons difficult.

Ragone-Calvo et al. (2003) examined a Delaware Bay strain (DEBY) that exhibited improved resistance to Dermo and MSX over successive generations. Comparisons with native stocks (F_0 Tangier Sound and Mobjack Bay) showed superior survival in the DEBY strain. The F_0 Tangier Sound stock used in their comparisons experienced high mortalities (~80%) in high salinity environments (15–25 ppt during periods of high Dermo prevalence). In contrast, the Tangier Sound stock used in our study survived better than an XB strain bred for multiple generations (9 MSX, 4 Dermo) for disease resistance in two moderate salinity environments (8–15 ppt). Clearly, the Tangier Sound population requires further examination, particularly with regards to genotype-environment interactions. However, comparisons of the F_0 CTS and CRB oysters indeed demonstrate intra-regional variability in Dermo resistance between Chesapeake Bay stocks.

Site Variation in Disease Dynamics

Site differences in *P. marinus* prevalence and intensity, although not directly compared, were apparent. Regent Point was

characterized as a site of higher disease exposure, because prevalence and intensity of *P. marinus* occurred earlier and at higher levels than at Kinsale. High prevalence and intensity of Dermo at the end of 2000 at Regent Point may have affected the number of overwintering infections and subsequent infection rates in 2001. Overwintering infections generally decrease with decreasing temperature and salinity (Ragone-Calvo & Burreson 1994). Evidence of a winter decline in prevalence was seen at Port Kinsale, but not at Regent Point, where prevalences remained high (70%) during the spring. Salinity was lower at Port Kinsale (8–12 ppt) than at Regent Point (15 ppt) during the early spring (March and April, 2001), and may have contributed to differences in prevalence. The high prevalence at Regent Point suggests that individuals retained high infections over the winter to initiate transmission of *P. marinus* when environmental conditions were favorable for development of the parasite. At Port Kinsale, prevalences and intensities of *P. marinus* infections were lower than at Regent Point in the spring, suggesting a lower number of overwintering infections. However, frequency distribution of infection intensities from May 2001 indicates that there were individuals with high infection intensities at Port Kinsale (Fig. 12). Such individuals may be responsible for initiating an epizootic, even when mean infection levels are low (Ford et al. 1999).

The presence of MSX disease also may explain differences in mortality between sites. MSX was initially detected in September 2000 at Regent Point and in the summer months of 2001, but was not detected at Port Kinsale throughout the study. Overall cumulative mortalities were higher at Regent Point than at Port Kinsale. Mortalities in the LOB stock were higher at Regent Point and similar to the CRB stock. MSX does not occur in the Gulf of Mexico therefore, the LOB stock would have had no resistance to *H. nelsoni*. Response to selection for resistance to *H. nelsoni* is apparently high, and can be attained in 1–2 generations (Ford & Haskin 1987). It would be expected that MSX resistance could be readily accomplished in Louisiana stocks through selective breeding. As demonstrated in existing Delaware Bay (DEBY)
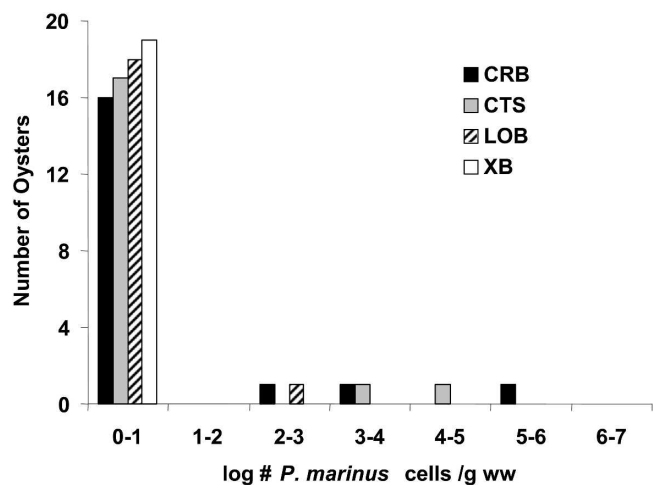

Figure 12. Frequency distribution of infection intensities of *P. marinus* during May, 2001 at Port Kinsale. Distribution of infection intensities among the CRB, CTS, LOB, and XB oysters illustrate the presence of individuals with high intensities of infection during a period when mean infection intensities are low. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

strains, dual resistance to Dermo and MSX can be achieved over several generations (Ragone-Calvo et al. 2003).

