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Biology of Mated Triploid Crassostrea ariakensis in Multiple Environments: Gametogenesis, Sex Ratio, Disease Prevalence, and Reversion

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BIOLOGY OF MATED TRIPLOID *CRASSOSTREA ARIAKENSIS* IN MULTIPLE
ENVIRONMENTS: GAMETOGENESIS, SEX RATIO, DISEASE PREVALENCE,
AND REVERSION

A Thesis

Presented to

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The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirement for the Degree of

Master of Science
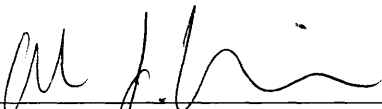
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Alan J. Erskine

2003

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This thesis is submitted in partial fulfillment of

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ABSTRACT

The decline of native *Crassostrea virginica* populations in Chesapeake Bay due to overharvesting, disease pressure, and habitat loss has been well documented. Since the mid 1900's, traditional solutions have included shell planting and reef construction with limited success, and selective breeding programs, concentrating on disease resistance, which has also not been completely successful.
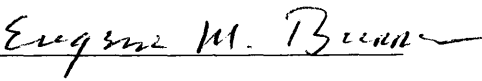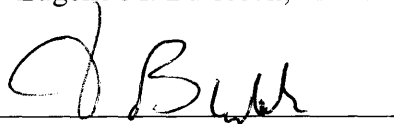
Since 1998, the Virginia Institute of Marine Science (VIMS) has been examining the performance of a non-native oyster, *Crassostrea ariakensis*, in triploid form, for possibly revitalizing the Virginia oyster industry. The triploid condition is advantageous because it renders the oyster sterile. *C. ariakensis* exhibited higher survival, disease resistance, and faster growth than *C. virginica* (Calvo *et al.* 2001). If *C. ariakensis* is to be considered as a candidate for commercial aquaculture, more data on the biology of this animal in Chesapeake Bay is critical.

I deployed 200 age 2-3 mated (tetraploid father x diploid mother) triploid *C. ariakensis* and several hundred age 1-2 diploid *C. virginica* at six industry field sites in September 2001. I sampled 25 triploid and 10 diploid oysters each month from January to December 2002. Histology was performed to determine gametogenesis, sex, and prevalence of *Haplosprodium nelsoni*. Ray's fluid thioglycollate medium assay was employed on all oysters sampled between July and October to determine *Perkinsus marinus* prevalence. All triploid oysters were tested for reversion using hemolymph tissue and flow cytometry (FCM). Mosaic individuals were sampled again for heart, gill, adductor, and gonad tissues to track reversion within the individual.

Histology revealed that triploid oysters did not mature uniformly like diploid oysters. Triploid gametogenesis was reduced and somewhat abnormal. Triploid males developed more completely than triploid females, but less than diploid males. Triploid males had the ability to produce a gradient of sex cells from spermatogonia to spermatozoa. Triploid females rarely arrest gametogenesis at an intermediate stage. Triploid female gametogenesis was generally aberrant, either producing numerous fully mature oocytes or arrested gonia and an occasional mature oocyte. Highly fecund mated triploid *C. ariakensis* females and males were observed.

P. marinus prevalence ranged from 20-100% in *C. virginica* compared to 0-44% in *C. ariakensis*. Infection intensities were rare to very heavy for *C. virginica* and rare to light for *C. ariakensis*. *H. nelsoni* prevalence in *C. virginica* ranged from 0-13% and the parasite was not detected in *C. ariakensis*.

Mosaicism was 1.2% in mated triploid oysters, occurring in 11 out of 930 animals. The percentage of diploid cells in mosaic individuals was less than 20% in all but two individuals that had 65 and 44% diploid cells. Hemolymph was the most sensitive indicator of reversion. Other tissues exhibited varying percentages of diploid cells.

The ability of triploid oysters to correctly segregate extra chromosomes and successfully produce mature gametes may be under genetic control and influenced by the environment. Unstable and stressful environments such as low salinity ones exhibited high numbers of mosaics, hermaphrodites, and fecund triploid oysters. Mating between triploid *C. ariakensis* gametes was estimated to produce thirty-four survivors that may be capable of reproduction out of a possible one million individuals.

RATIONALE

Previous field grow-out trials, from 1998 to 2002, have shown that *C. ariakensis* is a promising aquaculture or introduction species for Virginia's Chesapeake Bay. Research thus far has demonstrated that *C. ariakensis* has the ability to grow fast and will tolerate or resist local diseases (Calvo *et al.* 2001). Furthermore, consumers and industry members agree that the oyster tastes good (T. Mason, Mason Seafood, personal communication).

Continued research on *C. ariakensis* in Chesapeake Bay is needed to further understand the benefits and risks associated with this non-native. In the last year or so it has become clear that mated (tetraploid father x diploid mother) triploid oysters are the preferred experimental form of *C. ariakensis* to deploy in field trials. An important research question became what is the degree of gametogenesis in mated triploid *C. ariakensis*?

Ecologically, documenting the extent of gametogenesis provided insight into the possible limitations of biosecurity that we could expect from mated triploid *C. ariakensis* in Chesapeake Bay. Economically, future commercial-scale production of mated triploid oysters requires tetraploid brood stock which hinges on identifying fecund triploid female *C. ariakensis*. Clearly this is contradictory to the ecological goal. For biological control of a non-native species mated triploid *C. ariakensis* should be completely sterile. Revitalizing the oyster industry in Virginia via aquaculture of *C. ariakensis*, however, requires that at least a few mated triploid female *C. ariakensis* are identified to spawn tetraploid brood stock and future commercial-scale production of mated triploid *C. ariakensis*. Obtaining answers to these questions was one of my objectives in conducting these experiments.

The purpose of this research was to examine gametogenesis in mated triploid *C. ariakensis* in several environments across Virginia's Chesapeake Bay. In order to track a "normal" course of gametogenesis at each site, diploid *C. virginica* were deployed. Secondary objectives included measurements of shell height and wet weight in diploid and triploid oysters, *H. nelsoni* and *P. marinus* quantification in diploid and triploid oysters, and reversion in triploid *C. ariakensis*. All oysters were monitored and sampled from January 2002 until December 2002.

INTRODUCTION

Decline of native oyster population

The eastern oyster, *Crassostrea virginica* (Gmelin 1791), is an important bivalve of estuarine systems from Nova Scotia to the Gulf of Mexico. Undisturbed, they form three-dimensional reef structures that provide habitat for fish, crustaceans, and other bivalves. In the mid 19th century the Chesapeake Bay was the virtual definition of a productive estuary with *C. virginica* a keystone species. By the early 20th century, the future of the *C. virginica* population was in serious question (Brooks 1891, 1905). Constant harvests had flattened three-dimensional reefs to two-dimensional mounds well below the surface, rendering them vulnerable to sedimentation and reducing the overall productivity of the oyster life cycle.

Overfishing has been the historical explanation for the decline of *C. virginica*, although in the mid 20th century the fishery seemed to be maintaining through regulated success. More recent decline has been exacerbated by two oyster parasites, *Haplosporidium nelsoni* and *Perkinsus marinus* in Chesapeake Bay. Initial large-scale disease mortalities were associated with *H. nelsoni* in the early 1960's (Haskin and Andrews 1988). *P. marinus*, first detected back in the 1940's, was only associated with about 20% annual oyster mortality, not significantly affecting industry harvests (Andrews 1988). More recently, in the late 1980's and early 1990's, *P. marinus* spread throughout the Bay as a result of drought conditions from 1985-1989 (Burreson and Andrews 1988). Another drought from 1998-2002 seems to have led to increased prevalence yet again in the Bay.

Importance of oysters in Chesapeake Bay

Oysters occupy an ecological niche among the benthos. Newell (1988) estimated that pre-1900 oyster stocks could filter the Bay's entire volume of water in 3.3 days, whereas in 1988 it could take a year or longer. Stocks have declined since 1988. The oyster decline has indirect effects on ecological processes in the Bay. Remaining oysters are incapable of maintaining water quality, contributing to higher sedimentation rates and further disrupting the oyster life cycle. Abundant and healthy oyster populations would harvest phytoplankton, improve water quality, and provide essential fish habitat.

Oysters used to be the basis of a valuable industry. Pre-1900 Chesapeake Bay oysters were supplied to markets nationally and internationally. From 1875-1885 harvests were estimated to be ~20 million bushels per year for Chesapeake Bay (Hargis and Haven 1988). Human population growth and settlement along the Eastern seaboard maintained a high demand for oysters during this time period. This changed at the turn of the 20th century. An average annual oyster harvest for Virginia was ~3.5 million bushels from the early 1900's until the 1960's (Hargis and Haven 1988). Crude management plans were implemented during this period, essentially focusing the fishing season from October through June (Hargis and Haven 1988). The combination of fisheries management and economic recession may have resulted in sustainable harvests during the 1930's. Commercial landings of the Chesapeake Bay oyster were higher than any other region before 1960 and still supplied product to several markets worldwide. Unfortunately, demand quickly became higher than the product. Insufficient management strategies, infectious diseases, and overharvesting were all partly responsible for the decline in oyster harvest. Oyster landings in 1981 were estimated at

~1.1 million bushels, but 1989 estimates showed landings of ~270,000 bushels (VIMS 1996). The most recent estimate for oyster landings in Virginia was ~20,000 bushels in 2001 and 2002 (J. Wesson, Virginia Marine Resource Commission, personal communication). In Maryland, landings were ~175,000 bushels in 2001 but decreased to ~90,000 bushels in 2002, the lowest on record (C. Lewis, Maryland Dept. Nat. Resources, personal communication). Economically, loss of product means loss of jobs, infrastructure, and livelihoods. Currently, about twenty-one shucking houses operate in Virginia, compared to hundreds historically. Both shucked and whole products are imported predominantly from Louisiana and Texas stocks. Clearly there is need for a reliable supply of product.

Rehabilitation of the industry

Traditional Solutions

Early rehabilitation efforts focused on encouraging spat settlement by planting recycled oyster shell on the remaining two-dimensional reefs. Shell planting was effective, but after 1960 oysters survived only 1-2 years because of disease pressure. Disease management practices have focused on transplanting oysters from diseased areas to low salinity areas, where pathogen survival is lower. Transplanting oysters, however, also distributes pathogens carried by the host. Potentially, transplanted hosts could infect other oysters in the low salinity area. Transplantation is labor intensive. Transplanted stocks may spawn in the low salinity area to increase recruitment (Krantz and Jordan 1997). The use of low salinity areas for disease management has produced some harvestable oysters (Krantz and Jordan 1997). Unfortunately, historic low salinity areas suffer higher salinity in drought conditions, similar to recent conditions in the Bay.

In order to rehabilitate the native oyster industry, it is now clear that reef structure is necessary. Recent efforts have focused on rebuilding the existing flat reefs back into three-dimensional structures, especially in Virginia. Although this is very expensive, there has been some success in spat recruitment. Reconstructed reefs in small river systems have realized increased recruitment on the reef and surrounding areas (Wesson 1997; Southworth and Mann 1998). For example, in 1996, native oysters that were distributed in low-densities in Virginia were transplanted at a higher density onto a constructed reef. The following summer a 10 to 200-fold spatset increase was observed (Southworth and Mann 1998). An overall increase in spat around these constructed reefs has not however translated into sustained populations. New recruits generally succumb to disease before reaching full reproductive potential. Overall, the Bay's oyster populations continue to battle high disease pressure in spite of these restoration efforts.

