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Chromosome Set Instability in 1–2 Year Old Triploid *Crassostrea ariakensis* in Multiple Environments

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CHROMOSOME SET INSTABILITY IN 1-2 YEAR OLD
TRIPLOID *Crassostrea ariakensis* IN MULTIPLE ENVIRONMENTS

A Thesis
Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirement for the Degree of
Master of Science


by
Mingfang Zhou
2002


APPROVAL SHEET

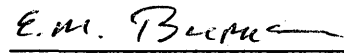
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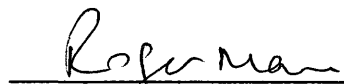

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
ABSTRACT.....	ix
INTRODUCTION.....	2
The Oyster Problem and Some Proactive Solutions.....	2
Triploid Oysters.....	5
Chromosome Set Instability in Triploid Oysters.....	8
Hypotheses and Objectives.....	9
MATERIALS AND METHODS.....	12
Animals.....	12
Study Sites.....	12
Experimental Design.....	13
Sampling Methodology.....	15
Statistical Analyses.....	17
RESULTS.....	19
Pre-screen of Triploid Stock.....	19
Deployment.....	19
Temperature and Salinity.....	20
Mortality.....	20
Shell Height Growth.....	21
Salinity Adaptation.....	22
Frequency of Mosaics.....	24
Frequency of Diploid Cells.....	24

Relationship between Frequency of Mosaics and Salinity.....	25
Relationship between Interval Frequency of Mosaics and Interval Mortality.....	25
Relationship between Interval Frequency of Mosaics and Relative Shell Height	25
Relationship between Frequency of Diploid Cells in a Mosaic and Salinity	26
Relationship between Frequency of Diploid Cells in a Mosaic and its Relative Shell Height Growth.....	26
DISCUSSION.....	27
Growth and Mortality.....	27
Frequency of Reversion.....	28
Decreased Frequency of Diploid Cells.....	30
Effects of Environmental Conditions.....	31
Heteroploid Mosaics.....	32
LITERATURE CITED.....	36
VITA.....	41

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LIST OF TABLES

Table	Page
1. Salinity regimes, abbreviations and full names of study sites.....	42
2. Temperature and salinity.....	43
3. Cumulative mortality of triploid <i>Crassostrea ariakensis</i>	44
4. Cumulative mortality of triploid <i>Crassostrea virginica</i>	45
5. Frequency of mosaics in triploid <i>Crassostrea ariakensis</i>	46

LIST OF FIGURES

Figure	Page
1. Study sites in Virginia.....	47
2. Study sites in North Carolina.....	48
3. Example histogram of flow cytometry on a triploid <i>Crassostrea ariakensis</i>	49
4. Example graph of flow cytometry test on a <i>Crassostrea ariakensis</i> mosaic.....	50
5. Cumulative mortality of triploid <i>Crassostrea ariakensis</i> by sites.....	51
6. Cumulative mortality of triploid <i>Crassostrea virginica</i> by sites.....	52
7. Cumulative mortality of triploid <i>Crassostrea ariakensis</i> by salinity regimes.....	53
8. Cumulative mortality of triploid <i>Crassostrea virginica</i> by salinity regimes.....	54
9. Interval mortality of triploid <i>Crassostrea ariakensis</i> by sites.....	55
10. Shell height of triploid <i>Crassostrea ariakensis</i> by sites.....	56
11. A comparative view of shell height accumulation of triploid <i>Crassostrea ariakensis</i> by sites.....	57
12. Shell height of triploid <i>Crassostrea virginica</i> by sites.....	58
13. A comparative view of shell height accumulation of triploid <i>Crassostrea virginica</i> by sites.....	59
14. Shell height of triploid <i>Crassostrea ariakensis</i> by salinity regimes.....	60
15. Shell height of triploid <i>Crassostrea virginica</i> by salinity regimes.....	61

16. Relative shell height growth of triploid <i>Crassostrea ariakensis</i> by sites.....	62
17. Significant noise of flow cytometry test of hemolymph.....	63
18. The effect of salinity adaptation.....	64
19. Frequency of <i>Crassostrea ariakensis</i> mosaics by sites.....	65
20. Frequency of <i>Crassostrea ariakensis</i> mosaics by salinity regimes.....	66
21. Interval frequency of <i>Crassostrea ariakensis</i> mosaics.....	67
22. Frequency of diploid cells in individual <i>Crassostrea ariakensis</i> mosaics until market size.....	68
23. Frequency of diploid cells in individual <i>Crassostrea ariakensis</i> mosaics until the end of experiments.....	69

ABSTRACT

The population of native eastern oyster *Crassostrea virginica* has been declining dramatically since the late 1880s, and in past decades, oyster diseases (MSX and Dermo) caused overwhelming mortality. One of the strategies developed to solve the disease problem and restore oyster populations in the Chesapeake Bay is introduction of a non-native oyster, *C. ariakensis*. This species showed lower disease susceptibility and superior survival and growth than *C. virginica* in a wide salinity regime. Triploidy was induced in *C. ariakensis* to control its reproduction. However, the recently documented phenomenon of reversion (development of diploid cells within a triploid) indicates the potential risk of triploid oysters becoming reproductively capable. This study was to monitor the frequency of reversion in triploid *C. ariakensis* from seed size to market size, and to examine the relationships between frequency of reversion and salinity regimes/mortality/shell height growth. Triploid *C. virginica* were used as the control of growth and survival.

Triploids were induced by cytochalasin B in April 1999. Triploid *C. ariakensis* were individually labeled before deployment. Seven sites were chosen under three salinity regimes: low (<15ppt), medium (15-25ppt), and high (>25ppt). From November to December 1999, three replicate groups of triploid *C. ariakensis* and two groups of triploid *C. virginica* were deployed to each site, with 52 oysters in each group. Every three months after deployment, all triploid *C. ariakensis* were biopsied for hemolymph to determine the occurrence and extent of reversion using flow cytometry.

As in other studies, growth and survival of *C. ariakensis* is superior to *C. virginica* in a wide salinity regime. *C. ariakensis* reached market size in medium and high salinity by June 2000, and in low salinity by December 2000.

Cytochalasin B induced a remarkable rate of triploid *C. ariakensis* (99%) in this study. Reversion was infrequent during the first year for triploid *C. ariakensis*. Totally 23 mosaics were found out of 919 living oysters (2.5%) by September 2000, when oysters in medium and high salinity were examined for the last time. Three more were found in low salinity in December 2000, when the study ended. Frequency of mosaics *C. ariakensis* ranged between 0% and 5% among the seven sites at the end. Frequency of diploid cells within individual mosaics was generally less than 10% although three of them contained 28%, 46% and 65% diploid cells. There was no significant relationship between frequency of reversion and salinity regimes, mortality or shell height growth. Frequency of reversion appeared to be age related and might have species variation. The low frequency of reversion demonstrated in this study suggests that the risk of reversion in commercial aquaculture of triploid *C. ariakensis* will probably be very low. The risk of reproductive recovery among triploids seems to reside in the unharvested and "lost" oysters that might remain in the Bay for long periods of time. Reproductive potential of mosaic *C. ariakensis* needs to be further studied to determine the extent of such risk.

**CHROMOSOME SET INSTABILITY IN 1-2 YEAR OLD
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INTRODUCTION

The Oyster Problem and Some Proactive Solutions

Since the late 1880s, the natural population of eastern oyster, *Crassostrea virginica* (Gmelin 1791), has been declining dramatically on the Atlantic Coast of the United States, due to over-harvesting, destruction of oyster reefs, deterioration of water quality, and prevalence of oyster diseases. This is a “tragedy of the commons” because oysters are significant in two aspects. Economically, they have been harvested since humans inhabited the Chesapeake Bay area and have supported commercial and recreational fisheries for over a century. Ecologically, oyster reefs are critical habitat for diverse ecological communities. They also play an important role in maintaining water quality of the Bay. Their extraordinary filtering capability helps to remove sediments, nutrients and algae from water, which in excessive amounts are harmful to the Bay’s ecosystem. By one estimate, the Bay’s entire water column could be filtered in three to six days by resident oysters before the decline (Newell 1988), while now, it might take a year or longer. Therefore, it is extremely important to restore oyster populations in the Bay.

Major impediments to oyster restoration have been the diseases, MSX and Dermo, which caused overwhelming mortality of eastern oysters in the past decades. MSX (protozoan parasite *Haplosporidium nelsoni*) was first documented in Delaware Bay in 1957 and two years later in Chesapeake Bay (Haskin et al., 1965; Andrews and

Wood 1967; Ford and Tripp, 1996). Dermo (protozoan parasite *Perkinsus marinus*) was first documented in the Gulf of Mexico in the 1940s (Ford and Tripp, 1996) and has been found in Chesapeake Bay since 1949. Virginia Institute of Marine Science (VIMS) and other groups along the mid-Atlantic coast developed several strategies to solve the disease problem. The two most proactive ones are selective breeding and introduction of non-native oyster species.

Selective breeding, or artificial selection, has been successfully applied to the eastern oyster to enhance its disease resistance. The principle is relatively simple. Oysters that survive disease(s) are selected as parents to propagate the next generation. After the first outbreak of MSX in Delaware Bay in 1957, the Haskin Shellfish Research Laboratory at Rutgers University began to examine the heritability of MSX resistance in eastern oysters. During the following decades, several pedigreed lines were established and they were up to 10 times more resistant to MSX than susceptible oysters (Haskin and Ford, 1979; Ford and Haskin, 1987). When Dermo invaded Delaware Bay in 1992, the “Haskin” lines were developed for dual disease (MSX and Dermo) resistance and are now called CROSBreed lines.

The process of selection, however, is very time-consuming. Twenty-five years of research and over eight oyster generations contributed to the establishment of “Haskin” lines (Ford and Haskin, 1987). Besides, genetic degradation is an ongoing concern since the process of selection is essentially inbreeding, which narrows the range of traits usually found in wild stocks, i.e., reduces overall genetic variability. Reduced genetic variability may cause the reduction of individual or population fitness (Allendorf and

Phelps 1980, Leary et al. 1983). This might account for decreased performance in early life stages. Recent efforts are focused on combining breeding with molecular genetics, which may greatly accelerate the process of selection.

Another proactive strategy to combat disease is introducing non-native species, since non-natives may be naturally resistant to diseases. To provide a science-based foundation for public policy decisions on this issue, VIMS formulated the “Rational Plan for Testing Application of Non-native Oyster Species” (“RP”) in 1996. The specific objectives are twofold. First, candidate species are being examined for their suitability, especially their disease susceptibility in Chesapeake Bay through a series of tests partly under quarantine conditions and partly in the field. Second, the results of these tests will provide information for assessment of environmental risks associated with their possible introduction. So far, two *Crassostrea* species have been evaluated: the Pacific oyster, *Crassostrea gigas* (Thunberg 1793), and the Suminoe oyster, *C. ariakensis* (Fujita 1913).

Stocks of the two species were derived from sources on the West Coast of the United States. Both were originally shipped from Japan. Seed of *C. gigas* was imported in the early 1900s and has been successfully cultivated on the West Coast since 1902 (Schaefer, 1938). Seed of *C. ariakensis* was inadvertently introduced with importation of *C. gigas* and *C. sikamaea* (the Kumamoto oyster). *C. ariakensis* has limited production on the West Coast primarily due to its requirement for lower salinity (15-20ppt) in the hatchery stage (Robinson and Landon, 1993).

C. gigas was the first species examined under “RP” since it exhibited superior resistance to diseases in preliminary laboratory testing (Meyers et al., 1991; Chu et al.,

1993) and has been well documented for successful aquaculture introductions around the world (Korringa, 1976; Chew, 1990; Menzel, 1991). During the field test in 1997-1998 using juvenile oysters at nine sites in Virginia, survival and growth of *C. gigas* were superior to *C. virginica* only in high salinity (>25ppt), which is not the condition commonly found within Chesapeake Bay (Calvo et al., 1999). *C. ariakensis* was then tested since it is generally acknowledged to tolerate lower salinity (Mann et al., 1991). The study of two-year old *C. ariakensis* at six sites from May 1998 to September 1999 demonstrated that its survival and growth were equal or superior to *C. virginica* over all salinities (Calvo et al., 2000). *C. ariakensis* has proven to be an excellent candidate for non-native oyster introduction in Chesapeake Bay.

Triploid Oysters

Introduction of non-native species is always fraught with potential ecological problems (Carlton, 1989), such as introduction of parasites and pathogens, competitive displacement of the native species (self-establishment), and genetic impact on the natives (hybridization). The best answer to control all these potential risks is induction of “triploidy.” Triploids are “sterile” so they cannot reproduce or hybridize with other oyster species. The secondary benefit of triploid induction is that the spawning and rearing procedure in hatchery minimizes potential parasite introduction. Therefore, triploidy has been applied in all field tests of non-native oysters. Actually, it is the only feasible mode of population control for aquatic organisms like oysters.

Why are triploids sterile? Triploid organisms contain three sets of chromosomes in their somatic cells instead of two sets in normal diploids. Diploids undergo meiosis to reduce the chromosome number by half in reproductive cells. The extra set of chromosomes in a triploid disrupts the intricate pairing of the original two sets during normal meiosis. A total lack of functional gametes or a greatly reduced production of functional gametes accounts for reproductive failure in triploids (Allen, 1988).

In a few species, such as certain gynogenetic fishes, triploidy is a natural mode of reproduction, but in most animal species, triploidy occurs infrequently and is considered a numerical mutation of chromosomes (Guo and Allen, 1994a). Stanley et al. (1981) made the first successful artificial production of triploid oysters by treating newly fertilized eastern oyster eggs with cytochalasin B (CB). CB is a cytokinetic inhibitor, which restrains normal cell division. The key to CB induction is the timing of meiosis. In a shellfish egg, meiosis normally arrests at the stage of chromosome duplication so the egg contains two sets of duplicated chromosomes. Fertilization reactivates the egg and meiosis continues. The first meiotic division results in the elimination of the first polar body (PB1) containing one set of duplicated chromosomes. The second meiotic division divides the remaining one into two haploid sets of chromosomes. One of them is eliminated as the second polar body (PB2). The other one, left in the egg, unites with the haploid sperm to restore the diploid condition. In the process of CB induction, CB is applied right before the second meiotic division. So, PB2 is kept in the egg and contributes the third set of chromosomes. However, the timing of PB2 elimination in newly fertilized eggs is subject to inherent variation: some eggs escape the CB treatment

and remain diploid. The efficiency of CB induction can be as high as 85-95% (S.K. Allen, Jr., VIMS, personal communication). 100% triploidy is unobtainable with induction techniques. However, induced triploid oysters may be individually examined by flow cytometry (FCM) to ensure utilization of 100% triploids before field tests.

In the summer of 1993, the successful creation of tetraploid *C. gigas* (Guo and Allen, 1994b) made the production of 100% triploid oyster brood stock possible by crossing tetraploids with diploids (Guo et al., 1996). Triploids made by this technique are called natural triploids. It is a crucial step to non-native oyster aquaculture. For *C. ariakensis*, tetraploids were not available for spawning at the start of this project. A stock of chemically induced triploid *C. ariakensis* was used instead.

Triploid oysters were successfully introduced to the West Coast and the reproductive potential of triploid *C. gigas* was studied extensively. Estimated by cross sectional area, gonad size of triploid males was about half of that in diploid males and gonad size of triploid females was about a quarter of that in diploid females (Allen, 1988). Also, gametogenesis in triploids was severely retarded. Follicles were few and incompletely formed. Gametes were in various stages of maturation within the same animal. However, both male and female triploid *C. gigas* did make significant numbers of gametes although the quality of the gametes varied widely among individuals (Allen and Downing, 1990). Further study revealed that the relative fecundity (measured by the amount of gametes) of triploid males was about 0.1% of diploid males and the relative fecundity of triploid females was about 2% that of diploid females (Guo and Allen, 1994a and S.K. Allen, Jr., VIMS, personal communication). Gametes from triploids were fully

capable of fertilization, but aneuploid progeny resulted and the survival of oyster larvae to metamorphosis and settlement was only about 0.0085% (Guo and Allen, 1994a).

Overall, reproductive capacity of triploids is practically zero.

Chromosome Set Instability in Triploid Oysters

In the first field trial of triploid *C. gigas* for disease resistance in Delaware Bay and Chesapeake Bay in 1993, a relatively high proportion (15% and 20% respectively) of chemically induced triploid oysters were found to be mosaic after nine months of disease challenge (Allen et al., 1996). The term mosaic refers to the coexistence of two or more genetically distinct cell populations derived originally from a single zygote. In our case, it is the presence of both diploid and triploid cells in the same organism. The gradual transition of a triploid individual to a mosaic is called “reversion”. The occurrence of mosaics was hypothesized as the disruptive effect of CB on early cell development. However, the frequency of mosaics in several triploid *C. gigas* populations increased over time, suggesting that there might be a tendency for chromosome loss in such populations.