Growth

In addition to increased survival, it was expected that disease-resistant stocks would exhibit improved growth because they would be better able to withstand the chronic effects of disease. Oyster strains that were MSX-resistant displayed an energetic advantage over susceptible strains (Barber et al. 1991a). The CRB stock, although highly susceptible to disease, reached market size (75-mm shell height) earlier than the other stocks. At Regent Point the CRB oysters reached market size in August 2000 at Regent Point and in November 2000 at Port Kinsale, before infection intensities became lethal. Comparisons of time to market size and cumulative mortalities at market size show that the CRB stock reached market size the fastest, and had the lowest mortalities at the time market size was attained (Fig. 13A, 13B). The rapid growth of the CRB stock indicates that this stock may be a useful aquaculture strain, particularly in areas where MSX intensities are low or absent. Fast growing oyster strains can be used to avoid the effects of Dermo disease, even in strains with no developed resistance to *P. marinus* (Allen et al. 1993). Disease resistance in this stock may also be improved through further selection, as has been the case with some MSX-resistant strains, which now grow faster than unselected strains (Matthiessen et al. 1990, Ragone-Calvo et al. 2003).

Dry weights declined in June 2001, whereas shell heights continued to increase, making growth comparisons difficult. In the present study we used shell height and dry weight as indices of overall growth. From a practical perspective, shell height is the most convenient method to compare performance among oyster strains. Determination of market size in the Gulf of Mexico and the Chesapeake Bay is based on shell height and so remains an important metric to oyster culturists in those areas. Additionally, despite the seasonal decrease in dry weight, log mean dry weights among all stocks correlated strongly with log mean shell heights at both sites from deployment to the end of the experiment (Fig. 14).

Condition Index

Demonstration of sublethal effects of disease on condition and energy reserves that can be ascribed to differences in disease resistance, remain equivocal. Differences in condition did not reflect patterns of survival (and presumably disease resistance), as condition index was highest in both resistant (XB) and susceptible (CRB) stocks (Fig. 8). In previous studies, condition index was reduced by *P. marinus* infection (Craig et al. 1989, Crosby & Roberts 1990, Paynter & Burreson 1991). However, high site-specific variation, attributed to differences in salinity, made it difficult to distinguish among several Gulf coast sites (Craig et al. 1989). Although statistically significant, intensity of infection explained less than 10% of the variablility in condition index in South Carolina oyster populations (Crosby & Roberts 1990). Paynter and Burreson (1991) compared the condition index between infected and uninfected oysters, not the relationship between intensity of infection and condition index. In their study, condition indices remained high during the months of increasing Dermo infections, in contrast to the present study.

In the present study, environmental influences (e.g., changes in temperature and food availability) on condition were likely greater than effects of disease. Seasonal variation in oyster condition exhibits remarkable inter-annual and intra-river consistency in the Chesapeake Bay (Austin et al. 1993). This may be related to consistency in the timing of phytoplankton blooms and food availability (Deslous-Paoli & Heral 1988). Because seasonal variation in condition index can be consistent from year to year, identifying effects of disease on condition may be difficult or unique to specific regions within the Chesapeake Bay.