Non-traditional Solutions

Selective breeding, or artificial selection, is an alternative approach to addressing the problems of oyster disease. Breeding for disease tolerance or tolerance involves selecting surviving offspring and using them as parents to spawn the next generation. Researchers at Haskin Shellfish Research Lab (HSRL) examined oysters that survived *H. nelsoni* after its outbreak in the late 1950's (Haskin and Ford 1987). They showed *H. nelsoni* tolerance was heritable and that the fifth generation offspring suffered only 30% cumulative *H. nelsoni* mortality after three seasons of exposure, compared to the unselected oysters with over 85% mortality over the same exposure period (Haskin and Ford 1987). In 1992, the onset of *P. marinus* in both Delaware and Chesapeake Bays required a new breeding strategy for the pedigree lines: selection for dual disease-

tolerance. The Cooperative Regional Oyster Selective Breeding (CROSBreed) Project, involving four mid-Atlantic research institutions, was developed in 1995 to develop dual disease-tolerance in oysters. A disadvantage of selective breeding is decreased crossbred variability because only desirable traits are selected for, which can reduce overall population or individual fitness (Allendorf and Phelps 1980). Combining efforts such as reef building and stocking of a disease tolerant oyster may provide a strategy for oyster rehabilitation. Ruzecki and Hargis (1989) reviewed estuarine circulation and retention of oyster larvae in James River estuary in Virginia and hypothesized that starter reefs may be beneficial to incubate larger oyster reefs downstream. Recently, collaboration between federal and state agencies in Virginia resulted in a strategy to increase restoration effort and effectiveness in the Chesapeake Bay (Allen *et al.* 2003). This integrated approach utilizes disease tolerant eyed larvae (competent to settle) that are released into a contained "starter" system with bags of shell substrate and raw seawater that is pumped over the system. After several months the "starter" system, full of disease tolerant spat, is placed in a pre-selected trap type estuary. Trap type estuaries (Andrews 1979) are beneficial because they retain larvae for settlement on local reefs. These new reefs, termed "incubator reefs", would hopefully provide new recruits to the surrounding two-dimensional shell reefs within the trap type estuary. In general, this aggressive approach relies on incubator reefs supplying surrounding reefs and surrounding reefs supplying main stem Chesapeake Bay estuary reefs. Success from this process may be realized if disease tolerant genes are incorporated and perpetuated in wild stocks and trap type estuaries continue to be successful.

An alternative solution has been consideration of non-native *Crassostrea* species, specifically the Pacific oyster, *C. gigas* (Thunberg 1793), and the Suminoe oyster, *C.*

ariakensis (Fujita 1913). Both oysters were introduced from Japan to the west coast of the United States in the early 19th century (Breese and Malouf 1977). Cultivation of *C. gigas* was tested in Chesapeake Bay first due to its overall success in the Pacific Northwest. Nearly all oyster production on the west coast is *C. gigas* (Mann *et al*. 1991). *C. ariakensis*, less suited to west coast conditions and therefore not widely cultivated, was the second test species.

In 1996 the Virginia Institute of Marine Science (VIMS) created "A Rational Plan for Testing Application of Non-native Oyster Species"(RP) in Chesapeake Bay. Under the RP, *C. gigas* was field tested from May 1997-May 1998 and exhibited higher disease tolerance compared to *C. virginica*, although survival and growth were unimpressive (Calvo *et al*. 1999). Due to limited success and market acceptance of the Pacific oyster in the Bay, *C. gigas* is not considered a suitable candidate for introduction. On the other hand, promising results were obtained from a field trial of *C. ariakensis* conducted from June 1998 through September 1999. This non-native oyster demonstrated higher survival, growth, and disease tolerance to both *H. nelsoni* and *P. marinus* compared to *C. virginica* (Calvo *et al*. 2001). These results, although promising, were only after one year of disease exposure. Overall superiority of *C. ariakensis* to *C. virginica* needs verification. In 2000 and 2001, *C. ariakensis* was released to qualified shellfish industry growers under the direction of the Virginia Marine Resources Commission (VMRC). High growth and survival in various environmental conditions were regularly reported by industry (Cowart Seafood Inc., Kellum Seafood, Inc., and Cherrystone Aquafarms, personal communication). *C. ariakensis* has emerged as the principal candidate for use in Chesapeake Bay. Potentially, Suminoe oyster harvest could relieve pressure on *C. virginica* stocks and work in concert with restoration and selective breeding programs. In

order to pursue this alternative a more complete understanding of *C. ariakensis* and its general biology is necessary.

Background on *C. ariakensis*

While the full extent of its native range is still in question (Zhou and Allen 2003), *C. ariakensis* has been reported to occur 12-34° N extending from southern Japan to the coasts of India (Kuroda and Habe 1952). Based on museum specimens Ranson (1967) described *C. ariakensis* as native to southern Japan, the coasts bordering the South China Sea, including Hong Kong, Vietnam, and Northern Borneo, Malyasia. Carriker and Gaffney (1996) reported *C. ariakensis* in Korea, Japan, China, and supposedly the coasts of India and Pakistan and further described *C. ariakensis* as an estuarine and warm water species. It is commonly found on intertidal hard grounds, in muddy creeks and brackish waters. Wide salinity and temperature ranges are characteristic in its native range. In Japan, *C. ariakensis* has been reported to occur from 9-30 ppt. (Amemiya 1928). Similarly, in China, occurrence of *C. ariakensis* seems to be from 10-30 ppt. (Lu 1994). According to Lu (1994), *C. ariakensis* can survive in a wide temperature regime, 2-35°C. Clearly *C. ariakensis* in Japan and China can tolerate wide salinity and temperature ranges, however possible limitations for *C. ariakensis* in Chesapeake Bay may be observed during extreme freezing winter temperatures (below 2°C) and prolonged high summer temperatures (above 35°C). Published accounts reported spawning ranging from 7-40°C with 30-40°C more common (*cf.* Mann *et al.* 1991). In the wild, spawning seems to be early in the late spring and synchronous with monsoon freshets that lower the salinity from 15 to 10 ppt. (S.K. Allen, Jr., VIMS, personal communication). *C. ariakensis* is well adapted to endure stressful estuarine conditions. Controlled laboratory

experiments may provide further insight to *C. ariakensis* biology. Growth and survival

of larvae under hatchery conditions has been reported optimal at 28°C and 20‰ (Breese

and Malouf 1977).

Hatchery rearing of *C. ariakensis* began on the west coast of the United States as

a means of expanding the oyster market. In the late 1970's and early 1980's, the west

coast oyster industry tested *C. ariakensis* as a possible "summer oyster". The heavily

cultured Pacific oyster undergoes gonadal maturation during the spring and summer, thus

softening the flesh. The Suminoe oyster delays gonadal maturation, at least in the Pacific

Northwest where it was tested, and maintains a firm, marketable condition during warm

summer months (Breese and Malouf 1977; Langdon and Robinson 1996, Perdue and

Erickson 1984). This led to small quantities of *C. ariakensis* being cultured on the west

coast, but *C. gigas* remains the primary commercial oyster due to its high productivity

and suitability to west coast conditions.

In Chesapeake Bay there have been relatively few studies with *C. ariakensis*, and

none on its effects as an introduced species. Clearly, there is a need for more complete

data about ecology, reproductive biology, and general physiology concerning this oyster.

Logically, there are environmental concerns with field testing non-natives in Chesapeake

Bay. To accommodate this and gain further information on *C. ariakensis* sterility has

been implemented.

Triploid *C. ariakensis*

A triploid oyster may be defined as an organism that has been genetically

manipulated to contain three sets of chromosomes. The triploid condition is important

for two reasons. Economically, triploidy offers a growth advantage over the normal

diploid condition because glycogen reserves typically used for reproduction are shunted to growth (Allen and Downing 1986). An oyster that grows fast would reduce the labor involved in culture, increase revenue in shucking houses, expand jobs, and increase marketing possibilities. Ecologically, triploid oysters are important because the extra chromosomes interfere with homologous pairing during meiosis, which eliminates or reduces reproduction among triploid oysters, ostensibly preventing colonization. Occasionally, triploid oysters overcome the obstacles associated with meiotic pairing and significant gamete production is observed. Triploid oysters that have the ability or mechanism to achieve fecund or ripe levels of sexual maturity are important for further crossbred manipulations (i.e. tetraploid oysters), but are problematic for biological control of *C. ariakensis* in Chesapeake Bay. Triploid oysters provide an effective means to conduct field experiments however some level of risk is involved. Since triploid oysters are rarely observed in nature, their production must be hatchery based.

One method used to achieve triploidy in oysters is by induction, i.e., preventing the elimination of the second polar body (PB2) during meiotic reduction. A popular inducer is cytochalasin B (CB). CB is a cytokinetic inhibitor that disrupts normal post-fertilization meiotic events and can produce large quantities of triploid oysters (Stanley *et al.* 1981; Allen 1988). The induction method can be effective and the offspring are usually 85-95% triploid. Failure to produce 100% triploid oysters arises from natural variation in the rate of egg maturation (personal observation). Since production of induced triploid oysters is not 100% effective, individual oysters must be certified triploid prior to any field deployment and therefore is labor intensive. Commercially, the oyster industry's objective is increased growth therefore 100% triploid is not required.

A second method to produce triploid oysters is mating tetraploid oysters with diploid oysters (Guo *et al*. 1996), so called mated triploid oysters. This method produces nearly 100% triploid oyster offspring and lacks the direct use of a harmful chemical, and so is preferred for its efficiency. Ecologically, the objective of population control is to eliminate colonization, therefore 100% triploid is required. The induction method cannot satisfy ecological requirements without individual certification.

Commercial triploid oyster aquaculture could utilize the mated method for triploid production to promote an industry as well as address ecological concerns. It is possible to envision triploid oyster aquaculture beginning the process of rebuilding Virginia's industry. For example, a "technical" hatchery may produce tetraploid oysters and disseminate this brood stock to other hatcheries where mated triploid oysters would be produced. Implementing several grow-out sites with different environmental conditions would increase the probability of obtaining "ripe" triploid oysters for use in tetraploid production. Ecologically the mated method is favored and the critical step for industry and research is the production of tetraploid oysters.

The Virginia Seafood Council has estimated that an industry based on mated triploid oyster aquaculture, in Chesapeake Bay or elsewhere, would gross about a million bushels a year (Virginia Seafood Council 2002).

Tetraploid production of *C. ariakensis*

Hatchery production of viable tetraploid oysters can be difficult. Guo (1991) hypothesized that blocking the first polar body (PB1) in eggs from triploid oysters using CB could produce tetraploid oysters. Guo and Allen (1994a) successfully produced tetraploid *C. gigas* using this method. The authors noted that zygotes produced this way

were largely aneuploid, but a small proportion of survivors to day seven post-fertilization were viable tetraploid oysters. The Aquaculture Genetics and Breeding Technology Center (ABC) at VIMS conducted 42 hatchery spawns in 2001 to produce tetraploid *C. ariakensis*. Millions of triploid oyster eggs were strip-spawned from both induced and mated triploid oysters. Fertilization of triploid oyster eggs and the inhibition of the first polar body by CB were successful; however, only about 15-30 viable putative tetraploid oysters were reared through metamorphosis (personal observation). None were tetraploid. In 2002, attempts to produce tetraploid *C. ariakensis* were more successful, and thousands are now available for breeding. Also in 2002, VIMS produced tetraploid *C. gigas* and *C. virginica* for the first time on the East Coast (S.K. Allen, Jr. personal communication). This is a significant development because tetraploid oysters from three different *Crassostrea* species are available to produce crossbred triploid oysters and conduct further experiments.

Tetraploid technology is unique in its requirement for triploid oyster eggs. The detection of "ripe" triploid female oysters seems counterintuitive. If triploid oysters are sterile, then how can they become ripe? Despite general reproductive sterility, triploid oysters do undergo some degree of gametogenesis (Allen and Downing 1990; Guo and Allen 1994a).

To make tetraploid oysters, triploid oyster eggs are fertilized with sperm from diploid oysters, followed by inhibition of PB1 using CB (Guo and Allen 1994a). Guo (1991) suggested that inviability of tetraploid oysters derived from diploid oyster eggs was caused by the cleavage of a normal egg with a large tetraploid nucleus yielding insufficient cytoplasm and resulting in too few cells at advanced embryonic stages. Guo and Allen (1994a) hypothesized that this deficiency in cell number could terminate

development in tetraploid embryos. Success can be realized from triploid oyster eggs because of the increased egg size and, presumably, the completion of embryonic development.