The origin and fate of reversion was further investigated in the spring of 1996, when three replicate spawns of both chemically induced and natural triploid *C. gigas* were tested in two separate sites: quarantine facilities near Delaware Bay and the natural waters of York River in Chesapeake Bay. Reversion was proved to be a regular feature of triploid *C. gigas*, as it occurred in all experimental groups. Furthermore, reversion was progressive at both individual and population level, i.e., once reversion started, the

frequency of diploid cells in an individual mosaic, as well as the frequency of mosaics in a population increases over time. Chemically induced triploids presented higher frequency of mosaics than natural triploids, and are therefore the more conservative indicators of reversion. (Standish K. Allen, Jr., unpublished data)

The equivalent examination of frequency of reversion at the population level on triploid *C. ariakensis* was integrated into the 1998-1999 “RP” field test mentioned before. At each sampling time, 16-35 oysters were randomly collected for ploidy tests from each site over the course of the study (Calvo et al., 2001). For examination of reversion frequency at the individual level, another 125 oysters from the same spawn were individually labeled and distributed at the same time. Similar results were obtained. Reversion was also a regular feature of triploid *C. ariakensis* and was progressive at both the individual and population level. (Standish K. Allen, Jr., unpublished data)

Reproductive potential of mosaics is still under investigation. It was reported that gametogenesis in mosaic oysters and triploid oysters was indistinguishable and that there was no evidence of normal (diploid) gametic activity in male mosaics (Chandler et al., 1999). Although data thus far has suggested that mosaics fail to develop haploid gametes, the ultimate appearance of haploid gametes cannot be ruled out. The appearance of mosaics and the process of reversion suggest that there are potential risks in applying triploidy for population control of non-native oysters.

Hypotheses and objectives

Now that triploid *C. ariakensis* has emerged as a potential aquaculture candidate in Chesapeake Bay, reversion of triploids complicates the issue. A risk assessment for self-establishment of triploid *C. ariakensis* in aquaculture is warranted and should be completed before any commercial scale introduction is conducted. Current information on reversion of triploid *C. ariakensis* is incomplete. One major limitation of the previous study on reversion was that oysters were two years old at the beginning of the experiment and their mean shell height was about 64 mm (Calvo et al., 2001). Normally harvesting occurs at about 75 mm. The main objective of this study is to quantify frequency of reversion in triploid *C. ariakensis* from seed size to market size, a more realistic assessment for commercial aquaculture. As in the previous study, sites were selected under low, medium and high salinity regimes. Instead of sampling oysters from within a group, each oyster was labeled and examined repeatedly and regularly throughout the course of the study.

The mechanism of reversion is unknown. The cause could be genetic or environmental, or both. I will address environmental effects in this thesis. The general hypothesis is that triploid oysters (or triploid cells) tend to revert more frequently under less favorable conditions. Specifically, salinity regimes, mortality and shell height growth will be examined. Salinity will be related to reversion because it is a crucial environmental factor in oyster aquaculture. Difference on mortality and shell height growth among sites of different salinities may also serve as an indicator of frequency of reversion because mortality and shell height are important indices of living conditions. Therefore, the second objective of this study is to examine the relationships between

frequency of reversion and salinity regimes, mortality or shell height growth. The null hypothesis is that such relationships do not exist.

MATERIALS AND METHODS

Animals

Stocks of *C. ariakensis* used in this study stemmed from sources on the West Coast. Triploid *C. ariakensis* were produced on April 22nd, 1999 by CB induction, a method described by Downing and Allen (1987) and Allen et al. (1989), and was the third generation of artificial spawn. Triploid *C. virginica* to be used as a growth and survival control were produced on the same day by the same process. Larvae of both species were reared through metamorphosis in ABC's (Aquaculture Genetics and Breeding Technology Center, VIMS) Gloucester Point Hatchery and then kept in the flow-through quarantine system until they were ready for deployment.

Study Sites

Eight sites were selected in Virginia and in North Carolina, and were broadly categorized into three salinity regimes according to previous records of their annual mean salinities: low (<15 ppt), medium (15-25 ppt), and high (>25 ppt). Each salinity regime had two or three replicate sites. Six sites were in Virginia and two in North Carolina. (Table 1, Figure 1 & 2)

Study sites were visited on a monthly basis after deployment for regular maintenance and temperature/salinity data records. Extra site visits were made in severe fouling or weather conditions. Fouling organisms such as barnacles, tunicates, sponges,

and seaweed were cleaned off the oyster bags and floating trays. Old oyster bags were replaced when necessary. Temperature was measured with a stem thermometer except at the two sites in North Carolina, where Hobo-Temp temperature loggers were used. Salinity was measured with a temperature-compensated refractometer. These data were used for reference.

Experimental Design

Three replicate groups of triploid *C. ariakensis* and two replicate groups of triploid *C. virginica* were deployed at each site, with about 52 oysters in each group. In total, about 156 triploid *C. ariakensis* and 104 triploid *C. virginica* were planted at each site.

Several steps were taken before deployment. First, every seed oyster of putative triploid *C. ariakensis* was examined for ploidy by flow cytometry (FCM). The principle of FCM and the procedure of hemolymph biopsy will be explained later. Then, the certified triploid oysters were individually labeled by fixing a numbered plastic tag on the oyster shell with Super Glue. Meanwhile, individual shell height was measured. According to these measurements, the oysters were sorted into three size classes (small, medium and large) and the oysters of each size class were distributed evenly and randomly into replicate groups ending up with the same average shell height in each group.

Seed of putative triploid *C. virginica* were not examined individually. A sample of 225 seed were randomly chosen and tested by FCM. Percentage of triploids in this

sample group was used to represent that of the whole brood stock. The whole stock of putative triploid *C. virginica* was then divided into replicate groups as described previously for triploid *C. ariakensis*. About half (27 individuals) of the randomly chosen *C. virginica* in each group were measured for shell height and labeled for repeated measurement.

Finally, oysters of each replicate group were put into a plastic ADPI bag (36cm x 44cm x 7 cm). A floating tray (2.3m x 0.5m x 0.3m) was used to hold three bags of triploid *C. ariakensis* and two bags of triploid *C. virginica*. Bags containing *C. ariakensis* were alternated with bags containing *C. virginica*, specifically, in the order of *C. a.*, *C. v.*, *C. a.*, *C. v.*, and *C. a.*. For sites in Chincoteague Bay, Chadwick's Bay and Wanchese, oyster racks were used instead of floating trays.

About three months after deployment, oysters were returned to the lab for reexamination. Fouling organisms were cleaned off the oyster shell. Mortality was counted in each bag. For triploid *C. ariakensis*, shell height was measured and ploidy was examined for each individual. For triploid *C. virginica*, only shell height was measured for the labeled oysters. Lost tags were replaced by reconstructing the previous and the current order of shell height measurements and matching the equivalents (Depending on the water flow in a specific site, tag loss ranged between 0-60% in one sampling interval. Due to the low incidence of reversion in this study, tag loss didn't interfere with an accurate trace record of reversion in individual oysters.) After all this was done, oysters were put back into their original bags and returned to their original sites. This procedure was repeated every three months until the end of the test.

Sampling Methodology

Principle of flow cytometry (FCM)

Our principle means of testing ploidy and analyzing mosaics has been FCM on a Partec CAII bench top model. Before testing by FCM, the tissue sample is stained by a fluorescent dye called DAPI (4', 6-diamidino-2-phenylindole). DAPI bonds covalently to nucleic acids and is absorbed by the nucleus in direct proportion to its DNA content. The stained nuclei are pumped through the FCM fluorescence detector in single file where a ultra-violet light source causes the bonded dye to illuminate. FCM measures the fluorescence intensity emitted by DAPI bonded nuclei, hence measures the relative DNA contents of each nucleus. In minutes, thousands or even tens of thousands of nuclei can be assessed.

The fluorescence measurement was shown as a frequency distribution graph on the display screen (Figure 3). The X-axis of the graph indicates fluorescence intensity, or relative DNA content, while the Y-axis indicates frequency. Since the cells of different ploidy levels contain remarkably different amounts of DNA, the graph displays them as discrete peaks (Figure 4).

The machine also calculates the mean and area of each peak. The mean represents the mean relative DNA content, hence the ploidy level; the area represents the number of cells detected at that ploidy level. If more than one peak is detected, the areas are used to calculate the proportion of cells at each ploidy level.

Standard fluorescent beads are used for correction before each use of FCM. The mean of the beads' fluorescence intensity is usually set at 22 units. The mean of diploid and triploid peaks of *C. ariakensis* is about 64 and 96 respectively (Figure 4).

Hemolymph Biopsy

Hemolymph was the tissue of choice for examining ploidy in this study for two reasons. First, hemolymph is believed to be the most sensitive indicator of mosaicism. According to a comparative study on six tissue types (gill, gonad, heart, adductor muscle, digestive gland and hemolymph) from two-year-old *C. ariakensis* and *C. gigas* mosaics, hemolymph contained the highest proportion of diploid cells (Chandler et al., 1999), thus was the most conservative indicator of mosaicism. Second, hemolymph biopsies can be sampled non-destructively, which is imperative for repeated sampling of the same individuals.

The procedure of hemolymph biopsy was as follows: oysters were notched on the margin of the dorsal area adjacent to the adductor muscle by a Dremel™ drill. The size of the notch was just big enough to insert a 23G (1 ½ inches) syringe needle. About 0.1 ml hemolymph was extracted from the adductor muscle and then expelled into a 1.5 ml plastic micro centrifuge tube containing about 1 ml DAPI. The sample prepared in this way can be stored for several weeks in a freezer of -80°C or tested immediately. The test results of both are equivalent. In order to save time on sampling over a thousand oysters so that a better comparison of reversion among the seven sites could be done, freshly made samples were immediately stored in the freezer until all sampling was done.

DAPI-hemolymph suspension, either freshly made or thawed, was aspirated several times with a syringe to disaggregate cells and to break cell membranes. The suspension was then filtered into a mini-test tube specifically designed for the Partec CA-II. DAPI and the hemolymph samples were kept on ice during the above processes.

Statistical Analyses

Interval mortality was calculated as the number of oysters that died during each sampling interval divided by the number of living oysters at the beginning of the interval. Cumulative mortality was calculated as the total number of dead oysters over a certain time divided by the number of living oysters at the beginning of the experiment.

Relative shell height growth for individual oysters was calculated as the overall shell height increment during the sample interval divided by the shell height at the beginning of the interval.

The overall comparison of final shell height between triploid *C. ariakensis* and triploid *C. virginica* was made by an F test. The extent of shell height difference between the two species among the three salinity regimes was examined by Mixed Model Analysis of Variance and Tukey's test. All these tests were conducted by the GLM (General Lineal Model) procedure in SAS (Statistical Analysis System) version 8.0 (SAS Institute Inc., 1990).

Frequency of reversion at the population level refers to the "frequency of mosaics." It was calculated as the total number of detected mosaics divided by the number of living oysters at each sampling date. Interval frequency of mosaics was

calculated as the number of newly detected mosaics during each sampling interval divided by the number of living oysters at each sampling date.

Frequency of reversion at the individual level refers to “frequency of reverted (or diploid) cells.” It was calculated from the frequency distribution shown on the FCM screen, as the area of diploid peak divided by the total area of both the diploid and triploid peaks.

Relationship between frequency of mosaics and salinity was examined by ANOVA (Analysis of Variance) in MINITAB, also by Logistic Regression Analysis and Contingency Table Analysis using CATMOD (CATegorical MODeling) procedure in SAS.

Relationship between interval frequency of mosaics and interval mortality was examined by Regression Analysis in MINITAB.

Relationship between interval frequency of mosaics and relative shell height growth was examined by Regression Analysis in MINITAB, and by Logistic Regression Analysis using CATMOD procedure in SAS.

Relationship between frequency of diploid cells in a mosaic and salinity was examined by ANOVA in MINITAB.

Relationship between frequency of diploid cells in a mosaic and its relative shell height growth was examined by Regression Analysis using GLM procedure in SAS.

The 5% significance level was chosen for data analysis. Percentage data were Arc-sin transformed.

RESULTS

Pre-screen of Triploid Stock

Oysters became suitable for hemolymph biopsy when their shell height reached about 40 mm. The brood of putative triploid *C. ariakensis* was examined for triploidy when they were five months old. From September 23rd to October 19th, 1999, a total of 1,512 seed were examined by FCM. Among them, 1,498 were triploid (99.07%), one was mosaic (0.07%) and 13 were diploids (0.86%). The mosaic had 3.98% diploid cells. The 1,498 certified triploids and the one mosaic were individually labeled. Shell heights were measured from Oct. 22nd to 24th, 1999 and 85 individuals were found dead (5.7% mortality). The remaining 1,412 living triploids and the one mosaic were divided into 27 groups in the way described in the method section, with about 52 oysters in each group. Three of the 27 groups were prepared for a test site in Maryland, but permission was denied. These three “Maryland” groups were kept at the Gloucester Point hatchery and tested for reversion only in March and June 2000. Seed of putative triploid *C. virginica* were examined by FCM on Oct. 7th, 1999. Out of a randomly chosen sub-group of 225 seed, 216 were triploid (96%), three were mosaic (1.3%) and six were diploid (2.7%).

Deployment

From November 15th to 17th, 1999, the oysters were deployed at the six sites in Virginia. Deployments at the two sites in North Carolina were done by December 10th,

1999. Oysters survived and grew to the end of experiments at seven of the eight sites. However, during the snowstorm in January 2000, the floating tray in Burton Bay was flipped over and stranded above water. Due to the freezing temperature and the jostling of the frozen oysters inside, almost all of them were killed. Oysters were recovered but no subsequent data were collected from that site.

In June 2000, triploid *C. ariakensis* reached market size (about 75mm) in medium and high salinity regimes, but not in low salinity. To collect more data for a better comparison of reversion and growth among different salinities, all the oysters were returned to their sites for another three months. In September 2000, oysters in low salinity were still below market size. At this time oysters in medium and high salinity were retrieved, while those in low salinity were monitored for another three months until December 2000.

Temperature and Salinity

Temperature and salinity data are listed in Table 2. Mean temperature during the study period ranged 12.2-19.8 °C among the seven sites. Mean salinity ranged 10.7-33.1 ppt among the seven sites: 10.7-15.1 ppt at low salinity sites; 20.1-20.8 ppt at medium salinity sites; 32-33.1ppt at high salinity sites.

Mortality

Cumulative mortality of triploid *C. ariakensis* at the seven sites ranged from 1.3% to 28.8% (Figure 5, Table 3), while cumulative mortality of triploid *C. virginica* at the

seven sites ranged from 5.8% to 96.2% (Figure 6, Table 4). On average, mortality of triploid *C. ariakensis* was lowest in medium salinity and highest in low salinity (Figure 7), while mortality of triploid *C. virginica* was lowest in low salinity and highest in high salinity (Figure 8). Among the four sampling intervals, interval mortality of triploid *C. ariakensis* was generally highest in summer (June – September 2000) (Figure 9).

Shell Height Growth

General data for triploid C. ariakensis and triploid C. virginica

Before deployment, mean shell height of triploid *C. ariakensis* at each site was about 52 mm, while mean shell height of triploid *C. virginica* at each site was about 36mm. After deployment, shell height varied more significantly in triploid *C. ariakensis* than in triploid *C. virginica* among different sites. Final shell height of triploid *C. ariakensis* ranged between 59.0 mm and 102.3 mm (Figure 10 and 11), final shell height of triploid *C. virginica* ranged between 57.3 mm and 74.4 mm (Figure 12 and 13). On average, triploid *C. ariakensis* at low salinity grew significantly slower than those in medium and high salinity (Figure 11 and 14), while growth rate of triploid *C. virginica* was similar in all three salinity regimes (Figure 13 and 15). In medium and high salinity, triploid *C. ariakensis* reached market size (about 75 mm) by June 2000. In low salinity, oysters took an additional six months or more to reach market size. Among the four sampling intervals, relative shell height growth of triploid *C. ariakensis* was generally greatest in spring (March – June 2000) (Figure 16).