Sublethal effects of Dermo disease may be important at critical periods of the eastern oyster's reproductive cycle. Condition and gonadal indices decreased with infection during periods prior to
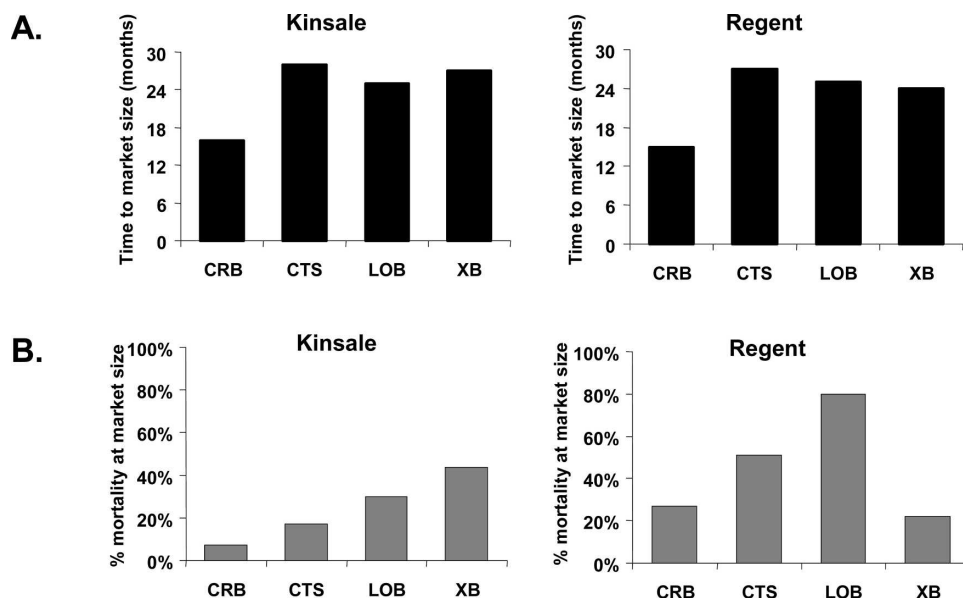

Figure 13. A. Months to market size among natural oyster stocks (CRB, CTS, LOB) and one hatchery strain (XB) at Port Kinsale and Regent Point. B. Cumulative mortalities (%) of oyster stocks at market size (75 mm) at Port Kinsale and Regent Point. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

A.

Kinsale

$y = 2.4417x - 4.4546$
$R^2 = 0.895$

B.

Regent

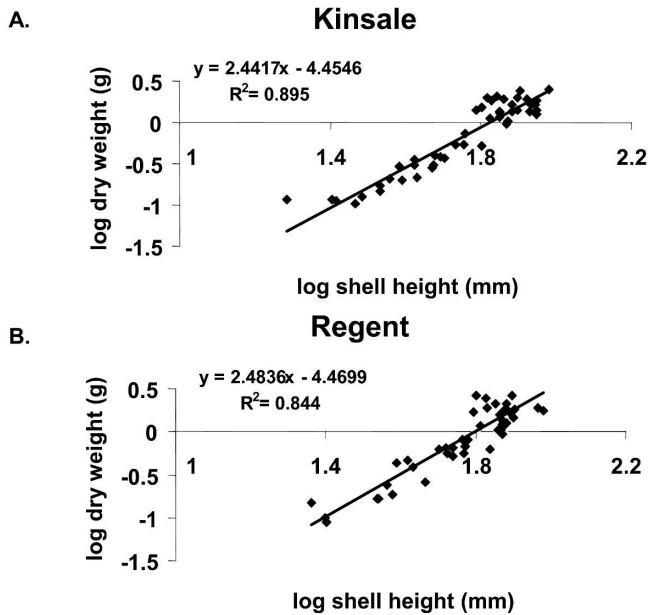$y = 2.4836x - 4.4699$
$R^2 = 0.844$

Figure 14. Comparisons of log shell height (mm) and log dry weight (g) at A. Port Kinsale and B. Regent Point. Shell height and dry weight data are the log of mean shell height and dry weight at each period sampled. The means are collapsed across oyster stocks. CRB = Chesapeake Bay, Rappahannock River, CTS = Chesapeake Bay, Tangier Sound, LOB = Louisiana, Oyster Bayou, XB = CROSBreed strain.

and during gametogenesis (Dittman et al. 2001). These relationships may be a reflection of prior exposure to *Perkinsus*, as negative effects on condition were only seen during periods when the parasite would be expected to be quiescent (winter to early spring). Their study implies that increased parasite loads hinder the ability of the oyster to undergo reproductive maturation. In the present study, effects of disease on condition may be more relevant during similar periods (late fall–winter and early spring) when condition index is high. During periods of low condition (summer, presumably post spawning) effects are not discernible because seasonal effects on condition mask any effects of disease. Spawning periods in the Virginia portion of the Chesapeake Bay are typically from June to September, with two spawning periods in the summer and fall (Andrews 1979, Hayes & Menzel 1981). A critical period may be during maturation prior to the second spawning in the fall, when oysters are still heavily infected. During this period, increases in dry weight were seen in the XB and LOB stocks at Kinsale, implying that these stocks were able to recover from the dual stresses of spawning and disease.