The successful production of tetraploid oysters depends on a number of other factors, such as proper timing of CB treatments, spawning temperature, and hydration time of triploid oyster eggs prior to fertilization. Eudeline *et al.* (2000a) suggested that in *C. gigas*, short CB treatments (i.e., 15-35 min post fertilization, corresponding to one-half of the time required for 50% PB 1 expulsion in triploid oyster eggs) yielded more tetraploid cells compared to long CB treatments (i.e., 7-43 min post fertilization, corresponding to approximately three-quarters of the PB 1 expulsion period). The authors also determined that tetraploid production was most consistent when treatments were performed on triploid oyster eggs from individual females compared to pooled eggs from several females. Eudeline *et al.* (2000b) determined that PB 1 release in *C. gigas* was generally slower for triploid oysters than diploid oysters at 26°C, but lowering the temperature to 19°C almost halted development of triploid oyster eggs. The amount of time triploid oyster eggs remain in seawater after being stripped and prior to fertilization (i.e., hydration time) also affects the rate of meiosis in *C. gigas* (Eudeline *et al.* 2000b). Generally, the longer the hydration time for triploid *C. gigas* eggs the shorter the duration of meiotic events (Eudeline *et al.* 2000b).

The above variables in tetraploid technology are moot if ripe triploid female oysters are unavailable. Spawns to produce tetraploid oysters yield far less than 1% of cultured eggs surviving to metamorphosis (Guo and Allen 1994a; VIMS, personal observation). Therefore, identifying fecund triploid female oysters is critical. Guo and Allen (1994a) determined triploid female *C. gigas* had between 0.4 and 8.2 million eggs,

whereas diploid female oysters would contain between 25 and 100 million eggs. Female triploid *C. ariakensis* can contain between 0.3 and 10 million eggs (personal observation). Very little is known about the biology of triploid *C. ariakensis*. Information on the degree of sterility in mated triploid oysters is rudimentary. There have been a few studies on induced *C. ariakensis* and *C. gigas* and mated triploid *C. gigas*.

Sterility of triploid *C. ariakensis*

There are concerns about testing *C. ariakensis* in the Chesapeake Bay, as there would be with any non-native species. To address this, all experiments in Virginia have used triploid oysters. Triploid *C. ariakensis* are useful because they generally impede gametogenesis due to the imbalance of three sets of chromosomes, effectively eliminating or reducing reproduction. As it pertains to triploid oyster production, sterility may be defined as incomplete gonad production or the production of abnormal gametes.

Reversion, the development of diploid cells in triploid oysters, has been observed in both induced and mated *C. gigas*. Induced triploid *C. gigas* showed higher reversion compared to mated triploid *C. gigas* (S.K. Allen, Jr. personal communication). Induced triploid *C. ariakensis* exhibited 5% or less overall mosaicism in field experiments (Zhou 2002). No research to date has focused on reversion in mated triploid *C. ariakensis*. Reversion seems to be a regular feature of triploid individuals of the genus *Crassostrea*, and the incidence of diploid cells in triploid oysters seems to increase with age (S.K. Allen, Jr. unpublished data). Higher reversion frequency in older oysters may be an important consideration for introductions, although from an aquaculture perspective, all

triploid oysters could attain market size rapidly and reversion may be an unimportant issue.

A thorough examination of gametogenesis in mated triploid *C. ariakensis* is important, both for obtaining tetraploid oysters and determining general sterility.

Gametogenesis

Normal gametogenesis

Gonadogenesis and gametogenesis in oysters generally is well documented. It includes the storage of energy reserves, accumulation of gametes by cell proliferation, the release of ripe gametes, and a resting or recovery period (Giese and Pearse 1974; Berthelin *et al.* 2000; Mann 1979; Cox *et al.* 1996). Identification of these stages is relatively easy using standard histological procedures. Kennedy and Battle (1964) outline in detail the cyclic changes of gonad development for *C. virginica*. The eastern oyster is protandric, i.e., initial maturation usually involves functional males and older oysters, functional females. A proportion of oysters in a population will usually change sex between spawning seasons, and as oysters grow the proportion of females in the population increases (Kennedy 1983; O'Beirn *et al.* 1998). Cox and Mann (1992) showed a significantly higher number of males than females in four populations of *C. virginica* possibly relating to the inclusion of small male oysters. Morales-Alamo and Mann (1989) found a sex ratio of 1.0 for oysters >60 mm shell height in the lower Chesapeake Bay possibly indicating the higher frequency of older female oysters in the population. Hermaphroditism (animals that have both eggs and sperm) is rare in most species of oyster, including the eastern oyster (Thompson *et al.* 1996). In a Georgia

estuary, O'Beirn et al. (1998) discovered only six hermaphrodites in 1,576 oysters examined (0.38%).

There has been only one study describing C. ariakensis gametogenesis. Perdue and Erickson (1984) compared the different gametogenic cycles of C. gigas and C. rivularis (=ariakensis) in Washington State. Although specific stages of gonad development were not described, C. ariakensis showed delayed spawning, compared to C. gigas, until early August and had only partially spawned by the end of October. In histological cross-sections, the percent of developed gonad was used as an indicator of gametogenesis in oysters. A higher percentage of gonad area indicated more extensive gonadal development. Perdue and Erickson (1984) demonstrated that C. gigas had a peak of 75% gonad area and C. ariakensis had a peak of 60%, however, C. gigas released all gametes developed by early September whereas C. ariakensis retained at least 30% gonad through late October. Sex ratios were found to vary greatly among cohorts of both species, but the majority of C. gigas were females and the majority of C. ariakensis were males (Perdue and Erickson 1984). Interestingly, C. ariakensis held in quarantine at VIMS (2000 and 2001) exhibited earlier sexual maturation (personal observation), possibly as a result of warmer Chesapeake Bay water temperatures in the early summer compared to Washington State. In their native range of China, it is clear that maturation occurs early in the year (Zhou and Allen 2003). Therefore, the report describing gametogenesis from Washington seems the exception, not the rule.

Triploid gametogenesis

Gonadal development in triploid shellfish, including soft-shelled clams, bay scallops, Eastern oysters, Pacific oysters, and hard clams, is abnormal (Allen et al. 1986;

Tabarini 1984; Barber and Mann 1991; Eversole *et al.* 1996). Varying degrees of abnormality have been observed depending on the species. Some triploid shellfish can undergo spawning events while other triploid shellfish are capable of producing viable offspring. Histological work from Allen and Downing (1990) suggested that some spawning occurred in triploid Pacific oysters. Guo and Allen (1994b) demonstrated that triploid Pacific oysters occasionally produce viable gametes, although progeny were mostly aneuploid. It is evident that triploid Pacific oysters can still be relatively fecund animals despite their triploid condition. On the other hand, Eversole *et al.* (1996) concluded that triploid hard clams had no viable reproduction or spawning due to severely abnormal gonads and the scarcity of sex cells. Lee (1988) described three-year old triploid *C. virginica* as developing through early stages of gametogenesis. Triploid male oysters had a proliferation of primary spermatocytes and triploid female oysters some oogonial production. As sex cells progress or are halted in various triploid shellfish species, glycogen reserves that would be used for gametogenesis are available for other physiological processes. Allen and Downing (1986) noted glycogen utilization in triploid *C. gigas* was significantly less than in diploid oysters during the spawning season.

Early phases of triploid gametogenesis tend to last longer and are more protracted compared to the later phases of gametogenesis (Allen 1987). Allen *et al.* (1986) described inactive, very early active, early active, middle active, late active, and ripe stages of development for triploid *Mya arenaria*. Based on the absence of maturing gametes, most triploid *M. arenaria* were inactive. Oocyte development was rare and abnormal when it did occur. Only two triploid *M. arenaria* sexually developed to the middle active stage (Allen *et al.* 1986). An occasional large oocyte was observed in triploid *Mercenaria mercenaria*, but in most cases the lumen was empty (Eversole *et al.*

1996). An indeterminate (=inactive) stage of development was most frequent in triploid

Sydney rock oysters, *Saccostrea commercialis* (Cox *et al.* 1996). Additionally, the

triploid follicle walls of male *S. commercialis* were lined with spermatogonia and

primary spermatocytes and gamete maturation was arrested between the secondary

spermatocyte and the spermatid (Cox *et al.* 1996). In contrast, Allen and Downing

(1990) described maturity in triploid male *C. gigas* as uniform. Specifically, most males

were capable of secondary spermatocyte (meiosis I) and spermatid (meiosis II)

production. In sharp contrast to males, Allen and Downing (1990) showed triploid

female *C. gigas* to mature very little. Some females produced numerous ova. The

majority of females had follicles with mature ova that were atretic or resorbing.

Therefore, most females were classified as resorbing, even though they were relatively

inactive (Allen and Downing 1990). Triploid *C. ariakensis* sacrificed during peak

gametogenesis and assessed using flow cytometry and histology found male follicles

contained some spermatogonia but the majority of sex cells were spermatids (Chandler *et

al.* 1999a). All mature follicles of triploid male oysters had spermatozoa present.

Generally, for most triploid shellfish, males can develop some spermatocytes and

spermatids, but few females produce developing oocytes. Bear in mind that all studies of

triploid gametogenesis to date have been with induced triploid oysters.

Sex ratio in triploid oysters is skewed compared to diploid oyster controls.

Older triploid oysters (>2 growing seasons) can have a higher abundance of females than

younger individuals (Allen *et al.* 1986; personal observation). Cox *et al.* (1996) found

that 2-3 year-old triploid *S. commercialis* female to male ratio was 2:1. Allen *et al.*

(1986) also demonstrated a higher female to male ratio (~3:1) in triploid *Mya arenaria*.

The authors went further to say that 77% of all triploid oysters had oocytes and 16%

more exhibited female-like characteristics, which may have been intersexes. The development of mature eggs in triploid oysters was rarely observed. Both triploid *M. mercenaria* and *C. gigas* showed a lower female to male ratio, 1:1.3 and 1:2, respectively (Eversole *et al.* 1996; Allen and Downing 1990). One study by Chandler *et al.* (1999a) on induced triploid *C. ariakensis* demonstrated 84% of five-year old oysters were males and the rest were hermaphrodites. In a separate study, induced triploid two-year old *C. ariakensis* were 66% male, 17% female, and 17% hermaphrodite (Chandler *et al.* 1999b). By working with triploid oysters in the hatchery (2001) I have observed that the *C. ariakensis* female to male sex ratio can be about 1:1 during summer months in two year-old oysters. But it also seemed that this sex ratio, as well as the extent of development in triploid female oysters, varied among sites. Possibly, environment plays a major role in sex ratios.

Effects of environment on gametogenesis and sex ratio

Gametogenesis in *Crassostrea* species varies with exogenous and endogenous factors (Thompson *et al.* 1996). Exogenous factors, such as temperature and salinity, affect gametogenesis and reproduction in oysters (Mann 1979; Shpigel *et al.* 1992; Stephen 1980). Elevated temperatures and fluctuations in salinity can also trigger gametogenesis in *C. gigas* (Mann 1979). Food abundance, absence, or quality can also influence gametogenic patterns (Kennedy and Krantz 1982). High food abundance and quality, elevated temperature, and high salinity correlate to more gonad development and spawning. Kennedy *et al.* (1995) observed no loss of reproductive capacity or abundance of eggs during low *P. marinus* infection. However, higher infections of *P. marinus* can alter the gametogenic cycle of *C.virginica* and actually inhibit development, maturation,

and spawning of gametes (Barber 1996). Endogenous factors such as genetic makeup also influence gametogenesis and reproduction (Barber *et al.* 1991.) Eastern oysters native to Long Island Sound that were inbred and then reared in Delaware Bay for six generations initiated gametogenesis and began spawning coincident with Long Island schedules and one month earlier than oysters native to Delaware Bay (Barber *et al.* 1991).