Comparison between triploid C. ariakensis and triploid C. virginica

Shell height was significantly different between the two species when data from all sites were combined ($P < 0.0001$, F tests based on data of September 2000). Furthermore, the extent of shell height difference between the two species among the three salinity regimes was also significant ($P = 0.01$, Mixed Model Analysis of Variance and Tukey's test, based on data of September 2000). The mean shell height of triploid *C. ariakensis* was larger than triploid *C. virginica* in all salinity regimes. The difference was 30.2 mm in high salinity, 22.2 mm in medium and 6.1 mm in low.

Salinity Adaptation

During the whole experiment, hemolymph biopsies in general generated clear FCM distribution graphs. In March 2000, however, graphs of most samples from Chadwick's Bay (CHAD) were indistinct. Noise signals were dominant. Normally sharp and clean triploid peaks were broadened, which often covered the position of the diploid peak and made distinguishing diploid peaks impossible (Figure 17). Three months later in June 2000, the noise signals were present in samples from both CHAD and Chincoteague Bay (CHIN). Interestingly, both sites were in the high salinity regime (annual mean salinity >25 ppt). Salinity in CHIN and CHAD was 30 and 34 ppt in March 2000, respectively, and 32 and 35 ppt in June 2000. It appeared that salinity might be the cause. And since the salinity in CHAD was higher than that in CHIN, the noise problem appeared earlier in CHAD.

To test that high salinity was the direct cause of the noise signals, on August 11th, 2000, I randomly took one bag of triploid *C. ariakensis* from CHIN at 32 ppt and put it into 21 ppt water in Gloucester Point Hatchery. Three days later on Aug. 14th, six out of 38 oysters had noise signals in their hemolymph samples. Another two days later, FCM graphs of those six became completely normal. On August 22nd, 18 oysters were randomly taken from CHAD with 32 ppt and were also transferred into 22 ppt water in the hatchery. About six days later, all samples generated normal FCM graphs.

To avoid the appearance of noise signals later, I did salinity adaptation again on Sep. 20th, 2000. One bag of triploid *C. ariakensis* was randomly chosen from CHIN at 34 ppt. Thirty-three out of 48 oysters in that bag showed noise signals in their samples before adaptation. The oysters were then put into 20 ppt water in the hatchery. Three days later on Sep. 23rd, noise signals were reduced significantly in samples from those 33 oysters. Only three of them still carried the noise. Six days later on Sep. 27th, the noise signals were totally gone (Figure 18).

The same procedure was applied to the rest of the oysters from CHIN and CHAD with equal success. What exactly happened to the hemolymph condition of the oysters living in high salinity waters is uncertain. One possibility was that the high salinity of the ambient water increased the salinity of hemolymph, which influenced the staining function of DAPI.

Frequency of Mosaics

Over the course of the study, reversion occurred at low frequency in triploid *C. ariakensis* at all seven sites. The final frequency of mosaics ranged between 0% (Great Wicomico, low salinity) and $5\% \pm 2.5\%$ SD (York River, medium salinity) (Figure 19 and Table 5). A total of 23 mosaics were found out of 919 surviving individuals (frequency: 2.5%) in September 2000, when the oysters in medium and high salinity were examined for the last time. Three more were found out of 356 (frequency: 0.8%) left in low salinity in December 2000, when the study ended.

On average, reversion occurred most frequently in medium salinity (Figure 20). Among the four sampling intervals, interval frequency of mosaics was generally greatest in summer (June-September 2000) (Figure 21).

No mosaics were found in the “Maryland” groups that were held at VIMS during the March and June 2000 test. These data were not included in figures and analyses but were used in discussion.

Frequency of Diploid Cells

Figure 22 and 23 shows frequency of diploid cells in individual mosaics at each sampling time. In Figure 22, the presentation of the data stops when the mean shell height in a site reached market size, a critical point in this study. Figure 23 shows all mosaic data that were collected. Most mosaics contained low proportion of diploid cells (less than 15%) over the course of the study. Three individuals contained a relatively high proportion of diploid cells at the end of the study (28%, 46% and 65% respectively).

Relationship between Frequency of Mosaics and Salinity

No significant difference in percentage of mosaics was found among the three salinity regimes ($P = 0.111$, ANOVA based on data of June 2000, $P = 0.439$, ANOVA based on data of September 2000). Probability of reversion was not a function of salinity ($P = 0.27$, Logistic Regression Analysis; $P = 0.25$, Contingency Table Analysis. Both based on data of September 2000).

Relationship between Interval Frequency of Mosaics and Interval Mortality

There was no relationship between interval frequency of mosaics and interval mortality ($P = 0.626$ for March-June 2000 interval, $P = 0.892$ for June-September 2000 interval, Regression Analysis).

Relationship between Interval Frequency of Mosaics and Relative Shell Height Growth

There was no relationship between interval frequency of mosaics and relative shell height growth ($P = 0.52$ for March-June 2000 interval, $P = 0.102$ for June-September 2000 interval, Regression Analysis). The probability of reversion was not a function of relative shell height growth ($P = 0.36$, Logistic Regression Analysis based on data of September 2000).

Relationship between Frequency of Diploid Cells in a Mosaic and Salinity

Frequency of diploid cells in a mosaic was not related to salinity ($P = 0.183$, ANOVA based on data of June 2000, $P = 0.061$, ANOVA based on data of September 2000).

Relationship between Frequency of Diploid Cells in a Mosaic and its Relative Shell Height Growth

Frequency of diploid cells in a mosaic was not related to its relative shell height growth ($p = 0.81$, Regression Analysis based on data of September 2000).

DISCUSSION

Growth and Mortality

My data supports the results from the previous study (Calvo et al., 2001), showing that growth and survival of *C. ariakensis* was generally superior to that of *C. virginica*. However, this trend was not consistent in all salinity regimes. For example, cumulative mortality of *C. ariakensis* was much higher than that of *C. virginica* in low salinity (Figure 7 and 8). Severe fouling by barnacles, sponges, and seaweed in low salinity Great Wicomico River and Coan River during summer and fall could be the cause of high mortality, since heavy fouling usually blocks the transportation of food and oxygen by inhibiting normal water flow. But why it caused significantly different mortality in the two species is questionable. Whether or not there was a disease effect is unknown. Also notice that before the deployment, the average shell height of *C. ariakensis* was 16 mm larger than that of *C. virginica*. Although the final mean shell height of *C. ariakensis* was 30.2 mm larger in high salinity, 22.2 mm in medium salinity and 6.1 mm in low salinity, *C. ariakensis* actually didn't outgrow *C. virginica* in low salinity.

The experimental design employed here was different from that of the previous study (Calvo et al., 2001). First, the animals were younger when I deployed them, as described in the introduction section. Second, triploid *C. virginica* was used as a control in this study instead of diploid. Third, the study sites covered a wider geographical area. Above all, this study extended the previous results and demonstrated that on the Atlantic

coast of Virginia and North Carolina, growth and survival of *C. ariakensis* is superior to *C. virginica* in a wide salinity regime.

Cumulative mortality of *C. virginica* in Chadwick's Bay was much higher than the other sites (Figure 6). Cumulative mortality of *C. ariakensis* in the same site was also relatively high (Figure 5). The cause of high mortality in Chadwick's Bay is unknown.

Over the course of the study, abnormally slow growth of *C. ariakensis* was observed at Wanchese. During the 14-month deployment, average shell height increased only about 8 mm, whereas the increase was 22 and 32 mm at the other two low salinity sites. The slow growth might be caused by strong wave action, which was observed only at Wanchese. It has been reported that high turbidity inhibits feeding efficiency of the oysters, restricting their growth (Loosanoff and Tommers, 1948). A high incidence of mud blisters inside dead oyster shells was also observed exclusively at Wanchese. Mud blisters might result from high turbidity in this case and might be an extra cause of slow growth because mud blisters reduces the ability of oyster to accumulate nutritional reserves (Wargo and Ford, 1993).

Frequency of Reversion

The brood of triploid *C. ariakensis* used in my study had a remarkably high efficiency of CB induction. Compared to expected efficiencies of 85-95% triploidy (S. K. Allen, Jr., personal communications), 99% of 1,498 putative juvenile oysters in this study were certified triploid in October 1999, six months after spawning. The efficiency of CB induction is mainly dependent on two factors: the synchrony of PB2 elimination in

newly fertilized eggs and the timing of CB treatment. The high efficiency of triploidy induction observed in my study suggests that there was little inherent variation in diploid *C. ariakensis* eggs and that the timing of CB treatment in this study was very accurate.

My study suggested that reversion of triploid *C. ariakensis* was infrequent during the first year. After the pre-screen, mosaics were first detected in June 2000, 14 months after birth, suggesting reversion first occurred sometime between March and June 2000. The overall frequency of mosaics in June 2000 was about 0.5% including data from “Maryland” groups, and the frequency of diploid cells in the seven mosaics remains mostly below 7% with only two exceptions of 26% and 31%.

Data from this study also suggested that reversion might be age related, since mosaics seemed to occur at a higher frequency in older triploid *C. ariakensis*. Interval frequency of mosaics was higher during June-September 2000 than during March-June 2000 (Figure 21). A comparison with the previous study on triploid *C. ariakensis* (Calvo et al., 2001) shows a similar trend. This study started with the juvenile oysters of six months old. After 11 months, the highest frequency of mosaics among seven sites was 5% and the overall mean was 2.5%. The previous study started with two years old animals (Calvo et al, 2001). After 12 months, the highest frequency of mosaics among six sites was 7.7% and the mean was 5.3%. However, the sites used for the two studies were not all the same. For both studies, environmental factors (salinity, temperature, currents, water contents, etc.) in every site were changing with time and their influences on reversion were uncertain. It is possible that the increase of the interval frequency of mosaics was caused by factors other than age.

Frequency of reversion might have species variation. In a previous study of CB induced triploid *C. gigas* that also started with juvenile oysters and deployed for a similar length of time of about nine months (S.K. Allen Jr., unpublished data), the final frequency of mosaics ranged between 3.6-10.7%, which is significantly higher than 0-5% in this study.

Decreased Frequency of Diploid Cells

Frequency of diploid cells increased continuously over time in most mosaic individuals. However, an opposite trend was observed in three mosaics from Coan River and one from Great Wicomico. In these four individuals, a frequency of 2.05%, 6.86%, 13.26%, and 2.14% diploid cells was detected respectively in September 2000 but all decreased to 0% in December 2000 (Figure 23). FCM artifact is negligible here because standard error was less than 0.5%, according to data from repeated sampling of the same mosaic individual either on the same day or within a few days.

There are two possibilities for frequency decrease of diploid cells in a mosaic. The first one is contamination during hemolymph sampling. For example, some of the ambient water held between oyster shells was sampled together with hemolymph and the contaminants generated a peak similar to that of diploid *C. ariakensis*. The contaminant could be self-illuminating or DAPI stainable. Another possibility is that a “stem cell” population of diploid cells developed, but then died for unknown reasons. Longwell and Stiles (1996) mentioned that in a mosaic, cells with deviant chromosome numbers are unlikely to contribute equally to development and growth, so their measured frequencies

are apt to change over time. One cell type, such as diploid cells in a mosaic, might win the competition for growing at one time and lose at another time.

Effects of Environmental Conditions

Results from this study demonstrated that there was no statistically significant relationship between the frequency of reversion and salinity regimes, mortality or shell height growth. This might be due to the overall low frequency of mosaics observed in this study. Although a low frequency of mosaics reduces reproductive risks associated with the appearance of diploid cells, the 26 mosaics out of 1275 individuals could not provide power for statistical analyses.

Results of this study suggest evidence of some environmental influences on reversion. Among the four sampling intervals, the third one, between June-September 2000, is interesting. Overall, this period showed the highest interval frequency of reversion (Figure 21). In addition, the highest interval mortality and the lowest shell height growth (Figure 9 and 16) were observed at most sites during this interval. In general this observation supports the hypothesis that reversion happens more frequently under less favorable conditions (Allen et al., 1999). These data were, however, insufficient for a solid conclusion because several exceptions to the general trend described above were observed at several sites. Furthermore, the levels of variance within each study site were in general very high due to limited numbers of replicates. Taken together, the data simply suggest that environmental effects on reversion might exist.

Another interesting observation is that a high salinity (>25‰ppt) seemed to be related to a low frequency of reversion. Frequency of mosaics at the two sites of high salinity was 1.3% and 1.9%, respectively, with the lowest average frequency of mosaics and smallest error bars among the three salinity regimes (Figure 20). In addition, the four mosaics found in high salinity presented lower frequency of diploid cells (1.7-6.5%) than those found in the other two salinities (up to 65%). Both this study and the previous one (Calvo et al., 2001) showed that the frequency of mosaics was highest in the medium salinity and lowest in high salinity, although no statistically significant differences were found among the three salinity regimes in both studies.

So far, this study is the only one addressing the environmental effects on reversion. Future studies should increase sample size, study period, or animal age to obtain more mosaic data that will in turn increase the power of statistical analyses.

Heteroploid Mosaicism

Mosaicism is an unusual and complicated issue. Its classic definition is the coexistence of two or more genetically distinct cell populations derived originally from a single zygote. These cells can be different in chromosome number or chromosome structure. The difference of chromosome number can be entire sets of chromosomes (heteroploid mosaicism), such as in the diploid-triploid mosaics in this study, or just a few individual chromosomes (aneuploid mosaic).

Heteroploid mosaicism has been reported in many species in both naturally occurring and artificially produced polyploid populations. In many cases of a natural

population, heteroploid mosaicism was found by chance and its cytogenetic mechanism remains unknown due to the lack of information about its origin (Bickham and Tucker, 1984; Kraus, 1991; Barsiene, 1992; Goddard and Schultz, 1993; Yamaki et al., 1999). In artificially induced populations, heteroploid mosaicism was mostly observed in early development stages like embryo, larvae, and juvenile fish, and its occurrence was related to direct impact of different induction methods. For example, Miller et al. (1994) examined heteroploids in chemically or physically induced polyploid salmonids and explained that chemical and physical treatments could induce chromosome separation errors during meiosis or mitosis: colchicine disrupts spindle formation, CB inhibits cytokinesis, and temperature shock or pressure shock does both. They also discussed that heteroploidy induced by saltwater exposure of salmonid eggs might be caused by polyspermy. Chromosome lagging, nondisjunction, and mono-, tri-, or tetra-polar division all contribute to mitotic error and have been observed in hybrids and pressure shock of salmonids (Yamazaki et al., 1989; Yamazaki and Goodier, 1993).

The diploid-triploid mosaicism found in recent studies of triploid oysters is a case different from those aforementioned. It was seen in artificially produced triploid oysters. However, it was not directly caused by CB treatment because the mosaics were certified as triploids at an early stage. Mosaics obtained in later developmental stages of oysters apparently are the result of chromosome set loss. In addition, the frequency of mosaics increased over time. To the best of our knowledge, there has been no similar report in other animals. This study is important not only because it is a crucial part of a risk



assessment of triploid *C. ariakensis* aquaculture, but also it represents a unique example of mosaicism.

Two tentative models of chromosome loss have been proposed to explain this type of mosaicism. One model assumes tri-polar spindle formation amidst mitoses of triploid cells (Allen et al., 1996). The other model, proposed in a recent research on mitotic metaphase spreads, suggests that chromosome elimination in triploid oyster cells may be caused by unusual chromosome clumping (Allen et al., 1999; Zhang et al, unpublished data). They demonstrated that mosaics with higher percentage of chromosome clumping tended to have higher percentage of hypotriploid cells in both *C. gigas* and *C. ariakensis*. This provided a clear link between chromosome clumping and chromosome loss. A possible explanation for this link is that clumped chromosomes are unable to undergo normal segregation. However, the question remains on how the chromosome clumping results in the loss of a whole set of chromosomes at one time.

In summary, the frequency of reversion in triploid *C. ariakensis* was very low through out the course of the study, especially during the first year. In June 2000 when the oysters were 14 months old, the overall frequency of mosaics was about 0.5% and the frequency of diploid cells in those individual mosaics usually remains below 7%. Final frequency of mosaics was less than 5% by sites and about 2.5 % in average. In individual mosaics, final frequency of diploid cells was usually less than 10%. There was no significant relationship between frequency of reversion and salinity regimes, mortality or shell height growth, which might result from the overall low frequency of mosaics observed in this study. The high salinity regime (>25‰ppt) seemed to be related to a low

frequency of reversion. Frequency of reversion appeared to be age related and might have species variation. This is the first time that we have documented the frequency of reversion in 1-2 year old triploid *C. ariakensis*. The low frequency of reversion demonstrated in this study suggests that the risk of reversion in commercial aquaculture of triploid *C. ariakensis* will probably be very low. The principle risk of recovery of reproductive capability among triploids seems to reside in unharvested and “lost” oysters that remain in the Bay for long periods of time. Reproductive potential of mosaic *C. ariakensis* needs to be further studied to determine the extent of such risk.