Despite the difficulty in detecting effects of *P. marinus* on physiologic condition, the observation that condition index varies among stocks grown under a common environment has important implications for aquaculture. Condition index is used as an indicator of meat quality (Lucas & Beninger 1985). Indicators of condition may be important criteria, along with growth and survival, in choosing a suitable strain for grow out. Stocks showed significant differences in condition. These differences were most significant at specific months. If these differences are consistent over successive growth seasons, then months when condition is highest may be targeted as optimal harvest periods, a typical practice of oyster growers (Brown and Hartwick 1988). Consistent differences

in condition among stocks or strains may be useful indicators of performance and criteria for selective breeding. Growth and reproductive patterns of genetically distinct eastern oyster strains can remain fixed over multiple generations (Loosanoff 1969, Barber et al. 1991b, Dittman et al. 1998), improving the predictability of optimizing strain selection. Condition index alone, however, may not be solely indicative of meat quality. In the first year of growth, condition index of all stocks decreased during the summer and was attributed to increases in shell weight, as dry weights continued to increase during this period. At Kinsale, condition index was comparatively low in the LOB stock, but their dry weights were highest among all stocks. It was more likely that changes in condition index of the LOB stock reflected differences in shell weight, because all Louisiana oysters grown at Kinsale developed noticeably thicker shells.

Energy Reserves

Glycogen and TAG values decreased during the summer months in a similar manner to the condition index, suggesting mobilization of nutrient reserves during gametogenesis and spawning (Engle 1951, Trider & Castell 1980). Seasonal influences on energy reserves may have masked effects of disease. Infection by *H. nelsoni* reduced condition index and energy reserves in *C. virginica* (Barber et al. 1988a, Barber et al. 1988b). In earlier studies, effects of *P. marinus* on biochemical composition were variable. Stein and Mackin (1957) showed in histochemical assays that glycogen was depleted in infected oysters. Glycogen, however, was higher in infected oysters than in uninfected oysters in studies by Wilson et al. (1988) and White et al. (1988). In these studies oysters were also parasitized by the snail, *Boonea impressa*, so glycogen levels may have been affected by this parasite as well. In Galveston Bay, glycogen concentrations were negatively correlated with infections that were greater than light (Soniat et al. 1989). Glycogen, however, also decreased with salinity, so environmental effects on glycogen could not be ruled out. Increases in lipid phosphate and fatty acids were observed in *P. marinus* infected oysters (Wilson et al. 1988), but no other recent studies, besides this one, have examined effects of Dermo disease on lipid content of the oyster. In the present study, effects of *P. marinus* on TAG were not apparent. As with glycogen, seasonal variation in TAG may make it difficult to detect any effects *P. marinus* might have on lipid stores. Individual variation in TAG and glycogen was also high. *Perkinsus marinus* was demonstrated to reduce reproductive output and gametogenic development (Kennedy et al. 1995, Dittman et al. 2001). Demonstration of other physiological effects has been less clear. Oxygen consumption in oysters was not reduced by Dermo infection (Newell et al. 1994, Willson & Burnett 2000). Seasonal cycles in energy reserves strongly influence the physiological state of disease resistant and susceptible oysters and must be taken into account when examining effects of disease in the field. As shown by Dittman et al. (2001), specific periods related to gametogenic phases of the oyster may be when effects of disease are most crucial. Processes of nutrient assimilation and storage must be examined in the context of these periods to determine the physiological effects of Dermo disease.

This study is the first field test demonstrating variation in Dermo-resistance of native stocks within (Chesapeake Bay—CRB and CTS) and between (Gulf of Mexico vs. Chesapeake Bay)

regions. We identified a native stock possessing resistance to *P. marinus*. The identification of the CTS stock as Dermo-resistant may be an important step in developing new disease-resistant hatchery strains. Furthermore, the CTS stock seems to be more resistant to MSX than the other stocks tested. The development of disease-resistant strains is a paramount objective in the Chesapeake Bay, for providing seed for oyster reef restoration and for developing commercial aquaculture. In addition to survival and disease resistance, performance related traits such as growth and condition must be considered when selecting suitable strains for aquaculture because parameters related to disease resistance and performance are not necessarily correlated.

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