Sex ratios are influenced by environment. Typically, insufficient food resource increases the percentage of male oysters in *C. gigas* and *C. virginica* (Thompson *et al.* 1996) due to higher energy requirements to produce eggs rather than sperm. Stress, such as parasitic diseases like *H. nelsoni*, also can increase the proportion of male oysters (Coe 1934) presumably because it saps energy from the gametogenic process. However, Ford *et al.* (1990) found that *H. nelsoni* infected *C. virginica* sampled in May had a higher ratio of females to males compared to subsequent sampling periods. The authors hypothesized that females have higher infection rates in May. Therefore, higher mortality of females in later samplings would equalize sex ratios.

Hypotheses and objectives

Assessing reproductive potential of triploid oysters is important for two reasons. The first reason is to gain knowledge of the best environmental conditions that produce ripe triploid female oysters for subsequent production of tetraploid oysters. The second reason is to document gametogenesis of mated triploid *C. ariakensis* for its impact on risk analysis and how it may influence triploid oyster aquaculture in Chesapeake Bay. These are the goals of my thesis research.

Associated risks involved with the introduction or experimentation of non-native oysters are numerous (Mann *et al.* 1991). As it pertains to triploid oysters, one such

aspect is the concept of fertility versus sterility. A major focus of this study is to determine the extent of gamete production. The results of this thesis could have far reaching implications for non-native oyster research in Chesapeake Bay. In the summer of 2003, the National Academy of Sciences (NAS) is expected to publish a report summarizing the risks and benefits associated with *C. ariakensis* in Chesapeake Bay. In addition, the panel will make a recommendation for the future of this non-native, the possibilities ranging from no field experiments, to triploid oyster aquaculture, to diploid oyster introduction.

Triploid oyster aquaculture in Virginia is partially an economic venture. The Virginia Seafood Council (VSC), an industry based group, gained federal and state approval to deploy one million mated triploid *C. ariakensis* in the waters of Chesapeake Bay during summer 2003. VSC has the specific intent of gathering economic data focusing on culture costs and marketability. Scientific investigations will take advantage of this deployment and study parasitic diseases, reversion, gametogenesis, shell disease, and molecular genetics in *C. ariakensis*. Mated triploid oyster aquaculture seems an obtainable goal for Virginia.

This project focuses on gametogenesis of mated (2n female x 4n male) triploid *C. ariakensis* in multiple environments within Chesapeake Bay. My primary objective was to qualitatively assess gametogenesis in triploid oysters. The general hypothesis was that gametogenesis in triploid oysters would be abnormal and reduced, although a few individuals would become ripe or fecund. I will be making the first full description of gametogenesis in these oysters.

As a secondary objective, I diagnosed disease prevalence and intensity. *H. nelsoni* and *P. marinus* prevalence was followed during the summer months when disease

pressure should have been at its peak. The study year, 2002, was the third season of exposure to these parasites, and presumably these large animals filtered voluminous amounts of water. Only one study has examined diseases in *C. ariakensis* (Calvo *et al.* 2001). The authors found no *H. nelsoni* and only rare or light *P. marinus* infections. That study used induced triploid oysters, however. This study is the first description of disease prevalence in mated triploid oysters. The general hypothesis was that disease prevalence would be absent in triploid oysters.

In another secondary objective, reversion was quantified using the proxy of percent diploid cells determined by flow cytometry (FCM). Zhou (2002) examined induced triploid *C. ariakensis* that exhibited on average 2.5% mosaicism and less than 10% diploid cells over the course of the study with a few higher percentages. The general hypothesis was that reversion in mated triploid *C. ariakensis* was similar across all sites. My study also is the first estimate of reversion in mated triploid oysters.

MATERIALS AND METHODS

Source of biological material

Nine diploid female *C. ariakensis* were obtained from US west coast stocks and quarantined at VIMS. These were used as female parent brood stock to produce mated triploid oysters. A single tetraploid male was obtained from a 1999 tetraploid spawn and used as the male parent. The mated triploid oyster mass spawn was done at VIMS/ABC on June 20, 2000 when approximately 45 million eggs were cultured. Larvae were raised in quarantined 210 L tanks at 26-27°C and 19-22‰ for approximately 20 days and fed a live algal diet of *Isochrysis spp.*, *Chaetocerous calcitrans*, *Chaetocerous galbana*, and *Tetraselmis chuii*. To increase setting efficiency, eyed larvae were epinephrine-set (Coon *et al.* 1986) with microcultch, placed in 212-micron downwellers and reared through metamorphosis. Spat were then transferred to the quarantine nursery system at ABC until deployment.

In fall 2000 VMRC allocated ~6,000 of these oysters to the VSC to run phase one of a project to test general field performance. The remaining animals were deployed in raceways and upwellers at other industry facilities to test the feasibility of obtaining winter growth in *C. ariakensis* seed. All animals were retrieved in April 2001 and ~60,000 were subsequently allocated to thirteen industry sites for deployment in June 2001 under the direction of VMRC as phase two of the VSC project. This research focuses on these mated triploid oysters at six industry field sites.

A native diploid oyster stock was used in this study because a non-native diploid oyster could not be deployed in Chesapeake Bay. The specific strain employed was a Delaware Bay (DEBY) *Crassostrea virginica* strain that has been selectively bred for

increased survival through tolerance to disease. Diploid *C. virginica* were mass spawned on May 10, 2001 using 20 males and 20 females as brood stock. The larvae were cultured in hatchery conditions for 20 days and fed a diet of *Isochrysis spp.*, *Chaetocerous calcitrans*, *Chaetocerous galbana*, and *Tetraselmis chuii*. Animals were epinephrine-set (Coon *et al.* 1986), to increase survival after metamorphosis and held in floats until deployment.

Study Sites

Refer to Table 1 for names and Figure 1 for a map of the location of each study site. This study was designed to test site effects on gametogenesis therefore all oysters had to acclimate to local conditions prior to first sampling. Diploid *C. virginica* were deployed from September 8th to 20th 2001, approximately six months prior to the first sampling period in January 2002.

Six commercial shellfish aquaculture farms were selected ranging from the high salinity Eastern shore (~35 ‰) to the lower salinity bayside (~10 ‰) of Chesapeake Bay, Virginia. Three sites were on the seaside of the Eastern shore and three sites were on the Western shore of Virginia's portion of the Chesapeake Bay. The experimental oysters were placed in separate, labeled ADPI mesh bags. Grow-out methods included floats and/or bottom cages. Diploid *C. virginica* and triploid *C. ariakensis* were deployed in the same manner at each site, although details of grow-out method varied among sites. Differences in grow-out method were considered trivial with respect to the influence of environment differences on gametogenesis. Criteria used to select participants at various study sites included salinity regime, past participation and experience with *C. ariakensis*

Design

Approximately 200 crossbred triploid oysters were deployed at each site, divided haphazardly among ¾" ADPI mesh bags (39x20x2") that were labeled. In addition, diploid *C. virginica* were deployed in ½" ADPI mesh bag (39x20x3") at each site to track a "normal" course of gametogenesis and disease pressure. Additional bags were used if necessary to maintain reasonable densities and decrease mortality. Two-year old *C. ariakensis* and one-year old *C. virginica* were used, hence the need for different size mesh bags.

Sampling

Refer to Tables 1 and 2 for field collection schedule and sample sizes. Winter dormancy of gonad was determined by paraffin histology in January 2002 by collecting 10 triploid oysters and 10 diploid oysters from each site. Subsequently, 25 triploid oysters and 10 diploid oysters were sampled from each site during May, June, July, August, October, and December. Unfortunately, the bag of *C. virginica* at TK (Figure 1) was lost soon after deployment and therefore no diploid *C. virginica* data was collected (Table 1). The June sample at site JH (Table 1) was not collected because the site was inaccessible at that time. For triploid oysters, individual shell height and shucked whole wet weight was recorded and paraffin histology was done to determine gametogenesis, sex ratio, and disease prevalence and intensity. Hemolymph tissue was taken to confirm triploidy.

Table 1. Sampling regime for diploid *C. virginica* and triploid *C. ariakensis* at all sites. na=not available. N=total numbers sampled in parentheses. Roman numerals indicate sampling period (January to December 2002). All sites are located within Virginia's portion of the Chesapeake Bay and Virginia's seaside of the Eastern Shore. TM=Tommy Mason, Chincoteague; JH=Jeff Hammer, Accomac; CS=Cherrystone Aquafarms, Cape Charles; TL=Tommy Leggett, Yorktown; TK=Tommy Kellum, Weems; BO=Bevans Oyster Company, Kinsale.

Triploid *C. ariakensis*

site	I	N	II	N	III	N	IV	N	V	N	VI	N	VII	N
TM	1/15/02	(10)	5/08/02	(25)	6/27/02	(25)	7/23/02	(25)	8/22/02	(25)	10/10/02	(25)	12/12/02	(25)
JH	1/15/02	(10)	5/08/02	(25)	na		7/25/02	(25)	8/22/02	(25)	10/10/02	(25)	12/12/02	(25)
CS	1/15/02	(10)	5/16/02	(25)	6/13/02	(25)	7/25/02	(25)	8/22/02	(25)	10/10/02	(25)	12/12/02	(25)
TL	1/12/02	(10)	5/10/02	(25)	6/23/02	(25)	7/21/02	(25)	8/20/02	(25)	10/6/02	(25)	12/1/02	(25)
TK	1/10/02	(10)	5/15/02	(25)	6/20/02	(25)	7/24/02	(25)	8/28/02	(25)	10/8/02	(25)	12/3/02	(25)
BO	1/08/02	(10)	5/14/02	(25)	6/20/02	(25)	7/22/02	(25)	8/27/02	(25)	10/8/02	(25)	12/3/02	(25)