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VITA

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Table 1. Salinity regimes, abbreviations and full names of the study sites. *During the snowstorm in January 2000, the oysters in this site were almost all killed. No data were collected from this site after January.

Salinity Regimes	Abbreviations of Study Sites	Full Names of Study Sites
Low (<15ppt)	WAN COAN GW	Wanchese, NC Coan River, VA Great Wicomico River, VA
Medium (15-25ppt)	EAST YORK	East River tributary, VA York River, VA
High (>25ppt)	CHIN CHAD BTON	Chincoteague Bay, VA Chadwick's Bay, NC *Burton Bay, VA

Table 2. Temperature and salinity by sites. Data were recorded monthly or more frequently. Sites are listed by abbreviations (See Table 1). *Data from CHAD were missing during the period of May '99—Sep'99.

Sites	Period	Temperature (°C)		Salinity (ppt)	
		Mean	Range	Mean	Range
WAN	Dec.'99—Oct.'00	19.8	1.3 - 30.5	10.7	4.0 - 20.0
COAN	Nov.'99—Dec.'00	19.3	3.5 - 31.0	11.6	9.0 - 16.0
GW	Nov.'99—Dec.'00	18.0	4.0 - 30.0	15.1	10.0 - 20.0
EAST	Dec.'99—Aug.'00	14.2	2.0 - 28.0	20.1	16.0 - 24.0
YORK	Nov.'99—Aug.'00	15.5	1.0 - 26.5	20.8	15.0 - 24.0
CHIN	Dec.'99—Sep.'00	16.2	2.0 - 26.0	32.0	29.0 - 34.0
CHAD*	Dec.'99—Apr.'00	12.2	2.5 - 24.0	33.1	27.0 - 38.0

Table 3. Mean and standard deviation of cumulative mortality of triploid *Crassostrea ariakensis* by sites. The oysters were six months old when deployed in October 1999. The three sites in low salinity were tested one more time December 2000 than the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Sites are listed by abbreviations (See Table 1).

Site	3/11/2000		6/17/2000		9/28/2000		12/15/2000	
	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
WAN	5.1	1.1	5.8	0.0	12.8	4.0	16.7	2.9
COAN	1.9	1.9	7.7	1.9	25.0	5.8	26.3	5.9
GW	3.2	4.0	5.8	5.1	28.2	9.7	28.8	8.8
EAST	0.6	1.1	1.3	1.1	1.3	1.1	/	/
YORK	1.9	0.0	6.4	4.0	8.4	2.9	/	/
CHIN	2.6	1.1	2.6	1.1	6.4	1.1	/	/
CHAD	4.5	2.9	12.2	5.6	27.6	4.0	/	/

Table 4. Mean and standard deviation of cumulative mortality of triploid *Crassostrea virginica* by sites. The oysters were six months old when deployed in October 1999. The three sites in low salinity were tested one more time December 2000 than the other sites. Two replicate groups were designated to each site. About 27 out of 52 oysters in each group were individually labeled for repeated measurement. Due to continuous tag loss and mortality, all remaining oysters in each group were measured during later experiment. Sites are listed by abbreviations (See Table 1).

Site	3/11/2000		6/17/2000		9/28/2000		12/15/2000	
	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
WAN	0.0	0.0	1.9	2.6	4.8	1.4	5.8	0.0
COAN	0.0	0.0	15.4	10.9	20.2	17.7	21.2	16.3
GW	0.0	0.0	1.9	0.0	12.5	9.5	18.3	6.8
EAST	0.0	0.0	4.8	6.8	36.5	16.3	/	/
YORK	1.8	2.5	4.8	4.1	10.6	4.1	/	/
CHIN	0.0	0.0	3.7	0.0	27.9	1.4	/	/
CHAD	0.0	0.0	64.8	7.9	96.2	0.0	/	/

Table 5. Mean and standard deviation of frequency of mosaics in triploid *Crassostrea ariakensis* by sites. The three sites in low salinity were tested one more time December 2000 than the other sites. Three replicate groups were designated to each site, with about 50 individually labeled oysters in each group. Sites are listed by abbreviation (See Table 1).

Site	3/11/2000		6/17/2000		9/28/2000		12/15/2000	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
WAN	0	0	0.7	1.2	0.7	1.3	2.3	2.3
COAN	0	0	0.7	1.2	4.3	3.0	2.6	0.2
GW	0	0	0	0	0.8	1.4	0	0
EAST	0	0	2.0	0	2.6	1.1	/	/
YORK	0	0	0.7	1.2	5.0	2.5	/	/
CHIN	0	0	0	0	1.4	2.4	/	/
CHAD	0.7	1.2	0.8	1.3	2.7	2.9	/	/

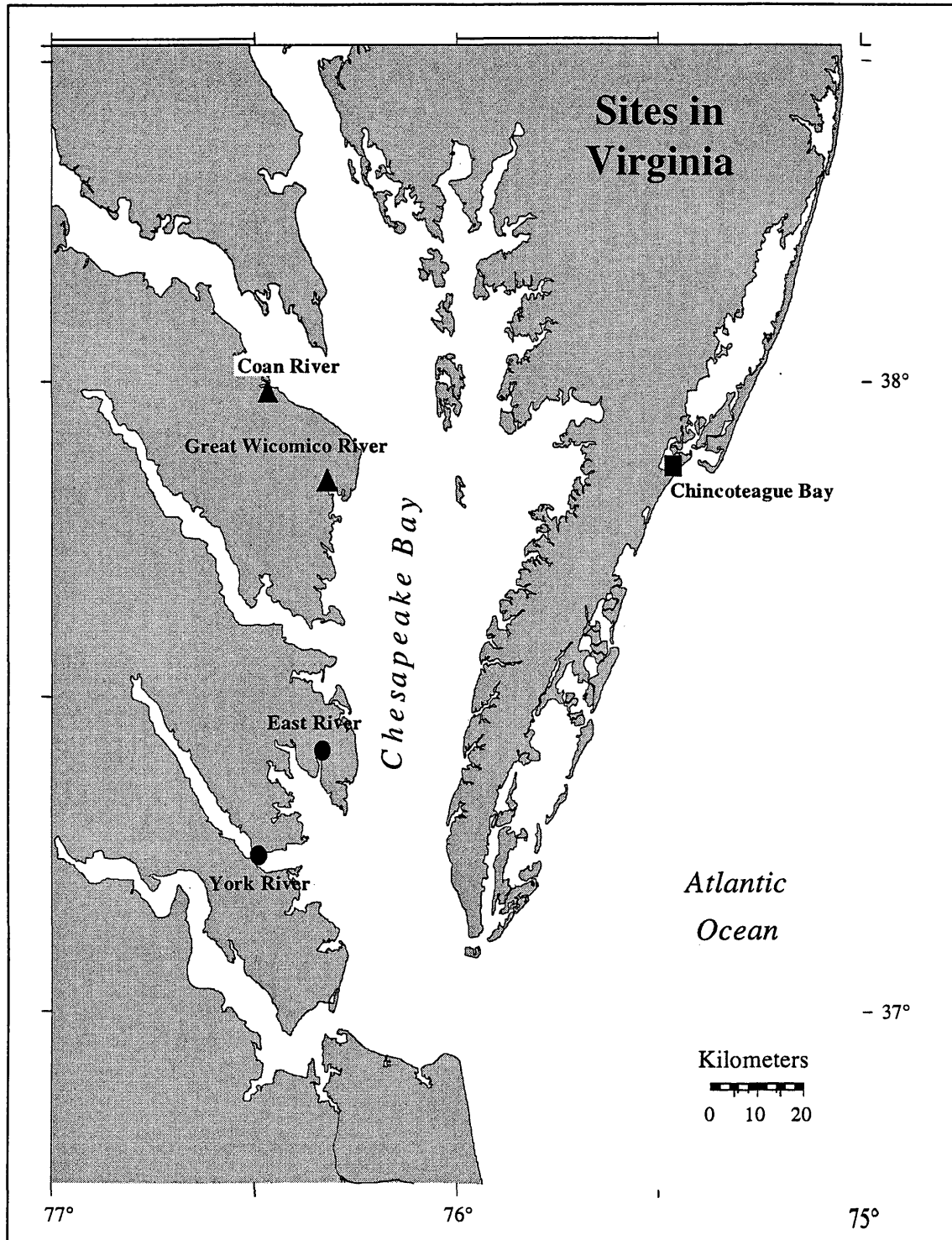


Figure 1. Study Sites in Virginia. • —low salinity (<15ppt); • —medium salinity (15-25ppt); • —high salinity (>25ppt).

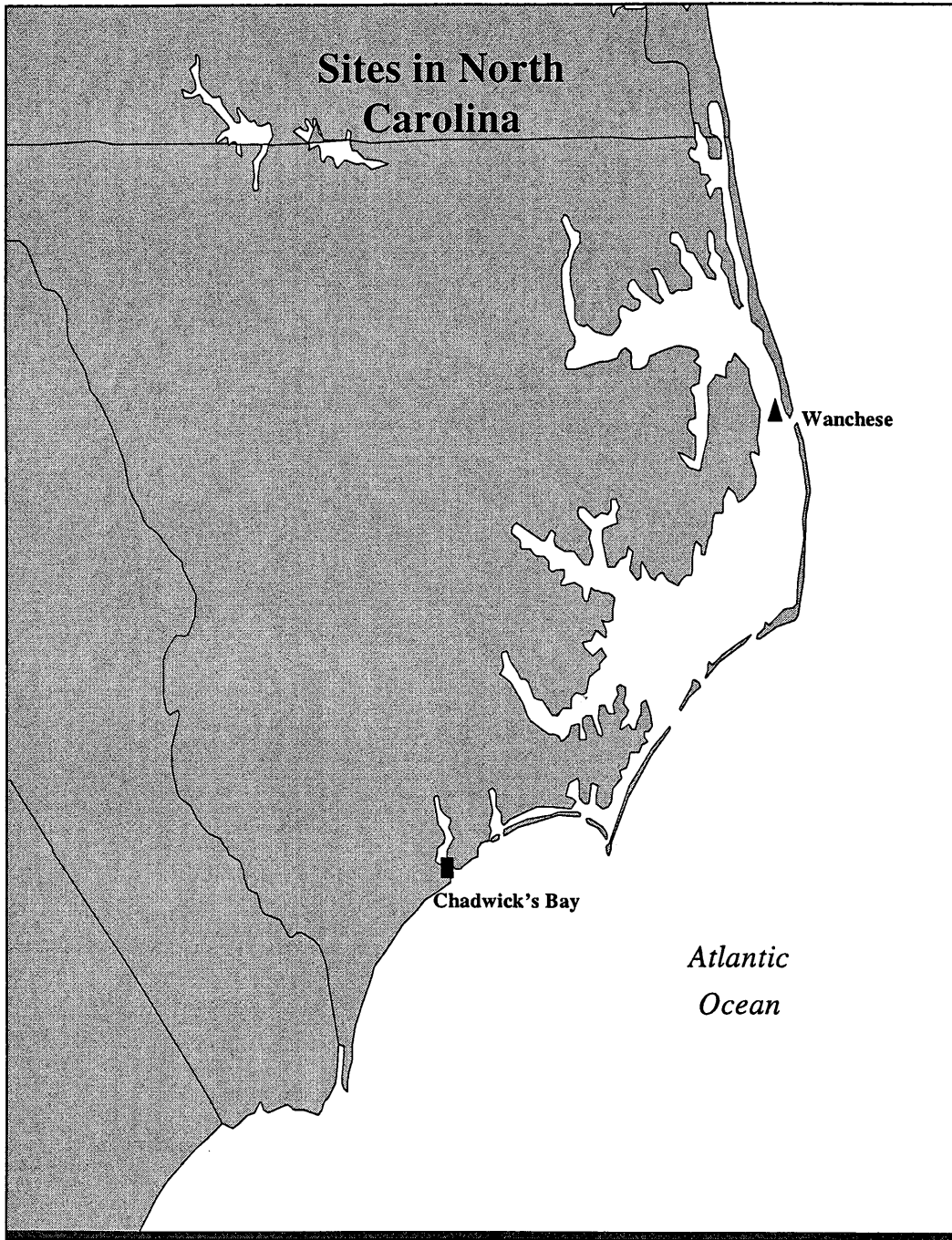


Figure 2. Study Sites in North Carolina. • —low salinity (<15ppt); • —high salinity (>25ppt).

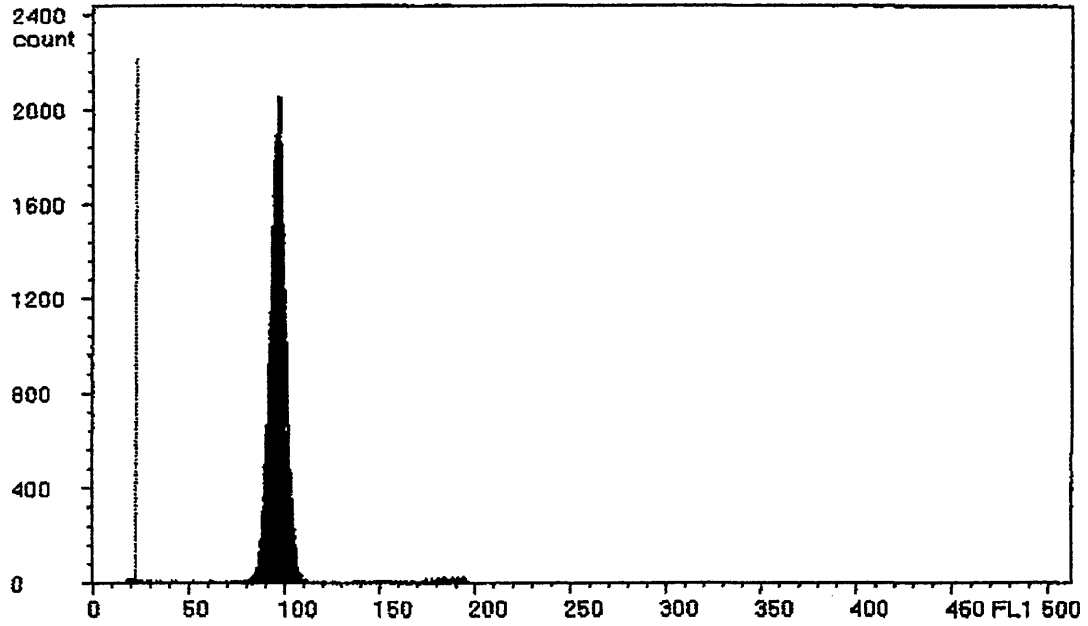


Figure 3. Example histogram of flow cytometry on a triploid *Crassostrea ariakensis*. X-axis indicates relative DNA content. Y-axis indicates frequency. Mean relative DNA content is 96.

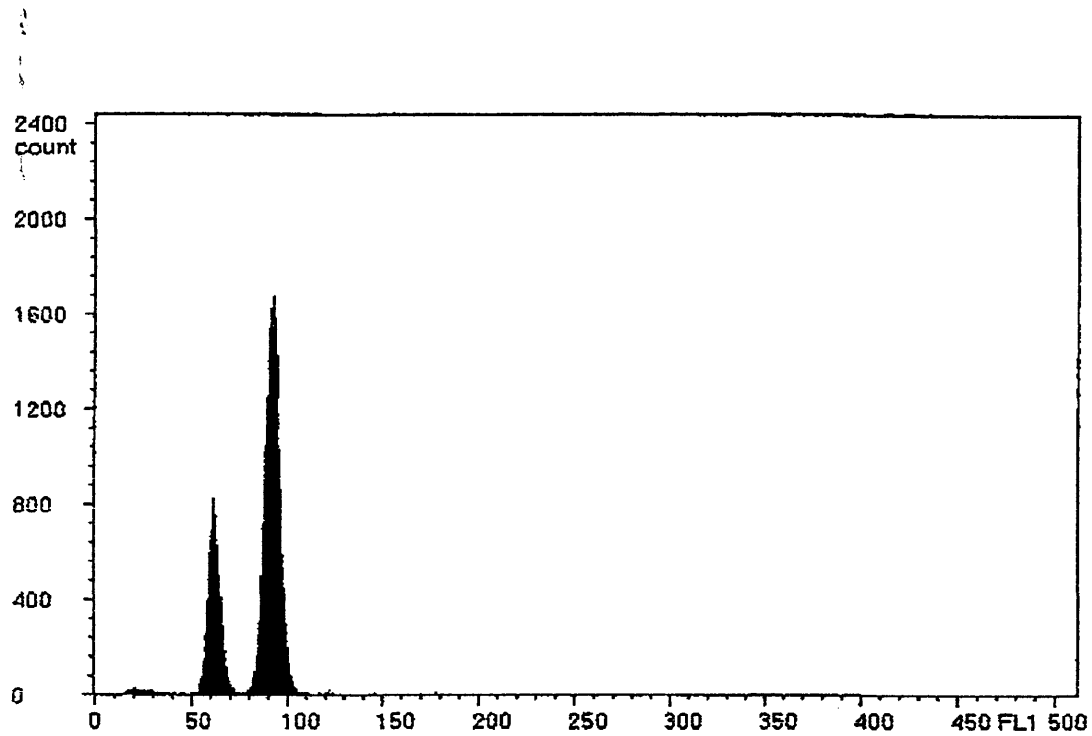


Figure 4. Example graph of flow cytometry test on a *Crassostrea ariakensis* mosaic. X-axis indicates relative DNA content. Y-axis indicates frequency. Mean relative DNA content at 3N peak is 93. Mean relative DNA content at 2N peak is 62.

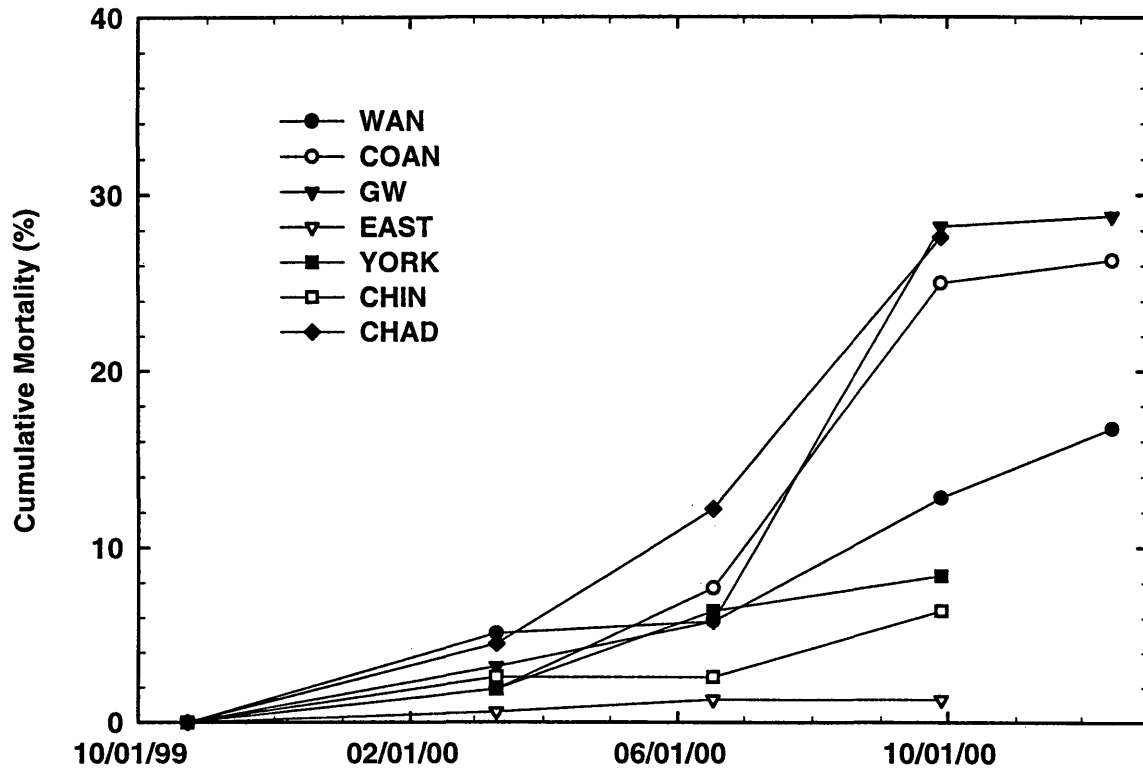


Figure 5. Cumulative mortality of triploid *Crassostrea ariakensis* by sites. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Symbols represent the mean from the three replicate groups. SD data are shown in Table 3. Sites are listed by abbreviation (See Table 1).

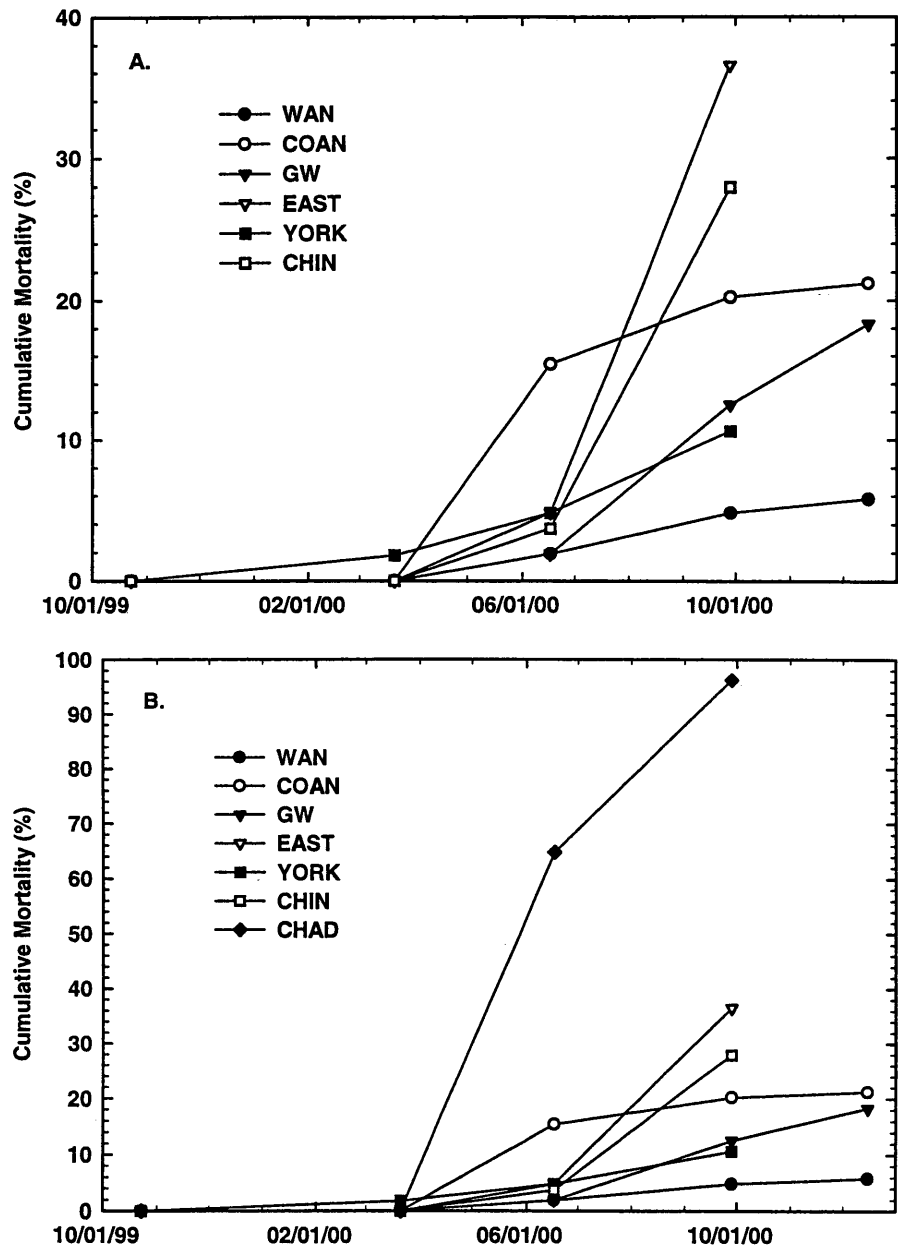


Figure 6. Cumulative mortality of triploid *Crassostrea virginica* by sites. A. Data from CHAD are not included. B. Complete data. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Two replicate groups were designated to each site. About 27 out of 52 oysters in each group were individually labeled for repeated measurement. Due to continuous tag loss and mortality, all remaining oysters in each group were measured during later experiment. Symbols represent the mean from the two replicate groups. SD data are shown in Table 4. Sites are listed by abbreviation (See Table 1).

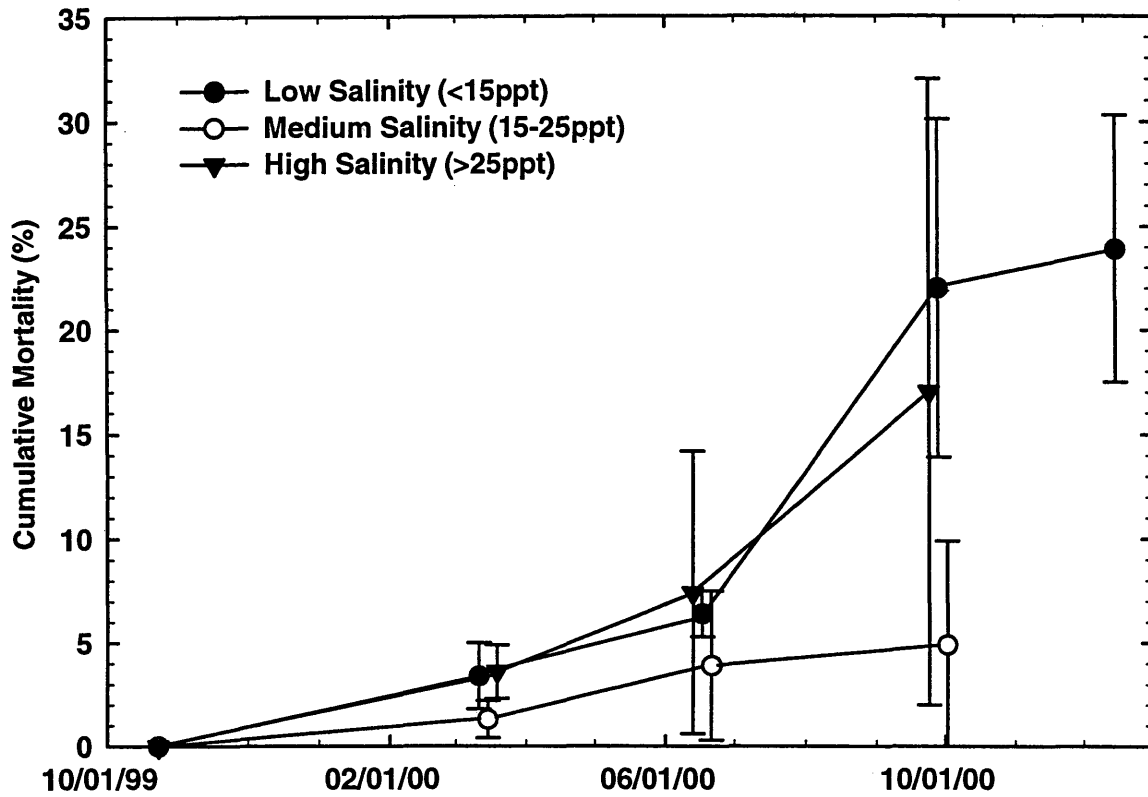


Figure 7. Cumulative mortality of triploid *Crassostrea ariakensis* by salinity regimes. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Symbols represent the mean of the two or three replicate sites in each salinity regime. Error bars represent one SD.

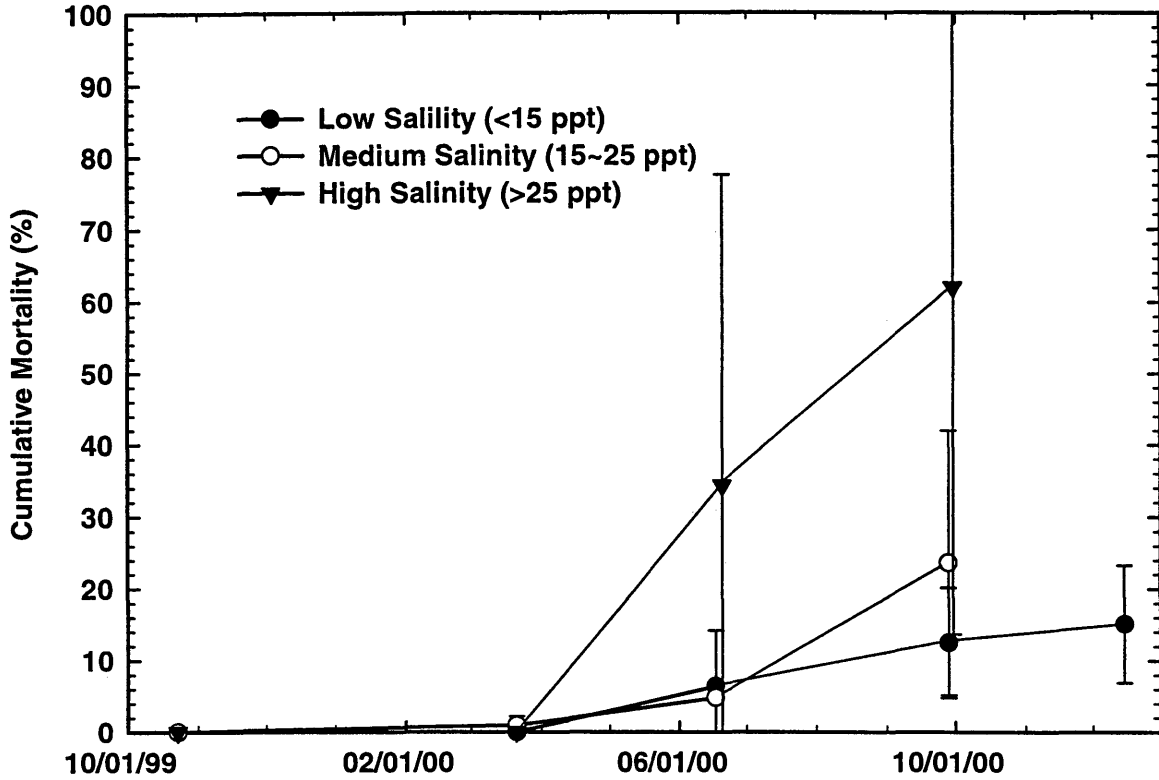


Figure 8. Cumulative mortality of triploid *Crassostrea virginica* by salinity regimes. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Two replicate groups were designated to each site. About 27 out of 52 oysters in each group were individually labeled for repeated measurement. Due to continuous tag loss and mortality, all remaining oysters in each group were measured during later experiment. Symbols represent the mean of the two or three replicate sites in each salinity regime. Error bars represent one SD.

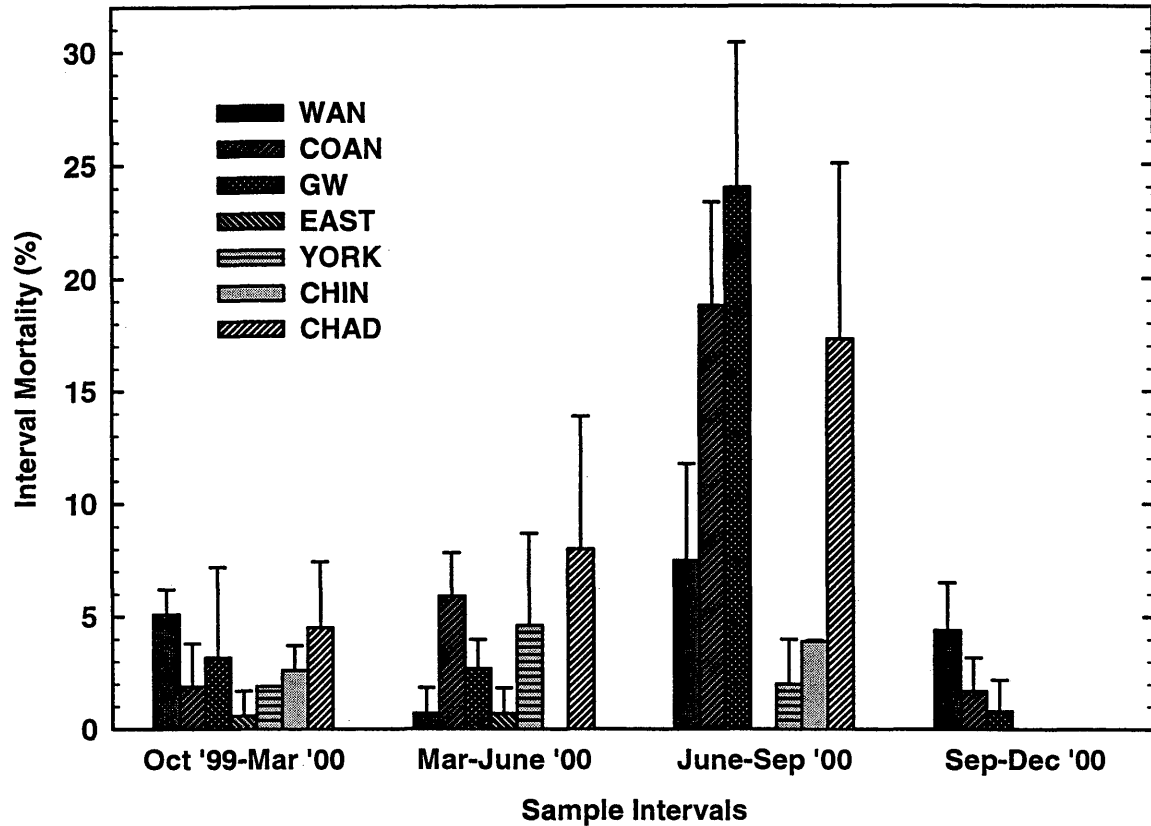


Figure 9. Interval mortality of triploid *Crassostrea ariakensis* by sites. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Columns represent the mean from the three replicate groups. Error bars represent one SD. See full name of study sites in Table 1.