Diploid *C. virginica*

site	I	N	II	N	III	N	IV	N	V	N	VI	N	VII	N
TM	1/15/02	(10)	5/08/02	(10)	6/27/02	(10)	7/23/02	(10)	8/22/02	(10)	10/10/02	(10)	12/12/02	(10)
JH	1/15/02	(10)	5/08/02	(10)	na		7/25/02	(10)	8/22/02	(10)	10/10/02	(10)	12/12/02	(10)
CS	1/15/02	(10)	5/16/02	(10)	6/13/02	(10)	7/25/02	(10)	8/22/02	(10)	10/10/02	(10)	12/12/02	(10)
TL	1/12/02	(10)	5/10/02	(10)	6/23/02	(10)	7/21/02	(10)	8/20/02	(10)	10/6/02	(10)	12/1/02	(10)
TK	na		na		na		na		na		na		na	
BO	1/08/02	(10)	5/14/02	(10)	6/20/02	(10)	7/22/02	(10)	8/27/02	(10)	10/8/02	(10)	12/3/02	(10)
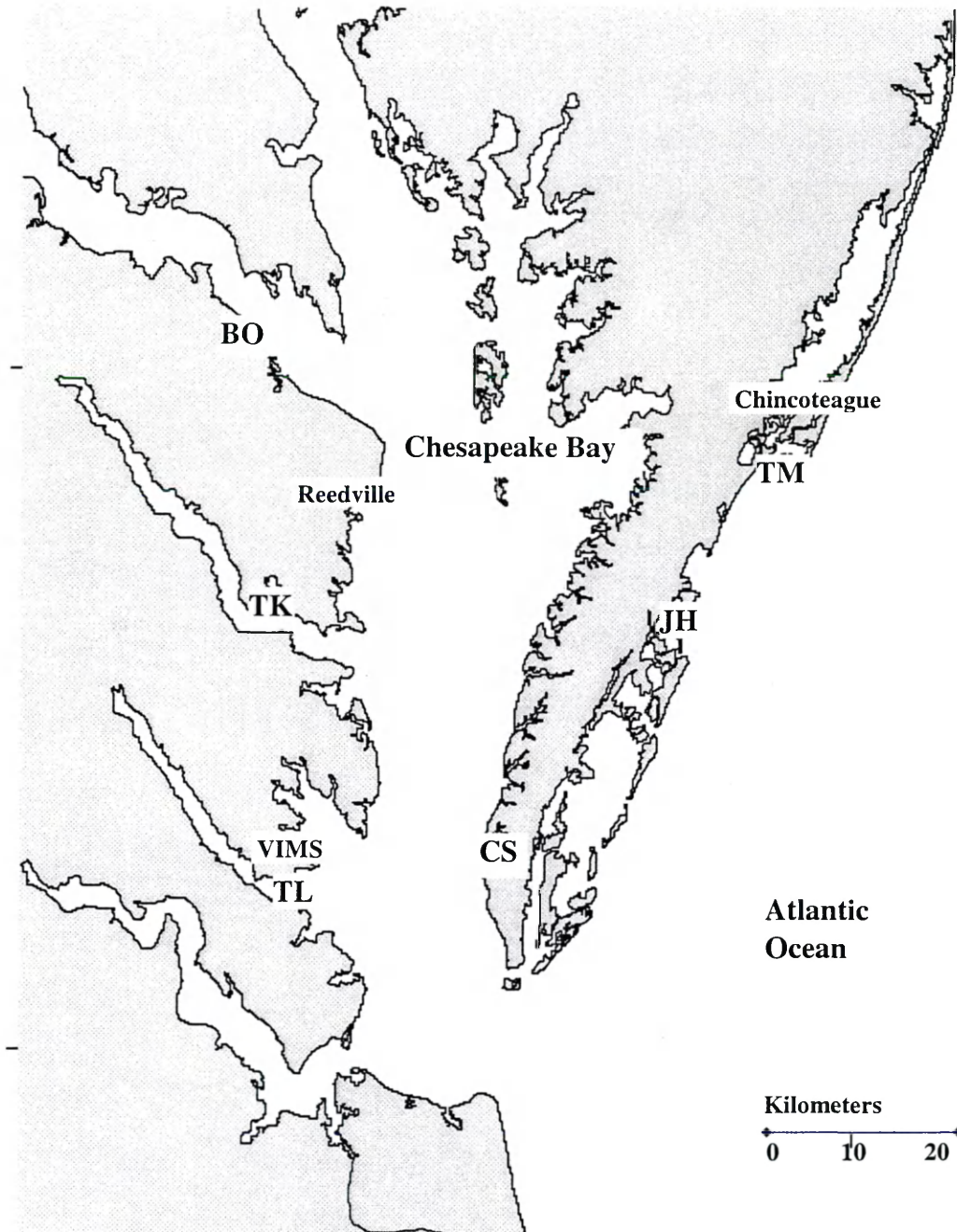
Figure 1. Map of study sites in Virginia. Diploid *C. virginica* and triploid *C. ariakensis* were deployed at each site for an assessment of gametogenesis. TM and JH are high salinity sites (>30ppt); CS and TL are medium salinity sites (20-30ppt); TK and BO are low salinity sites (<20ppt). TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

Table 2. Timeline of deployment, sampling (numbers of diploid *C. virginica* and triploid *C. ariakensis*), and laboratory processing.

Sept. 2001	Jan. 2002	May 2002	Jun. 2002	Jul. 2002	Aug. 2002	Oct. 2002	Dec. 2002
deployment	10 diploids	10 diploids	10 diploids	10 diploids	10 diploids	10 diploids	10 diploids
	10 triploids	25 triploids	25 triploids	25 triploids	25 triploids	25 triploids	25 triploids
	histology	histology	histology	histology	histology	histology	histology
	reversion	reversion	reversion	reversion	reversion	reversion	reversion
				RFTM	RFTM	RFTM	

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If mosaic oysters were found, adductor, heart, gill, and gonad were sampled to determine the extent of reversion. For diploid oysters, individual shell height and shucked whole wet weight was recorded and paraffin histology was done to determine gametogenesis, sex ratio, and disease. For both diploid and triploid oysters, whole wet tissue weights were recorded to the nearest tenth of a gram immediately after shucking and shell heights in millimeters were measured from the hinge axis to the outermost posterior edge (Carriker 1996). During high disease prevalence months (i.e., July, August, and October) Ray's fluid thioglycollate medium (RFTM) analysis (Ray 1952) was done on all diploid and triploid oysters from each site.

Diploid *C. virginica* and triploid *C. ariakensis* gametogenesis

Diploid *C. virginica* served as a baseline for normal gametogenesis at each site. *C. virginica* gametogenesis was classified following similar criteria outlined by Kennedy and Krantz (1982) and Mann (1979).

Gametogenesis, sex ratio, and hermaphroditism were documented for diploid and triploid oysters. Although triploid gametogenesis has been described for several *Crassostrea* species, no study has focused on gametogenesis of mated triploid *C. ariakensis*. A common theme among other triploid oyster species seems to be that gametogenesis is predictably retarded. Description of triploid gametogenesis in *C. ariakensis* followed those used by Allen (1987) and Allen and Downing (1990) for *C. gigas*, and Allen *et al.* (1986) for *Mya arenaria*. Individual oysters were categorized according to the following stages of maturity: Inactive, Early Active, Middle Active, Late Active, Ripe, Spawn Partially, Spawn Completely, Resorbing, Indifferent, and "New"

Early Active. Gonadal maturation, especially for triploids, was based on the kind, rather than degree, of gametogenesis.

Males

Inactive: Undifferentiated gonia cells line immature follicles. Follicles are concentrated in small clusters with little to no growth or branching. Gender in typically inactive gonads is not distinguishable.

Early Active: Growth and branching of follicles can be evident, but not required. Mitotic proliferation of spermatogonia is apparent. Majority of follicles are filled with spermatogonia or primary spermatocytes, occasionally a few secondary spermatocytes.

Middle Active (Plate I): Actively branching follicles begin to invade interfollicular (glycogen bearing) tissue. Small follicles are filled with primary and secondary spermatocytes. A gradient of cell types can be seen as spermatogonia line the follicle wall and spermatids begin to occupy the lumen.

Late Active (Plate II): Mature follicles containing mostly spermatids or differentiating spermatozoa. Nearly all interfollicular tissue is occupied by gonad. Large follicles dominate with a few still growing and branching. Spermatozoa, with pink staining flagella, orient toward the lumen of mature follicles.

Ripe (Plate III): Follicles are swollen primarily with spermatozoa, but a few spermatids remain. Orientation or alignment is haphazard in preparation for spawning. No interfollicular tissue remains at this stage.

Spawn Partially (Plate IV): Interfollicular tissue is evident but its organization is loose. Gonoducts contain mature spermatozoa or spermatids.

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Plate I. Middle Active diploid male *C. virginica* at site TM in June 2002. Spermatids have differentiated into the lumen of follicles (arrow). Follicles have branched into the interfollicular space. Spermatocytes remain at the walls of follicles. No spermatozoa are present. 100x
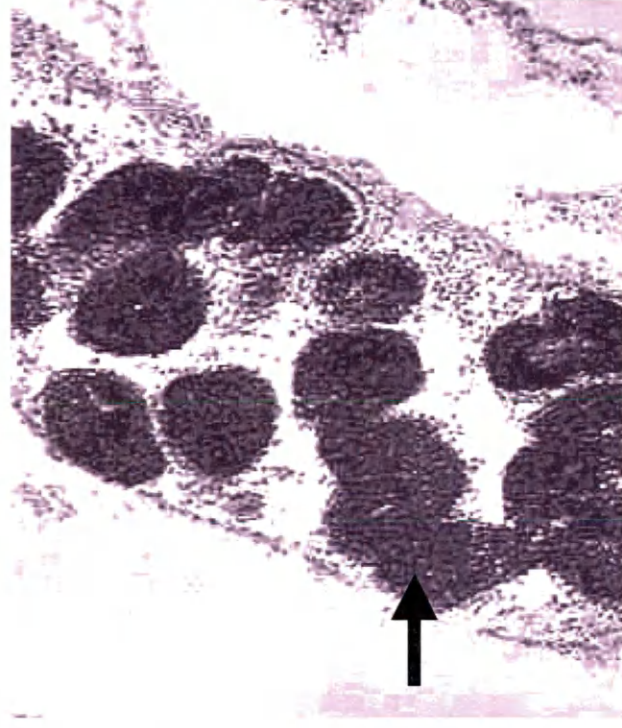
Plate II. Late Active diploid male *C. virginica* at site TL in June 2002. Spermatids predominate and have begun differentiating into spermatozoa in the lumen (arrow). Follicles are well branched and occupy most of the interfollicular space. A few spermatocytes may be observed at the follicle walls, but are not dominant. 100x

30
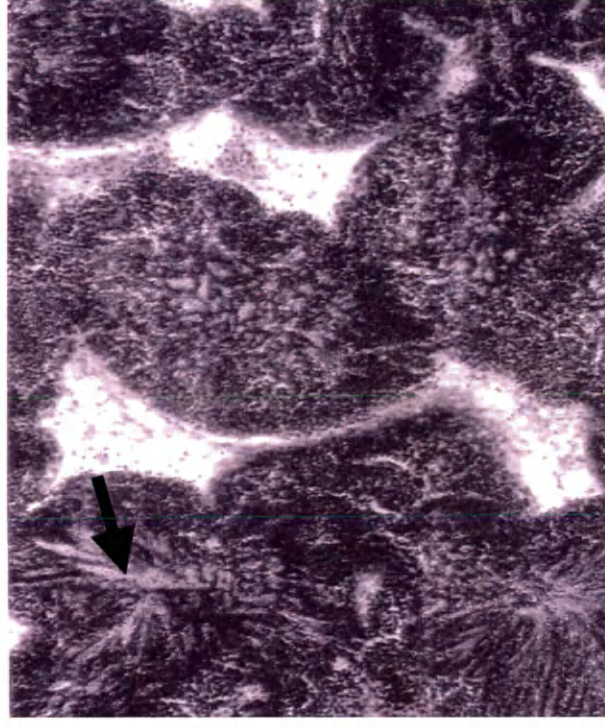
Plate III . Ripe diploid male *C. virginica* at site TM in July 2002. Spermatids and spermatozoa dominate well branched follicles. Little interfollicular tissue remains (small arrow). Spermatozoa begin to fill the gonoducts (large arrow). No spermatocytes are observed. 100x
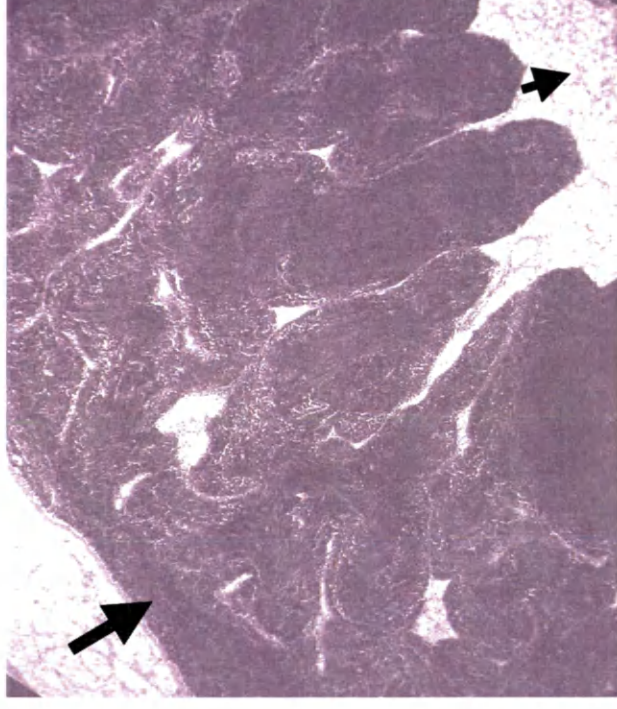
Plate IV . Spawn Partial diploid male *C. virginica* at site JH in August 2002. Few spermatozoa can be seen in the follicles (small arrow) and gonoducts (large arrow). 40x

31
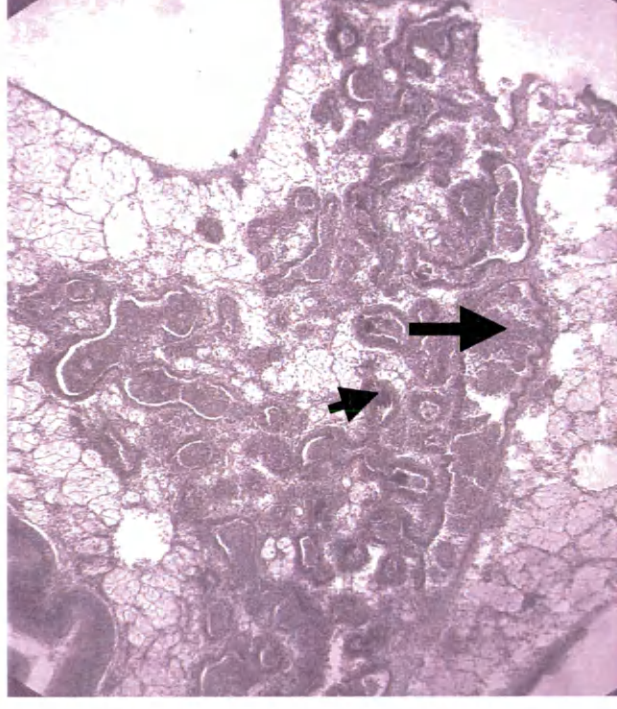
Spawn Completely (Plate V): Follicles are empty. Few mature spermatozoa or spermatids remain in the gonoducts. Interfollicular tissue is disorganized.