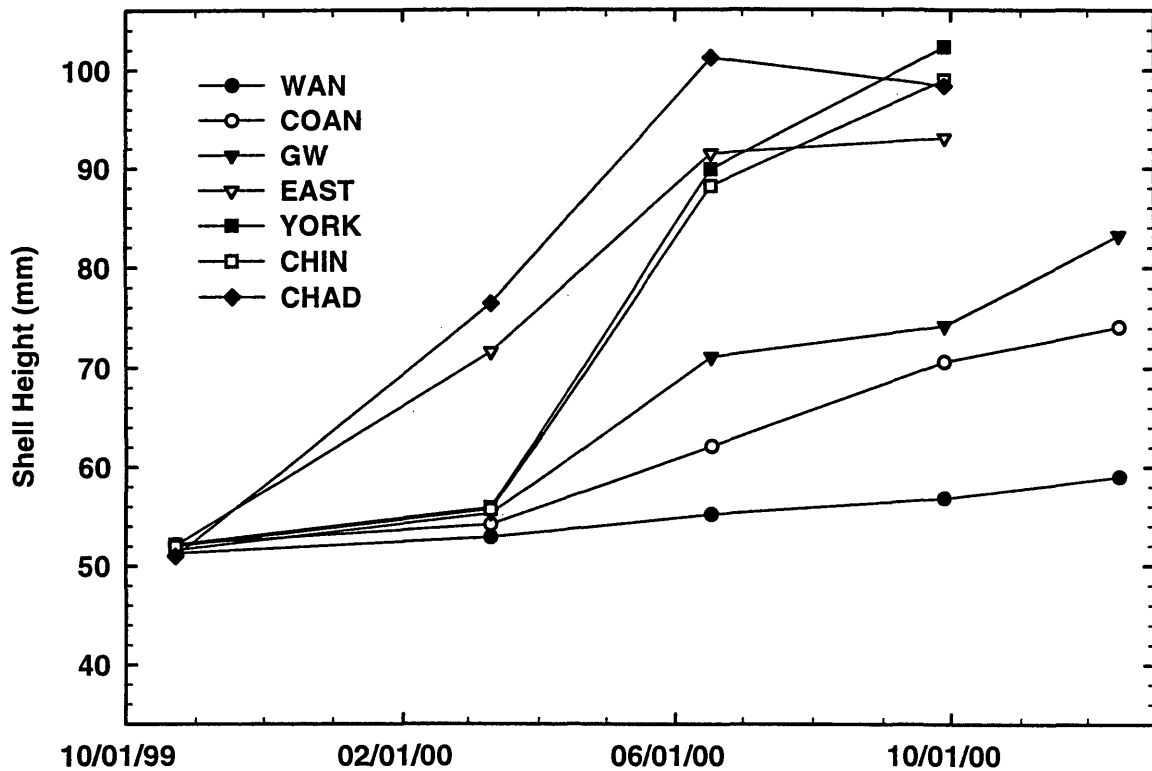


Figure 10. Shell height of triploid *Crassostrea ariakensis* by sites. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Symbols represent the mean from the three replicate groups. Sites are listed by abbreviation (See Table 1).

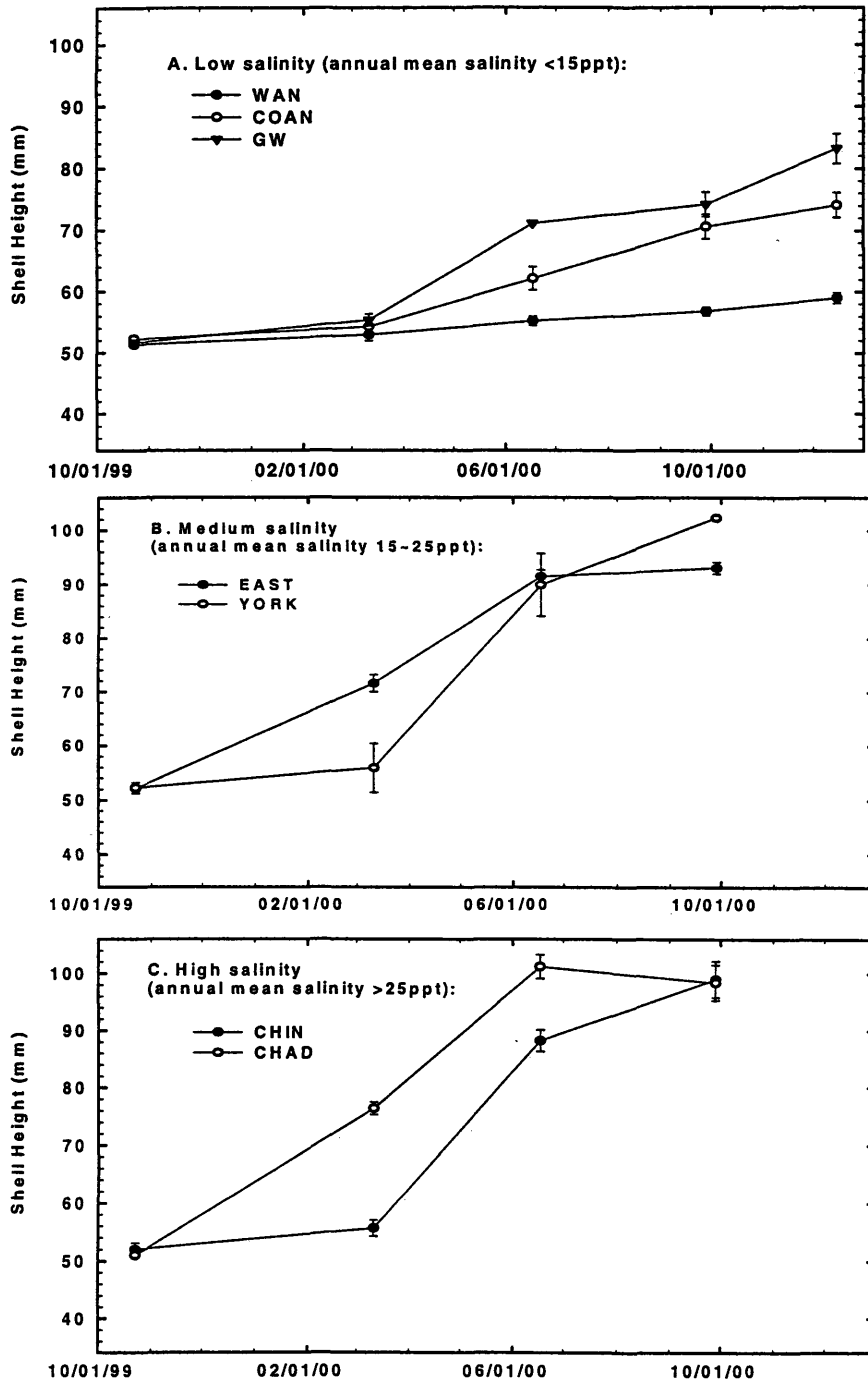


Figure 11. A comparative view of shell height accumulation of triploid *Crassostrea ariakensis* among sites of low (A), medium (B), and high (C) salinity regimes. See Figure legend 10 for detail. Symbols represent the mean from the two replicate groups. Error bars represent one SD. Sites are listed by abbreviation (See Table 1).

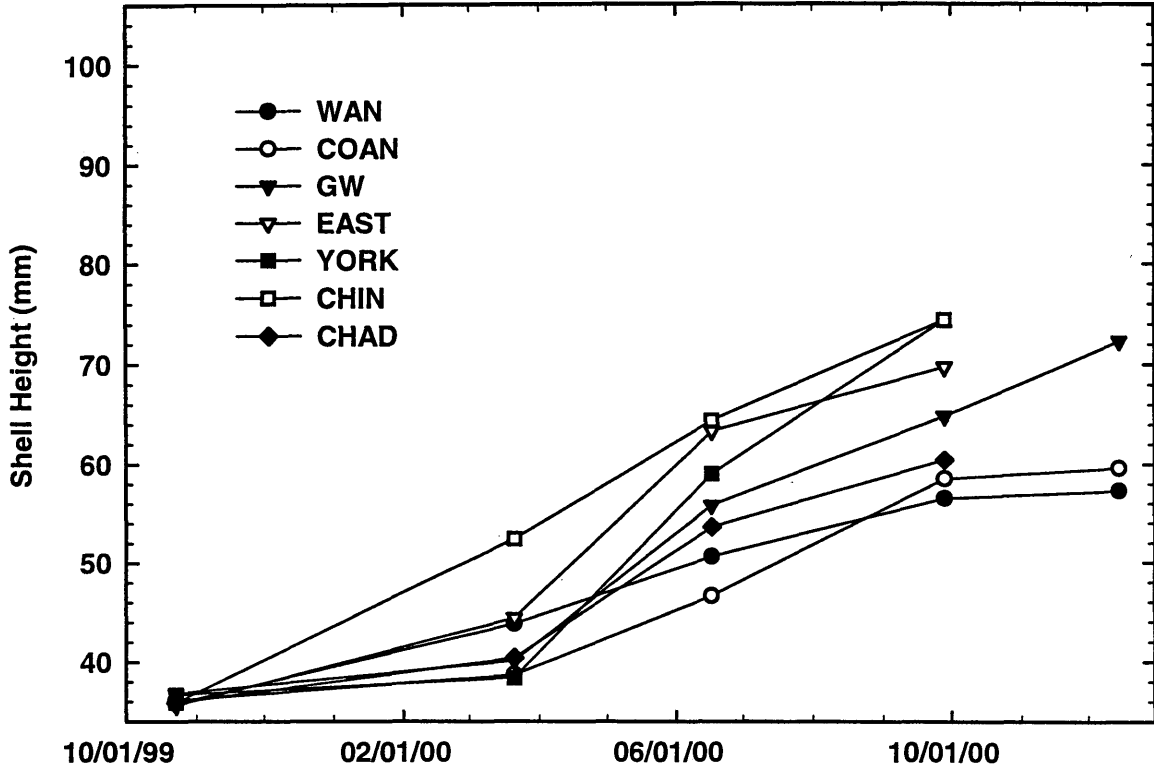


Figure 12. Shell height of triploid *Crassostrea virginica* by sites. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Two replicate groups were designated to each site. About 27 out of 52 oysters in each group were individually labeled for repeated measurement. Due to continuous tag loss and mortality, all remaining oysters in each group were measured during later experiment. Symbols represent the mean from the two replicate groups. Sites are listed by abbreviation (See Table 1).

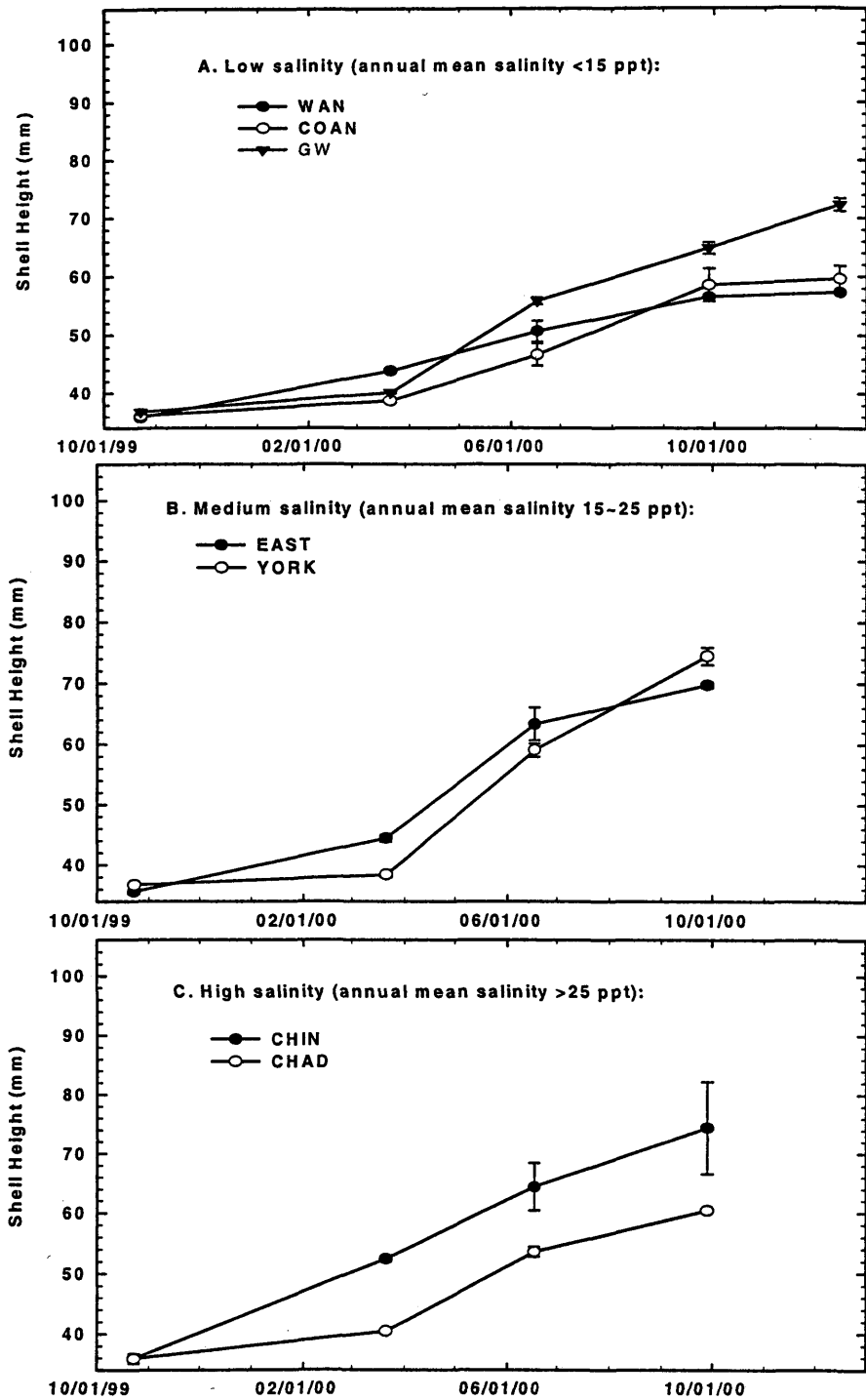


Figure 13. A comparative view of shell height accumulation of triploid *Crassostrea virginica* among sites of low (A), medium (B), and high (C) salinity regimes. See Figure legend 12 for detail. Symbols represent the mean from the two replicate groups. Error bars represent one SD. Sites are listed by abbreviation (See Table 1).

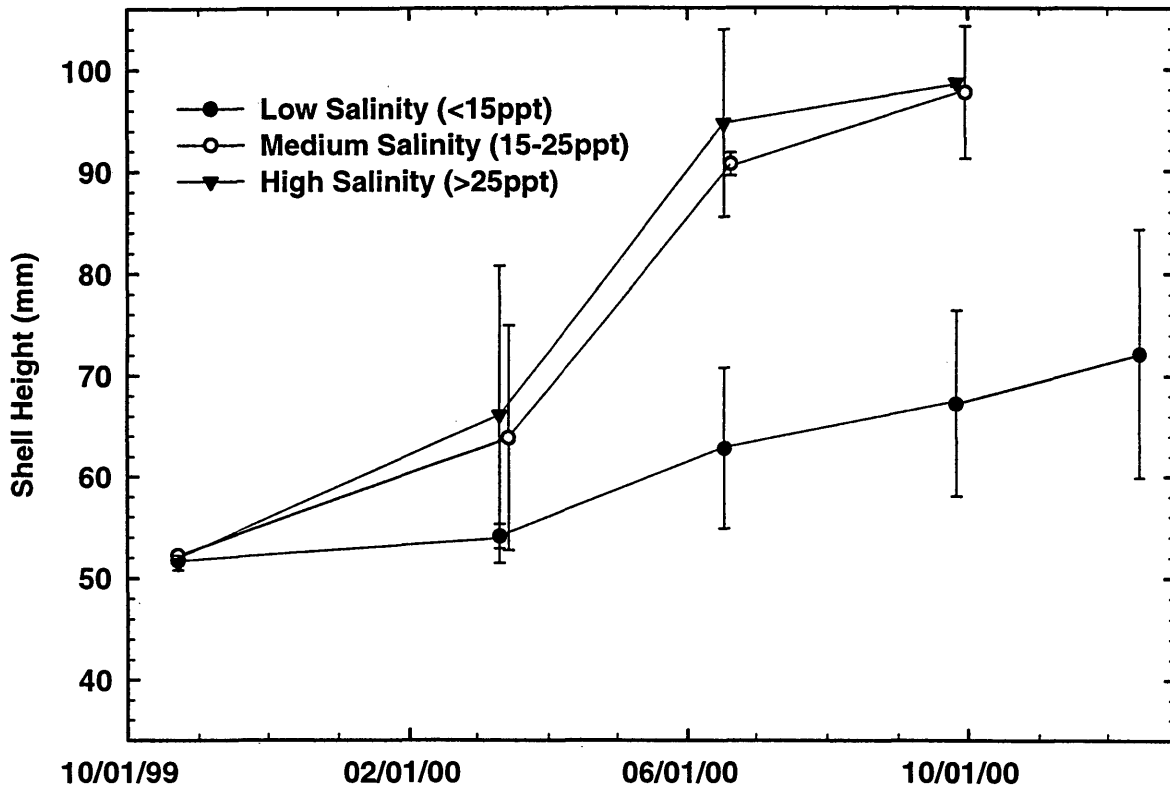


Figure 14. Shell height of triploid *Crassostrea ariakensis* by salinity regimes. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Symbols represent the mean from the sites in each salinity category. Error bars represent one SD.

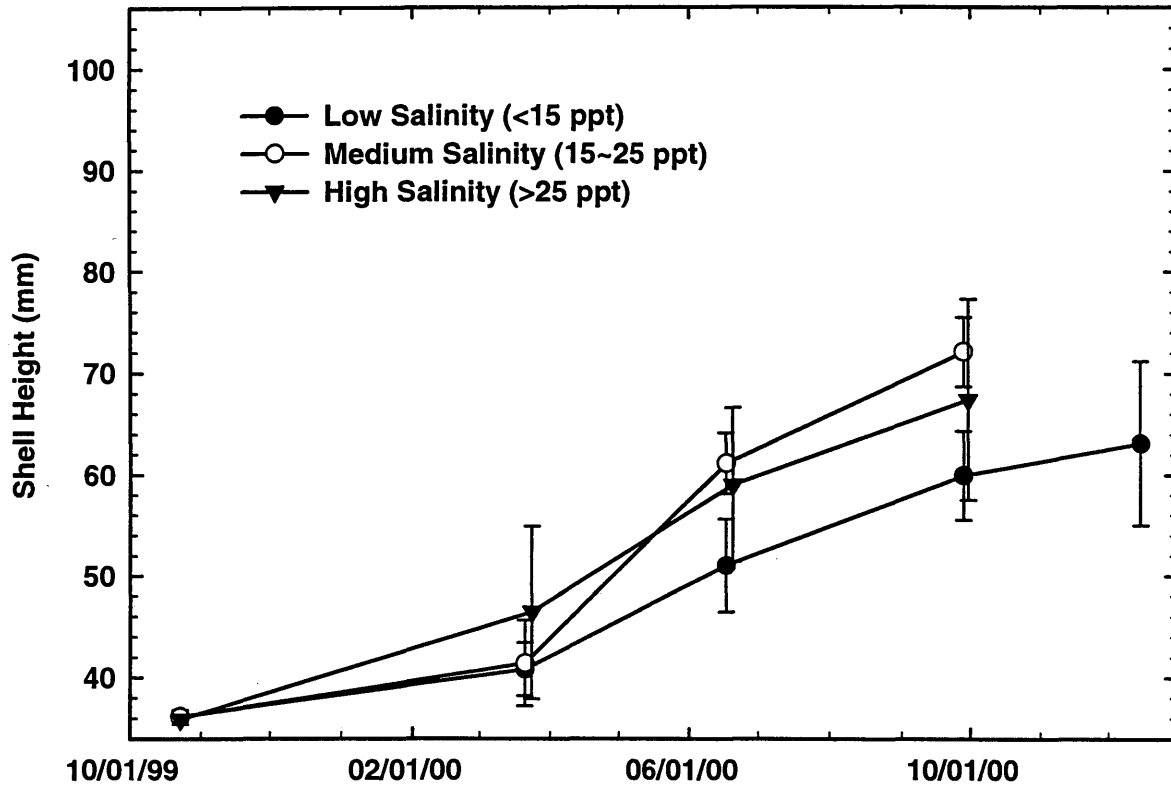


Figure 15. Shell height of triploid *Crassostrea virginica* by salinity regimes. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Two replicate groups were designated to each site. About 27 out of 52 oysters in each group were individually labeled for repeated measurement. Due to continuous tag loss and mortality, all remaining oysters in each group were measured during later experiment. Symbols represent the mean of the two or three replicate sites in each salinity regime. Error bars represent one SD.

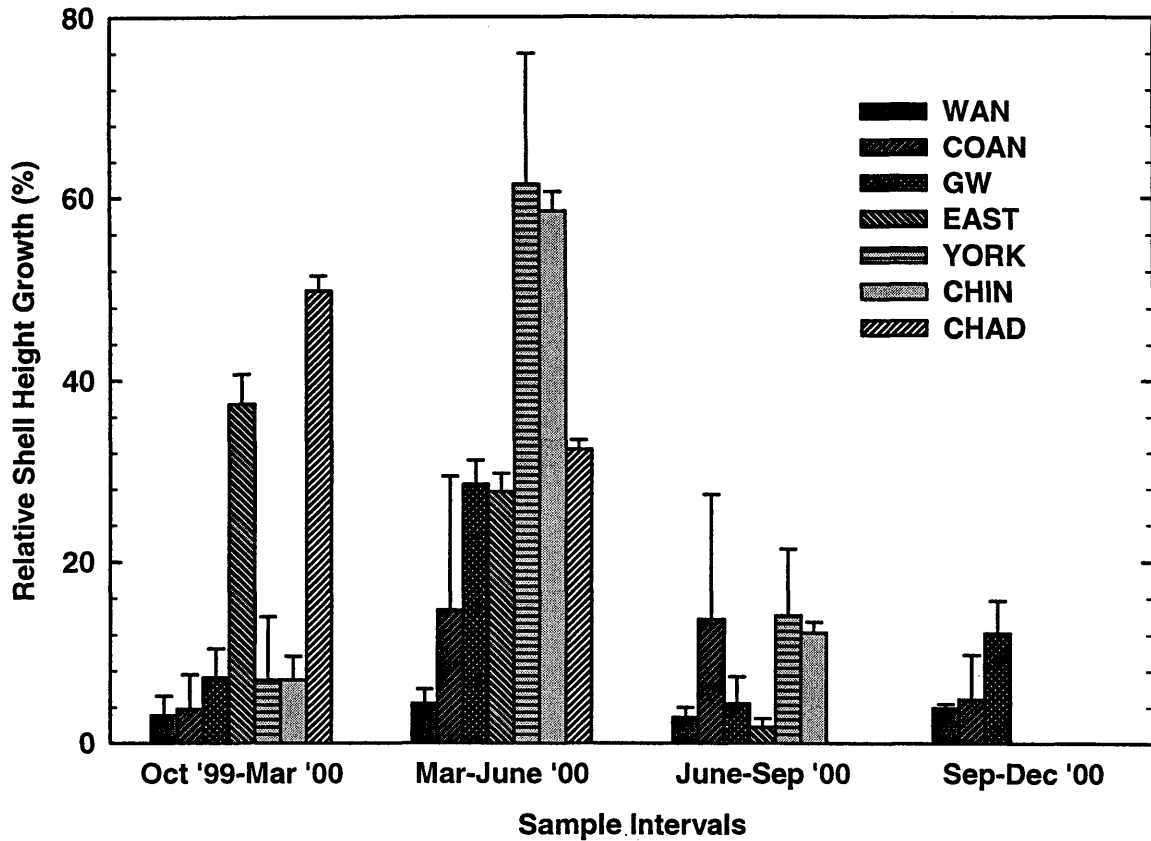


Figure 16. Relative shell height growth of triploid *Crassostrea ariakensis* by sites. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Columns represent the mean from the three replicate groups. Error bars represent one SD. See full name of study sites in Table 1.

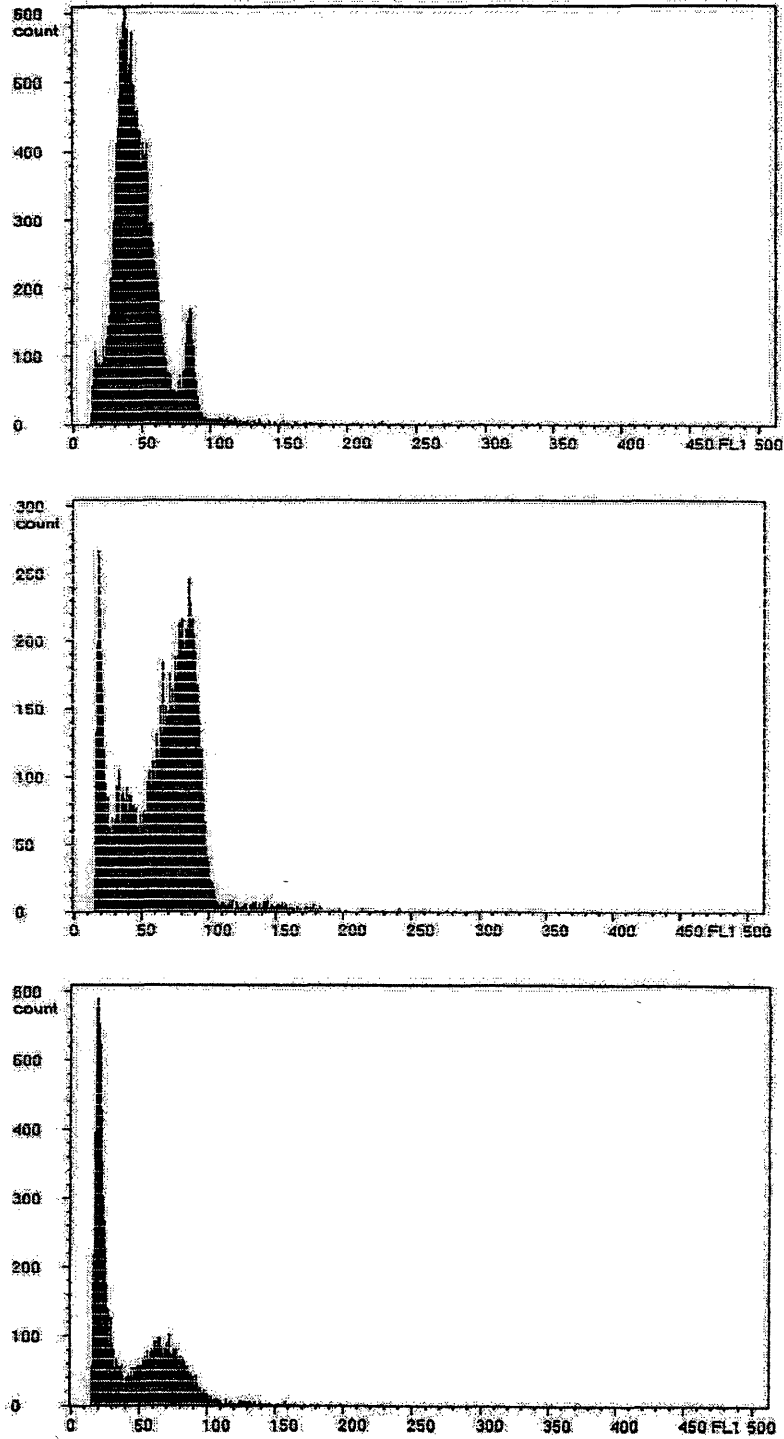


Figure 17. In March 2000, most of the hemolymph samples of triploid *Crassostrea ariakensis* from Chadwick's Bay showed significant noise when tested by flow cytometry. Several example graphs are shown above.

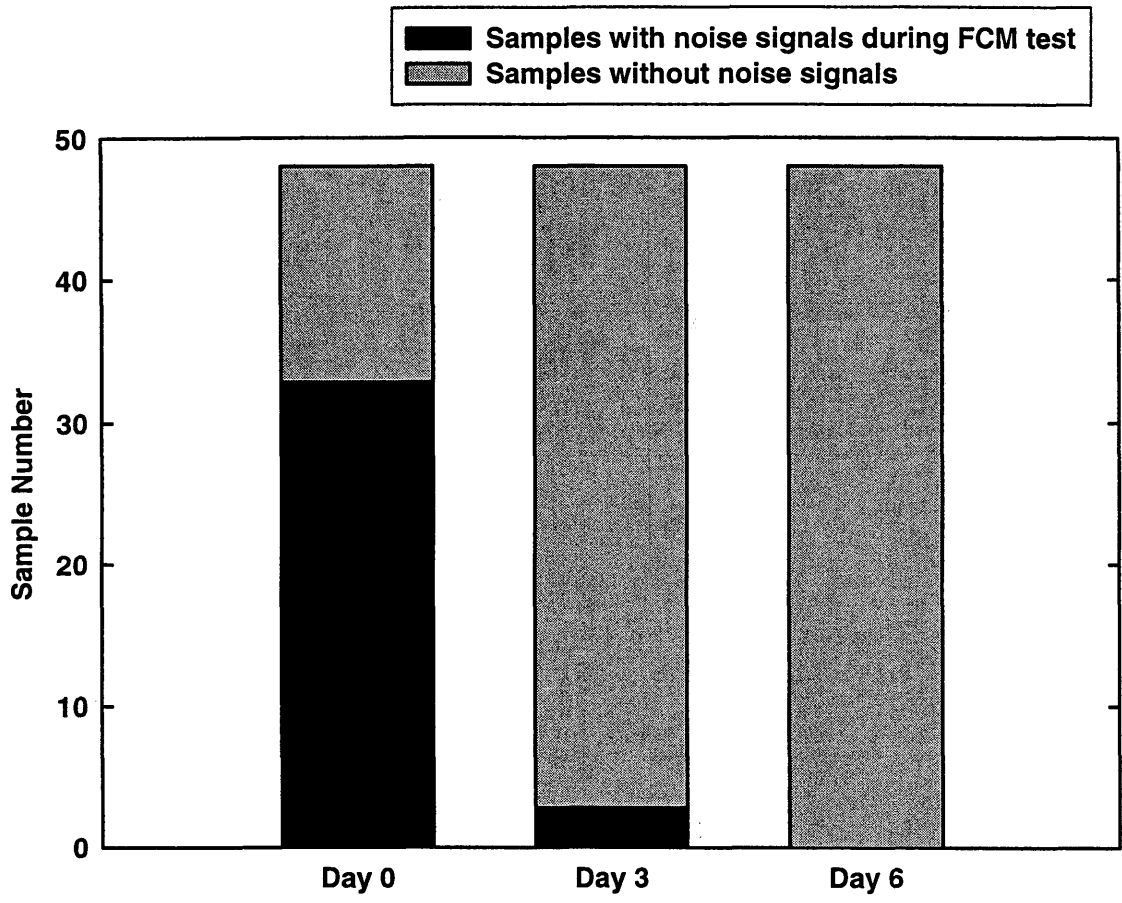


Figure 18. The effect of salinity adaptation, a test to see if salinity is the cause of noise signals found in FCM test of hemolymph sample from oysters in high salinity sites. One bag of triploid *Crassostrea ariakensis* was randomly chosen from Chincoteague Bay at 34 ppt on Sep. 20th, 2000 (Day 0). The oysters were tested by flow cytometry and then acclimatized to 20ppt water at Gloucester Point Hatchery on the same day. Samples with noise signals were reexamined every three days until Sep. 27th, 2000 (Day 6).