Resorbing: Loosely organized interfollicular tissue remains and is invaded by hemocytes, which begin consuming residual gametes. Empty follicles can still be seen, often filled with hemocytes.

Indifferent: This stage is seen several months after the reproductive season, e.g., December. Some residual follicles may be apparent, but most are completely collapsed. Males may be distinguished only if unresorbed spermatozoa are present.

"New" Early Active (Plate VI): This stage occurs following completion of gametogenesis in winter and is characterized by mitotic proliferation of spermatogonia at the follicle walls. Newly forming follicles are small and immature.

Females

Inactive: Usually sex is not distinguishable at this stage. Follicles are immature and small with undifferentiated gonia cells line the walls, looking similar in males and females.

Early Active (Plate VII): Small follicles with numerous immature oocytes that have dark staining nuclei and basophilic cytoplasm are evident. All oocytes are attached to the basal walls of the follicles. Long peduncles have yet to form.

Middle Active: Ooctyes enlarge as the follicles continue to grow and occupy more interfollicular tissue. Follicle centers develop large lumens as the auxocytes (a cell that is destined to enter meiosis) become less basophilic and more acidophilic. Maturing oocytes are forming peduncles anticipating release into the central lumen of the follicle.

Plate V. Completely spawned diploid male *C. virginica* at site JH in October 2002. Only a few residual sperm can be seen in the swollen gonoduct (arrow). Follicles are empty and/or constricting. Hemocytes may be observed invading follicles. 100x
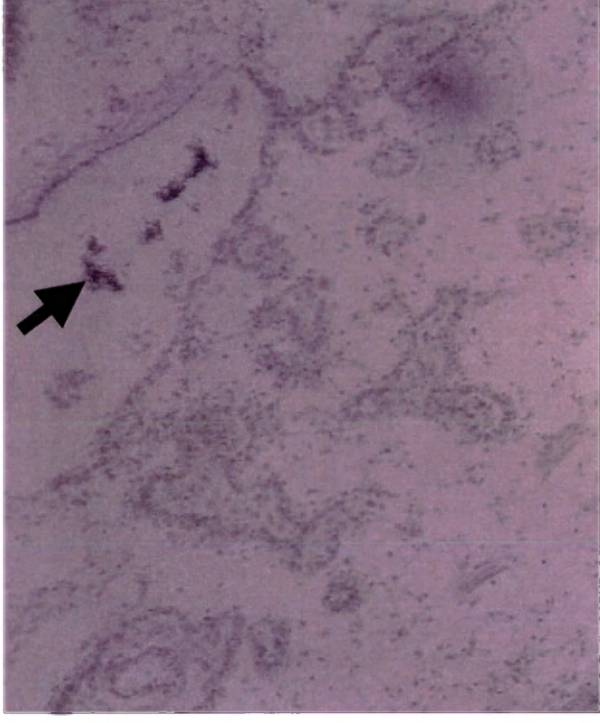
Plate VI. "New" Early Active diploid male *C. virginica* at site BO in January 2002. New follicles begin to grow and are occupied by spermatogonia (large arrow) and undifferentiated gonia (small arrow). 100x
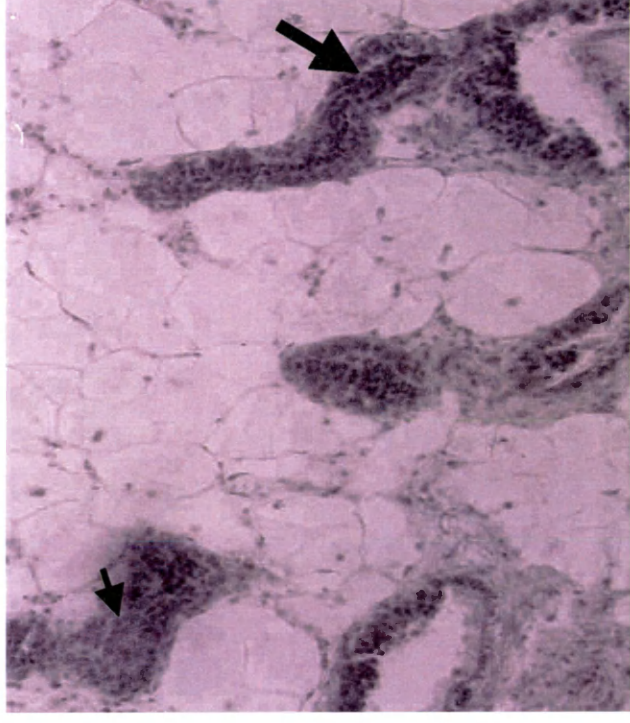
Plate VII. Early Active diploid female *C. virginica* at site TL in May 2002. New follicles begin to grow and are occupied by oogonia (large arrow) and undifferentiated gonia (small arrow). 100x
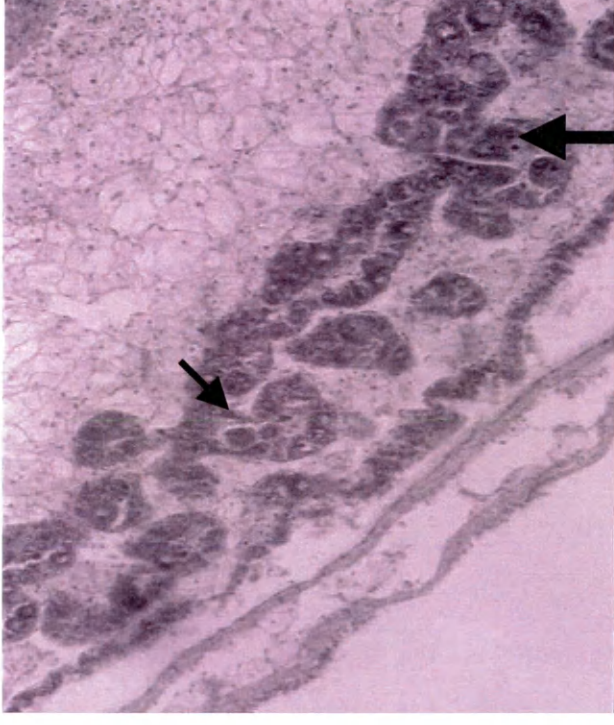
Plate VIII. Late Active diploid female *C. virginica* at site TL in August 2002. Follicles have filled the interfollicular space. Predominantly follicles contain mature oocytes (large arrow) yet some developing oocytes may remain (small arrow). 200x

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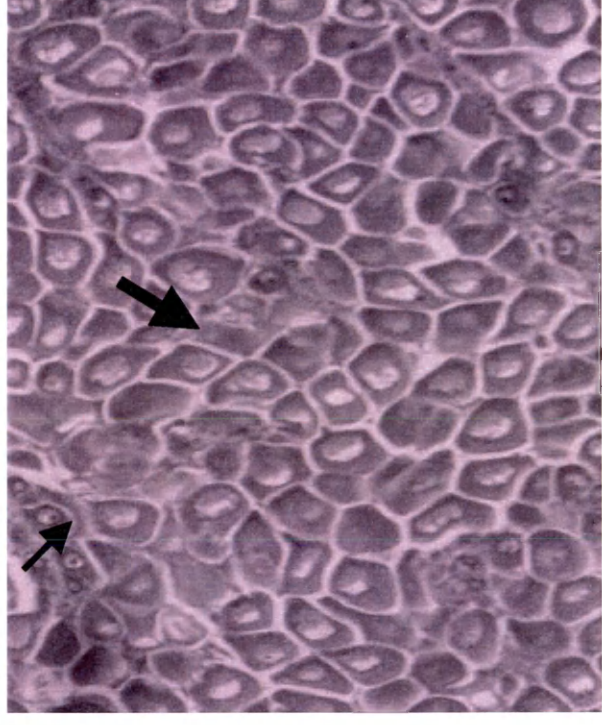
Late Active (Plate VIII): Many oocytes are mature and have reached maximum size. Some ova are free in the central lumen of follicles. Little to no interfollicular tissue remains.

Ripe (Plate IX): Most ova are free in the central lumen of follicles and cytoplasm is acidophilic. Few ova remain attached with peduncles to the basal membrane of follicles.

Spawn Partially: Most follicles are somewhat empty, primarily with mature ova free in the lumen or gonoducts. Interfollicular tissue is loosely organized. Follicle walls become thin and distended.

Spawn Completely (Plate X): Follicles are empty, distended, and thin-walled. Only a few mature ova remain in swollen gonoducts. Interfollicular tissue is disorganized.

Resorbing (Plate XI): Hemocytes invade remaining follicles, empty spaces, or gonoducts to begin phagocytosis of remaining ova.

Indifferent: Follicles are absent or collapsed. Hemocytes are absent. There is no apparent differentiation of gonia. Interfollicular tissue begins to reorganize. Typically sex at this stage is not distinguishable.

"New" Early Active (Plate XII): Indifferent gonads begin to differentiate giving rise to oogonia along the walls of small, immature follicles, indistinguishable from male follicles.