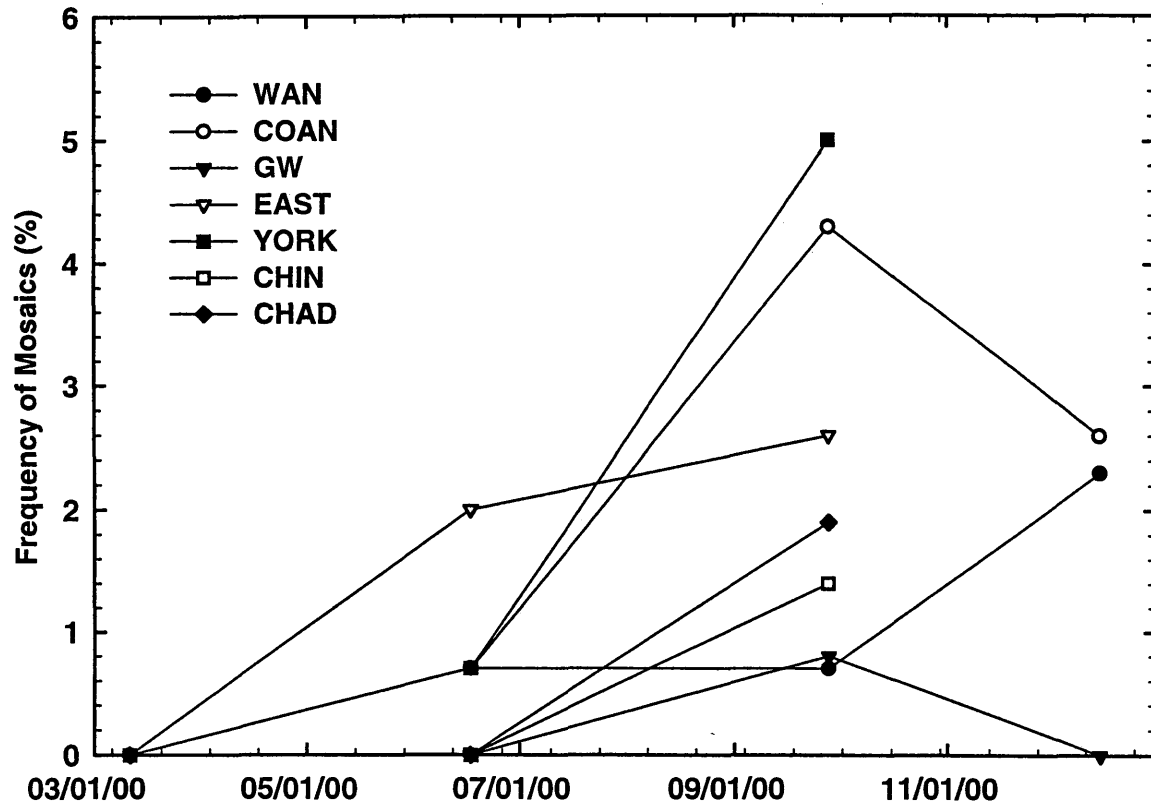


Figure 19. Frequency of *Crassostrea ariakensis* mosaics by sites since March 2000, when a total of 1059 triploid oysters were first reexamined for ploidy after their deployment, till the end of the experiment in December 2000. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Symbols represent the mean from the three replicate groups. SD data are listed in Table 5. See full name of study sites in Table 1.

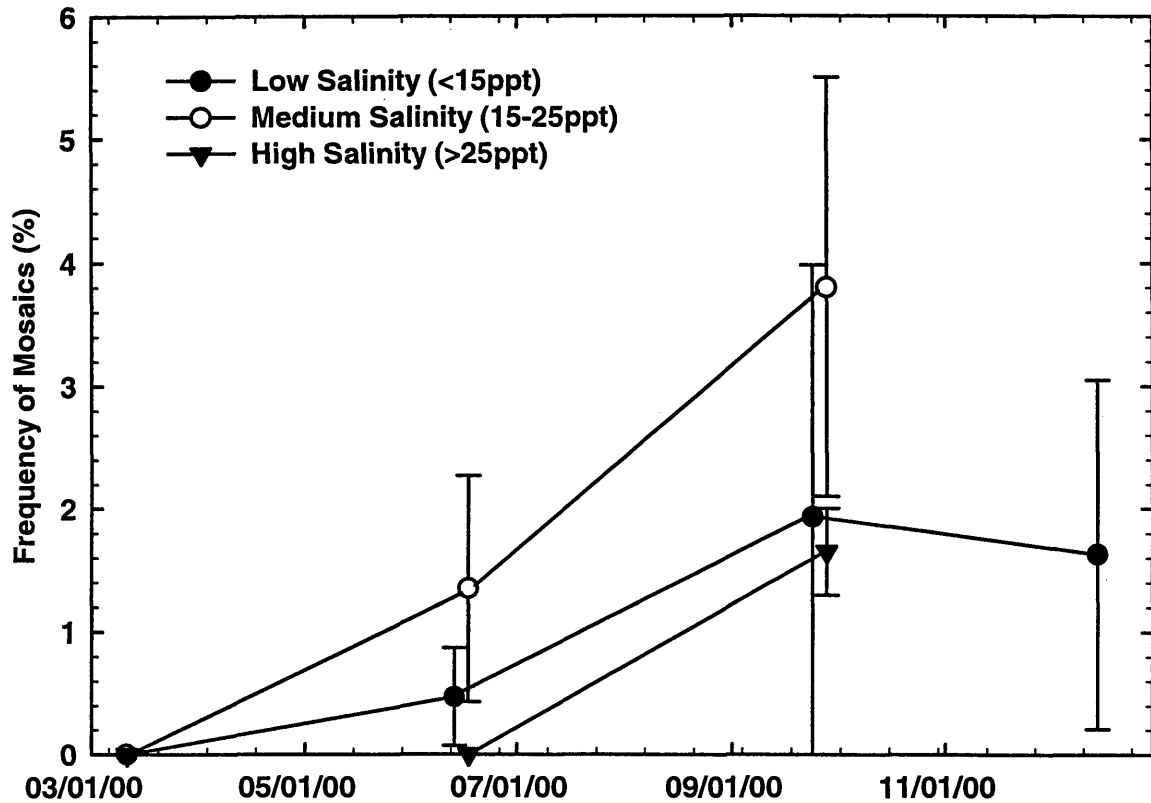


Figure 20. Frequency of *Crassostrea ariakensis* mosaics by salinity regimes since March 2000, when a total of 1059 triploid oysters were first reexamined for ploidy after their deployment, until the end of the experiment in December 2000. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 52 individually labeled oysters in each group. Symbols represent the mean from the three replicate groups. Error bars represent on SD.

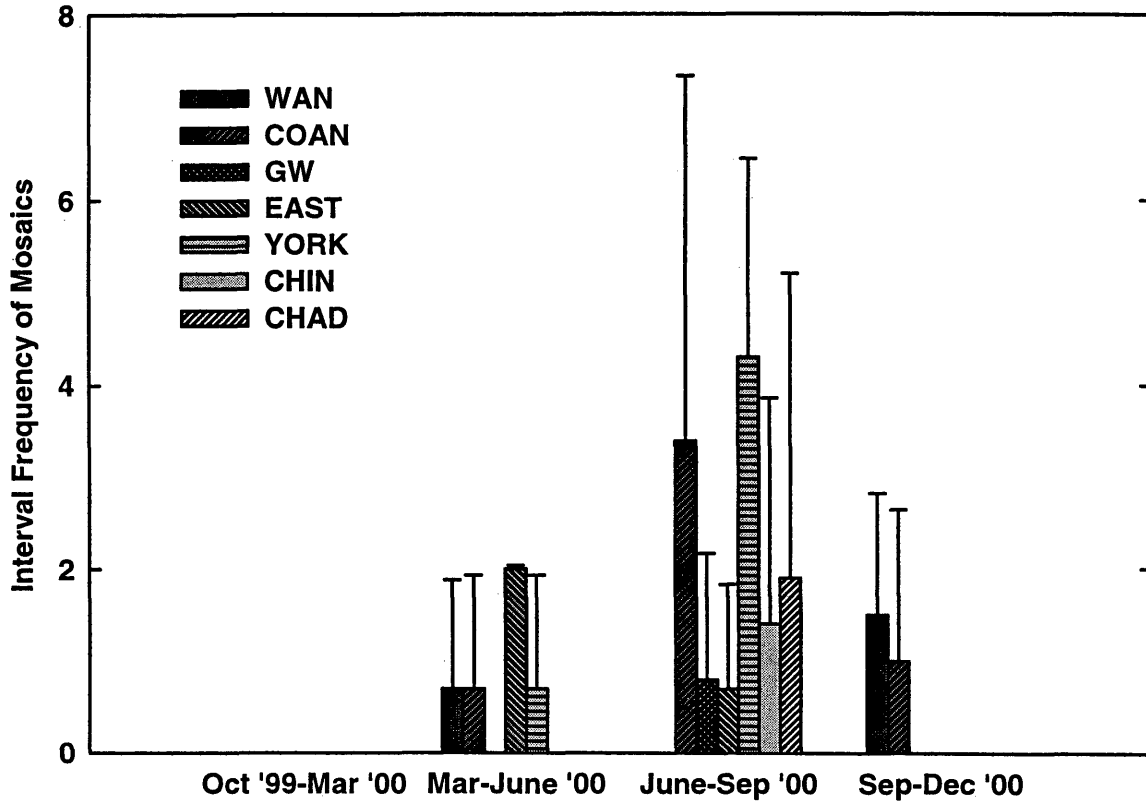


Figure 21. Interval frequency of *Crassostrea ariakensis* mosaics by sites since March 2000, when a total of 1059 triploid oysters were first reexamined for ploidy after their deployment, till the end of the experiment in December 2000. Oysters were six months old when deployed in October 1999. Oysters at low salinity sites were tested one more time December 2000 than those at the other sites. Three replicate groups were designated to each site, with about 50 individually labeled oysters in each group. Columns represent the mean from the three replicate groups. Error bars represent one SD. See full name of study sites in Table 1.

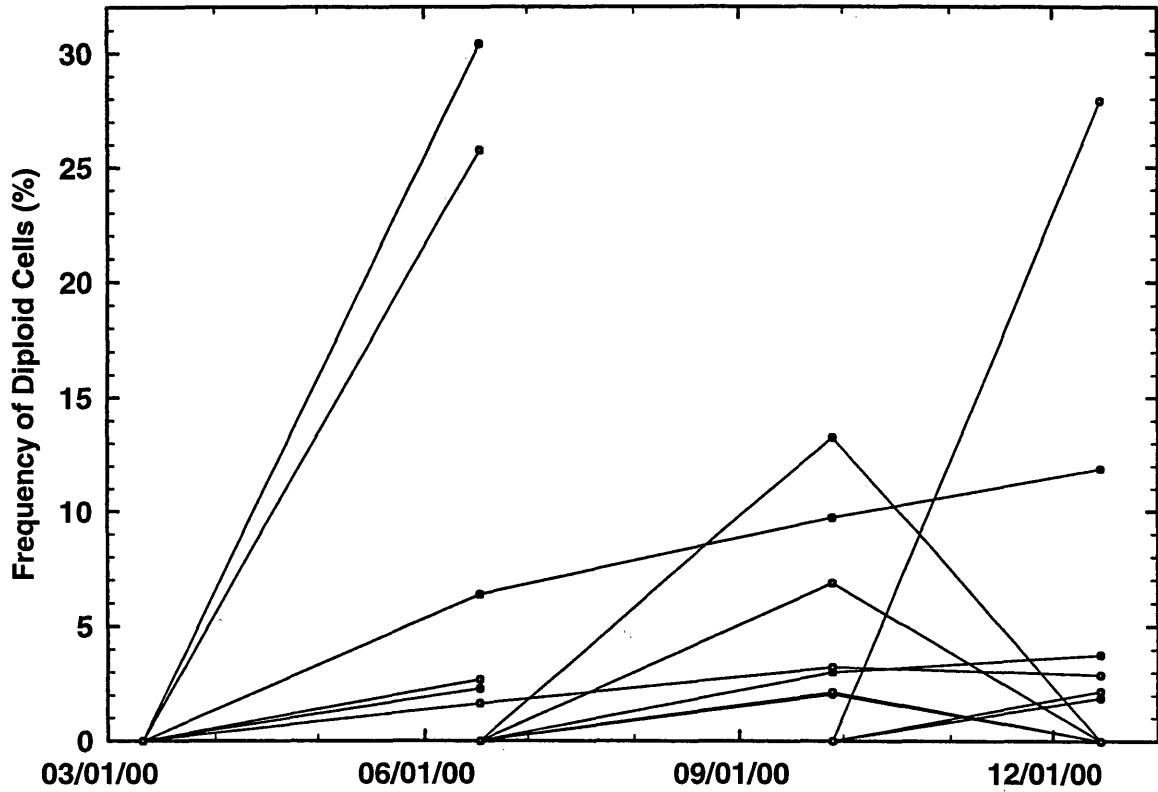


Figure 22. Frequency of diploid cells in individual *Crassostrea ariakensis* mosaics that were found in multiple sites since March 2000, when 1059 living triploid oysters were first reexamined for ploidy after their deployment, till they reached market size (around 75mm). Oysters were six months old when deployed in October 1999. Each connected line represents the percentage change of diploid cells in each mosaic.

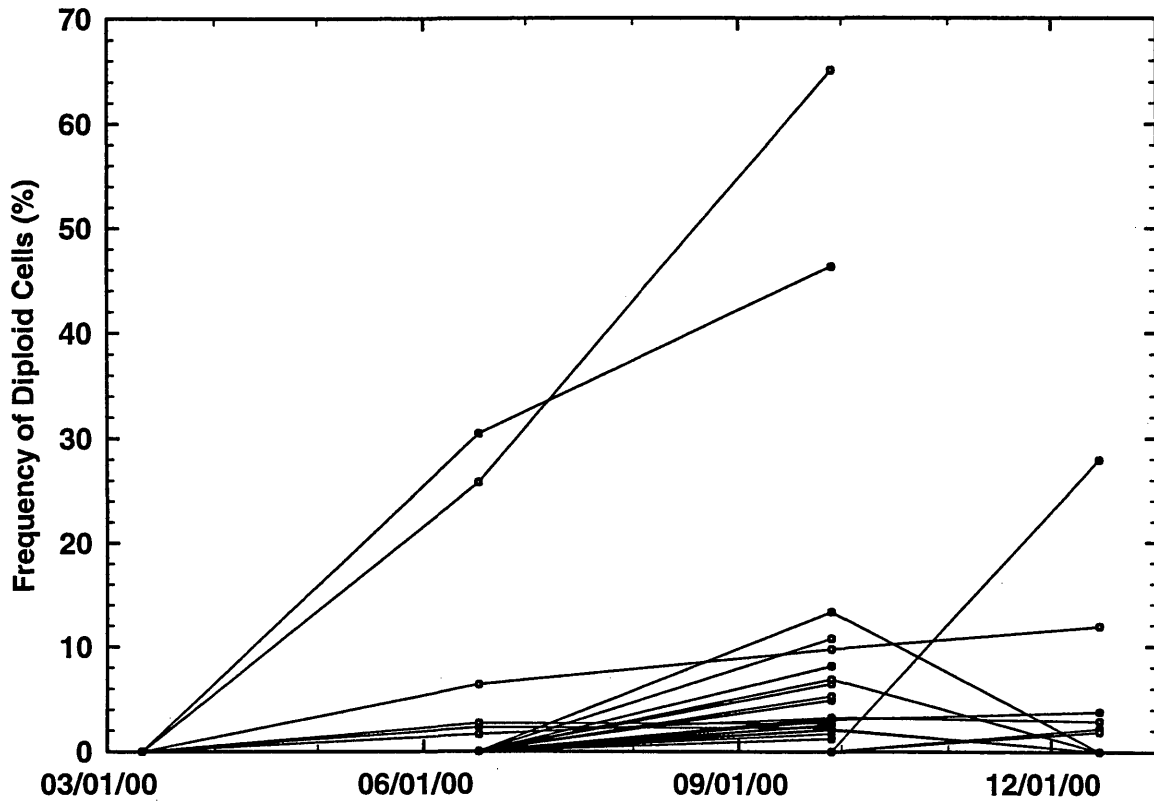


Figure 23. Frequency of diploid cells in individual *Crassostrea ariakensis* mosaics that were found in multiple site since March 2000, when 1059 triploid oysters were first reexamined for ploidy after their deployment, till the end of the experiment in December 2000. Oysters were six months old when deployed in October 1999. Each connected line represents the percentage change of diploid cells in each mosaic.