Hermaphrodites

Stages of maturity used to classify hermaphrodites were the same as outlined above. Male and female hermaphrodites (i.e., majority of the gonad is predominantly

Plate IX . Ripe diploid female *C. virginica* at site TL in July 2002. No interfollicular can be seen. Follicles contain mature oocytes (large arrow) and begin to empty into the swollen gonoduct (small arrow). 100x
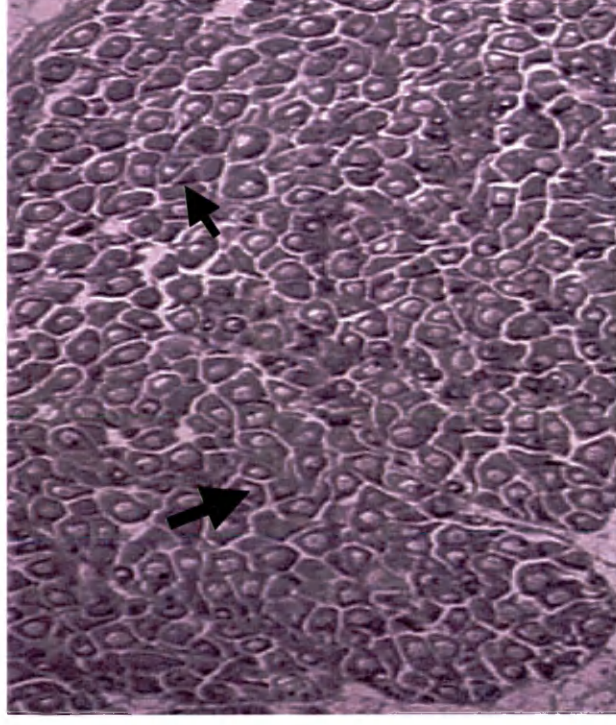
Plate X . Spawned diploid female *C. virginica* at site CS in August 2002. Follicles have collapsed and only residual mature oocytes remain in either remnant follicles (arrow) or gonoducts. 100x
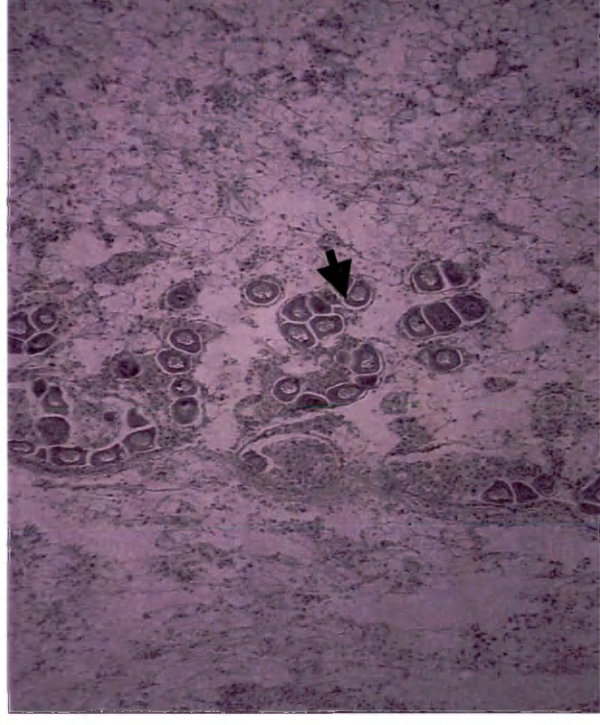
Plate XI . Resorbing diploid female *C. virginica* at site CS in October 2002. Follicles are invaded by hemocytes (small arrow) as mature oocytes (large arrow) are being digested. 200x
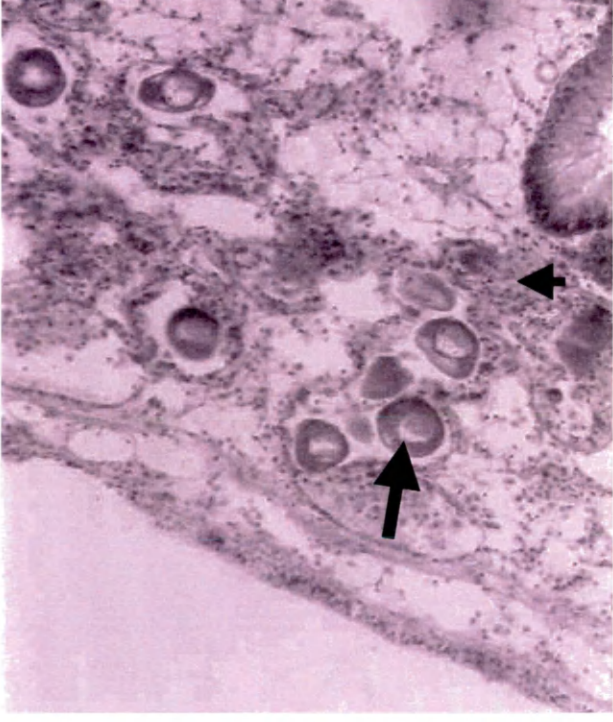
Plate XII . "New" Early Active diploid female *C. virginica* at site CS in December 2002. New immature follicles have begun forming with oogonia (large arrows) and undifferentiated gonia (small arrow). 100x
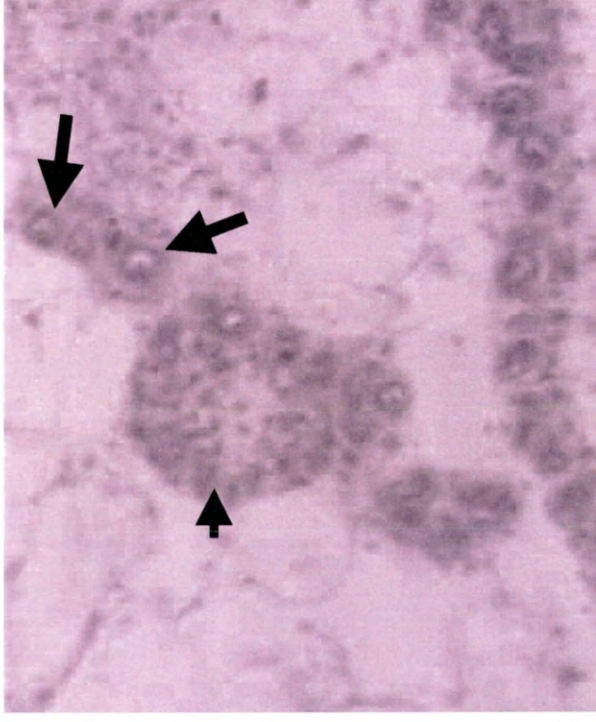
Plate XIII . Middle Active diploid hermaphrodite *C. virginica* at site TL in June 2002. Maturing follicles mostly contain developing oocytes (large arrow). Spermatogonia are found at the follicle walls (small arrows). 100x
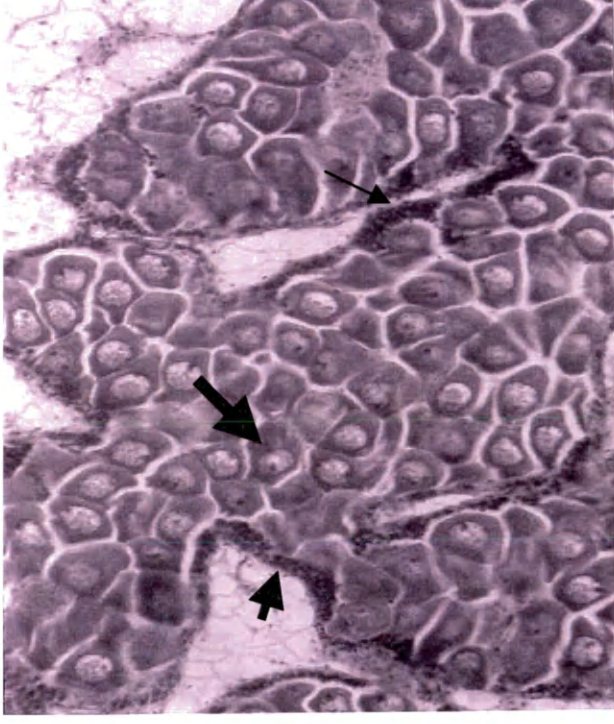
Plate XIV . Ripe diploid hermaphrodite *C. virginica* at site CS in July 2002. Developed follicles mostly with maturing oocytes (large arrow) and spermatids and spermatozoa (small arrow). 100x

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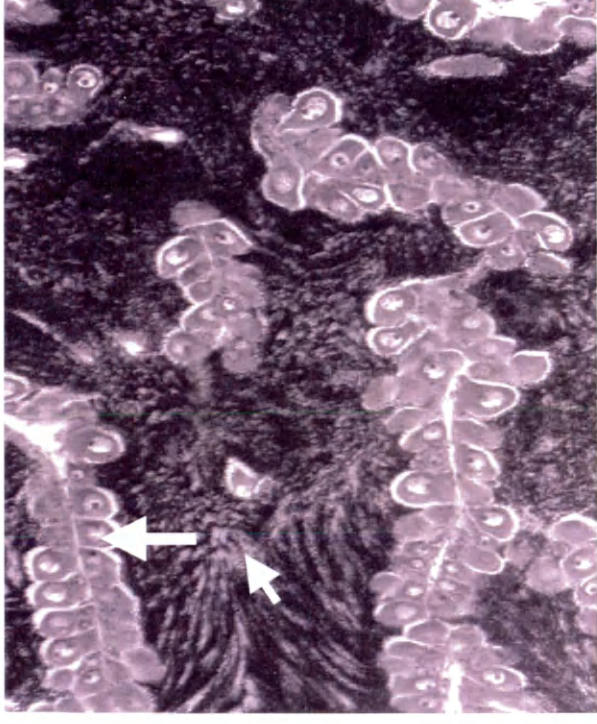
either male or female with the other sex also present; Plate XIII, Plate XIV) were

categorized according to the dominant kind of sex cells observed (Allen 1987).

Both masculine hermaphrodites (i.e., the majority of mature or developing sex

cells were male) and feminine hermaphrodites (i.e., the majority of mature or developing

sex cells were female) were observed in this study. Diploid hermaphrodites were rare

and triploid hermaphrodites were relatively more common.

Histology

Stages of development in diploid *C. virginica* and triploid *C. ariakensis* were

determined by paraffin histology, which also allowed assessment of disease prevalence.

Standard histology procedures for marine bivalves were followed according to the

NOAA Technical Memorandum NMFS/NEC-25, "Histological Techniques for Marine

Bivalve Mollusks" (1983) and Burreson *et al.* (1988). Transverse 4 to 6mm cross-

sections were taken just posterior to the labial palps for both *C. virginica* and *C.*

ariakensis. Individual sections were placed in labeled cassettes and fixed in Davidson's

Solution for 24 to 48 hours. Due to the large size of some oyster sections, up to a 72-

hour fixation period was necessary. After fixation, cassettes were placed in 70% ethanol

for at least 24 hours. Tissues were then dehydrated in 95% ethanol for 25 minutes prior

to processing through the Hypercenter XP tissue processor (Shandon Instruments,

Pittsburgh, PA). Tissues were transferred to the Tissue-Tek embedding station (Miles

Scientific, Inc., Naperville, Ill) and processed tissue was individually embedded in

paraffin using labeled embedding rings. Paraffin blocks were sectioned at 4 to 6 microns

using the Olympus Cut-4055 Microtome (Olympus America, Inc). Sectioned tissue

ribbons of paraffin were floated in a heated water bath of 42°C. A clean slide was used

for one to several oyster cross-sections depending on size. The slides were then stained

with Harris' hematoxylin and eosin stains using Varistain Gemini (Thermo Shandon,

Pittsburgh, PA). To preserve the integrity of the stained slides, a coverslip was mounted

using a coverslip-mounting medium.

Disease examination by histology

Diseases endemic to the Bay have been reported to cause high mortality in *C.*

virginica (Haskin and Andrews 1988; Andrews 1988) but *C. ariakensis* appears to be

more resistant or tolerant (Calvo *et al.* 2001). Prevalence of *H. nelsoni* and *P. marinus*

was examined in diploid and triploid oysters. Historically, the heaviest months of *P.*

marinus and *H. nelsoni* infections are July, August, and September (Ragone Calvo and

Burreson 1999). Histological preparations made for gametogenesis were used for

determining *H. nelsoni* infections according to Burreson *et al.* (1988). *P. marinus* was

diagnosed using RFTM assay and infection intensity determined following criteria from

Ray (1954) and Mackin (1962).

Reversion (*C. ariakensis* only) by flow cytometry (FCM)

Reversion is defined as triploid cells that lose chromosomes, reverting most often

to a diploid condition. The result is a mosaic oyster that has both diploid and triploid

cells. Examination of reversion in crossbred triploid *C. ariakensis* was conducted by

FCM using a Partec CCAII bench top model (Munster, Germany). FCM is a technique

that determines fluorescence intensity of stained samples. Before testing by FCM, tissue

samples are stained with the fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI).

DAPI bonds to nucleic acids in direct proportion to the DNA content. FCM measures this DNA content when ultra-violet light causes the DAPI to illuminate. FCM gives a graphic output of discrete peaks with fluorescence units (intensity) on the X-axis that corresponds to relative DNA content. The Y-axis is the frequency of stained cells at a given intensity. The area under discrete peaks allows determination of relative percentage of cells by ploidy.

Hemolymph Biopsy

Hemolymph was extracted from every triploid oyster sampled to confirm triploidy. A Dremel™ tool was used to notch the triploid oysters just next to the adductor muscle. A syringe, 23G (1½ inches) needle, was inserted and approximately 0.1 ml of hemolymph was removed and placed in a microcentrifuge tube containing 1 ml DAPI. The DAPI-hemolymph samples were read immediately after being aspirated several times with a syringe to disaggregate cells and break cell membranes, then filtered, and finally placed in a test tube specifically designed for the Partec CCA-II. Both DAPI and samples were kept on ice during analysis. If reversion was detected in the hemolymph sample, then adductor, heart, gill, and gonad tissues were sampled to determine the level of reversion in each. The same biopsy procedure outlined above was used for all tissue samples.

Statistical Analyses

This research was largely descriptive, limiting statistical analyses. Statistical procedures were performed in MINITAB, SPSS, and SAS.

Growth

Shell heights and whole wet weights were plotted for diploid and triploid oysters at each site. Correlations were tested for significance using Spearman's Rho and Pearsons correlation. As an overall comparison, Wilcoxon Signed Rank tests were used to examine the percent shell height and wet weight difference in diploid and triploid oysters.

Disease

Refer to Table 3 for numerical transformation of disease results. A ranking of no parasites (none or 0) to very heavy infections (very heavy or 8) was assigned to each oyster for *H. nelsoni* and *P. marinus*. Prevalence and weighted prevalence were calculated for both diseases at each site and sampling. Disease prevalence was plotted for each species at each site. 'Infected' and 'uninfected' diploid and triploid oysters were tested for significant differences using Chi-square tests.

Reversion

The frequency of percent diploid (reverted) cells was calculated using the distribution from the FCM screen by dividing the area of the diploid peak by the total area of both the diploid and triploid peaks. Reversion by tissue type was plotted for each mosaic oyster. The number of mosaic oysters through time was plotted for each sampling period. Tissue samples from hemolymph and gonad were plotted to determine the extent of correlation.

Table 3. Transformation of variables for graphical representation and statistical analysis. Note there is not a "sex" that is transformed to zero. Range of categories for gametogenesis is 0-9; Dermo intensity is 0-8; MSX intensity is 0-4; Sex is 1-4.

Numerical category	Gametogenesis	Dermo intensity	MSX intensity	Sex
0	Inactive	none	none	
1	Early Active	rare	rare	male
2	Middle Active	very light	light	female
3	Late Active	light	moderate	hermaphrodite
4	Ripe	light to moderate	heavy	unknown
5	Spawn Partially	moderate		
6	Spawn Completely	moderate to heavy		
7	Resorbing	heavy		
8	Indifferent	very heavy		
9	"New" Early Active			

Gametogenesis

Gametogenesis categories were transformed (Table 3) to numerical categories (0-9) according to the stage of development for individual oysters. Graphically, mean gametogenesis across each sampling interval was compared between diploid and triploid oysters for each site. The difference between the two means, termed gametogenesis lag, was determined for the species at each site during each sampling.

Sex Ratio / Hermaphroditism

The sex of individual oysters was determined by histology. Overall triploid and diploid sex ratios (male:female:hermaphrodite:unknown) at each site was tested using Chi-square analysis. Male:female:hermaphrodite ratios were tested for triploid oysters at each site and male:female sex ratio was tested for differences in each species and at each site using Chi-square analysis.

RESULTS

Site characterizations

TM was located in Tom's Cove, Chincoteague, Virginia and mean salinity was 35 ppt (Table 4). Grow-out methods at TM consisted of floats and off-bottom cages. The surrounding area was tidal mud flat with a maximum depth at high tide of approximately 6 feet. Oysters were sampled from both systems.

JH was located in Foley's Creek, Accomac, Virginia and mean salinity was 33 ppt (Table 4). Grow-out consisted of floats. The area was a small creek system that was approximately 30 feet wide and 15 feet deep.

CS was located in Cherrystone Creek, Cape Charles, Virginia and mean salinity was 28 ppt (Table 4). Grow-out consisted of off-bottom cages. The area was characterized by a shallow (less than 5 feet at high tide) mud flat.

TL was located in York River, Yorktown, Virginia and mean salinity was 25 ppt (Table 4). Grow-out consisted of off-bottom cages. The area was an open river embankment that was approximately 10 feet deep at high tide.

TK was located in Corrotoman River, Weems, Virginia and mean salinity was 21 ppt (Table 4). Grow-out consisted of hanging bags suspended from the dock. The area was an open river embankment that was approximately 15 feet deep at high tide.

BO was located in Yeocomico River, Kinsale, Virginia and mean salinity was 17 ppt (Table 4). Grow-out included off-bottom cages and then floats. The area was a wide river system and a creek embayment that had maximum high tide depths of 20 feet and 10 feet, respectively. Oysters were sampled from both systems.

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C. virginica growth and disease

Table 4 shows the shell height measurement at first sampling (January) and shell height differences from first to last sampling (December) for all sites. Mean shell heights ranged from 37.3 mm at JH to 61.7 mm at BO at first sampling and the differences ranged from +15.7 mm (+25%) at BO to +25.5 mm (+60%) at TL. Mean shell height across all sites at first sampling was 47.4 mm and mean difference was +19.2 mm.

Table 4 also shows the wet weights at first sampling (January) and wet weight differences from first to last sampling (December) for all sites. At site BO wet weights were not taken in January and therefore the May wet weight measurement was used as the first sampling. First sampling for wet weights is May at BO, not January, and is therefore not used in first sampling calculations. Mean wet weights ranged from 1.0 g at JH to 2.6 g at CS at first sampling and the differences from –5.3 g (–38%) at BO to +4.6 g (+177%) at CS. Mean wet weight at first sampling was 2.1 g and mean difference was +1.4 g. Mean whole tissue weights across all sites fluctuated by sample date (Figure 2). In January diploids weighed 2.1 g, reached a peak in June at 6.5 g, decreased to 3.6 g in August, and subsequently increased to 5.8 g by December.

Four of the five sites (TM, JH, CS, TL) had positive shell height and wet weight differences. However, one site (BO) had a negative wet weight difference (-5.3) over time.

Mean diploid shell heights versus wet weights were plotted to examine correlations at each site (Figures 3-5). Confidence intervals (95% CI) and predicted confidence intervals (PI from the regression equation) are included for each graph. TM (Figure 3) had a correlation coefficient (R^2) of 42.5%; JH (Figure 3)—59.2%;

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Table 4. Summary of overall growth in *C. ariakensis* and *C. virginica* from first to last sampling at all sites and for both species. Increase or decrease in initial shell height (mm) and whole wet weight of tissues (g) and mean salinity (ppt) at each site. Percents in parentheses. Mean is given for the percent difference in shell height and whole wet weight. na=not available. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

site	salinity	Triploid *C. ariakensis*				Diploid *C. virginica*			
		starting sh. ht.	difference (%)	starting wet wt.	difference (%)	starting sh. ht.	difference (%)	starting wet wt.	difference (%)
TM	35	96.5	+29.7 (31)	20.6	+17.1 (83)	48.1	+15.9 (33)	2.4	+2.5 (104)
JH	33	133.7	+5.5 (4)	33.3	+4.6 (14)	37.3	+20.7 (55)	1.0	+2.3 (230)
CS	28	85.8	+18.2 (21)	21.0	+23.6 (112)	47.4	+18.1 (38)	2.6	+4.6 (177)
TL	25	78.3	+46.4 (59)	19.8	+27.5 (141)	42.6	+25.5 (60)	2.5	+2.7 (108)
TK	21	95.8	+24.6 (26)	35.4	+2.9 (8)	na	na	na	na
BO	17	85.7	+17.6 (21)	32.1	-2.7 (-8)	61.7	+15.7 (25)	13.7	-5.3 (-38)
Mean			27%		58%		42%		116%
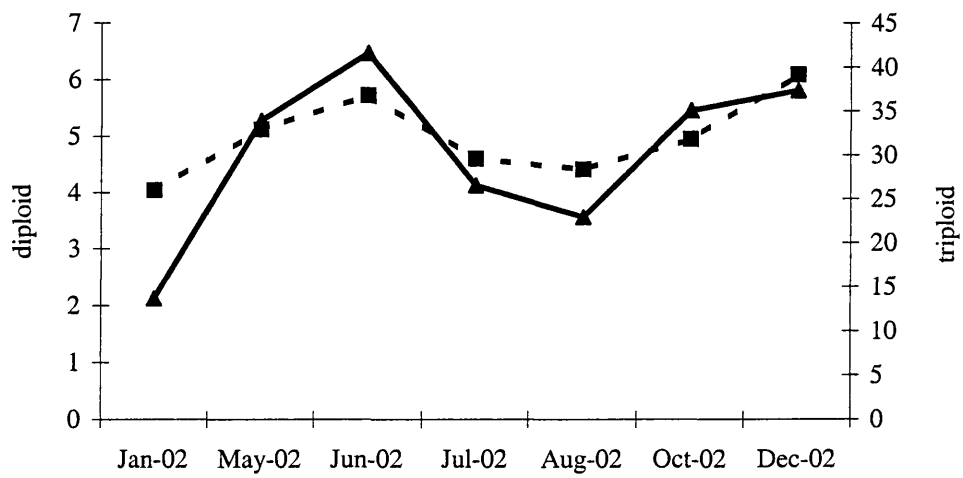
Figure 2. Seasonal mean whole wet tissue weight (in grams) for diploid *C. virginica* and triploid *C. ariakensis* by sample date. Solid line=diploid; dashed line=triploid.
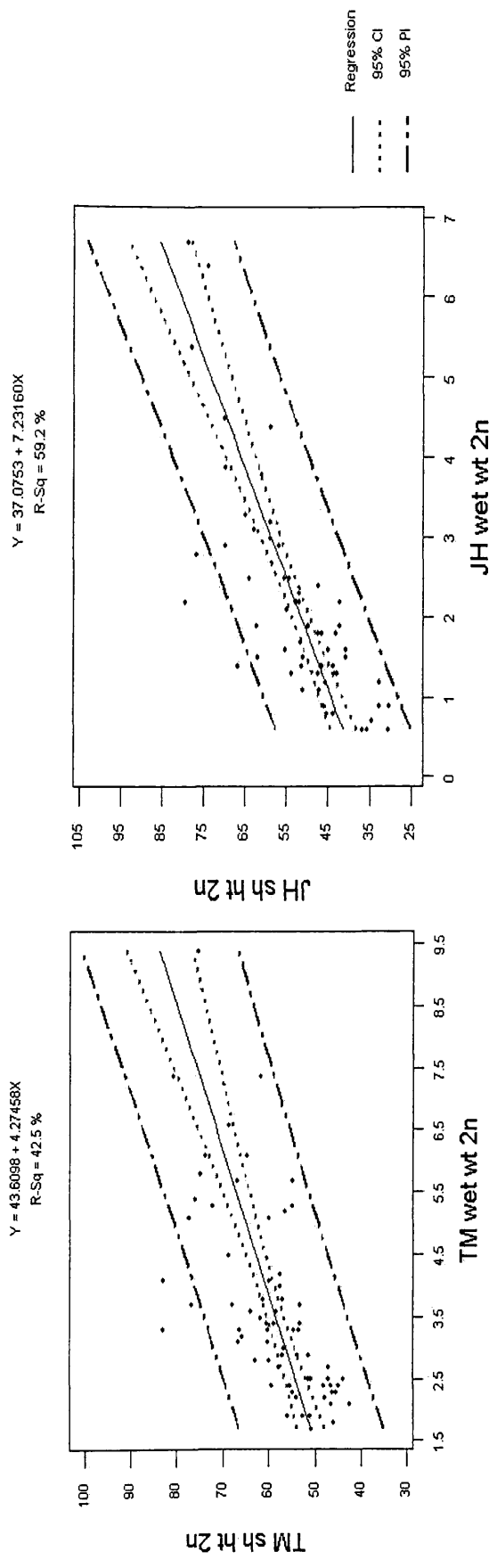
Figure 3. Diploid *C. virginica* growth. X-axis is shell height (mm) and Y-axis is whole wet weight (g). Equation and R-squared are included. CI=95% confidence interval; PI=predicted confidence interval based on linear regression equation. TM=Tommy Mason; JH=Jeff Hammer.
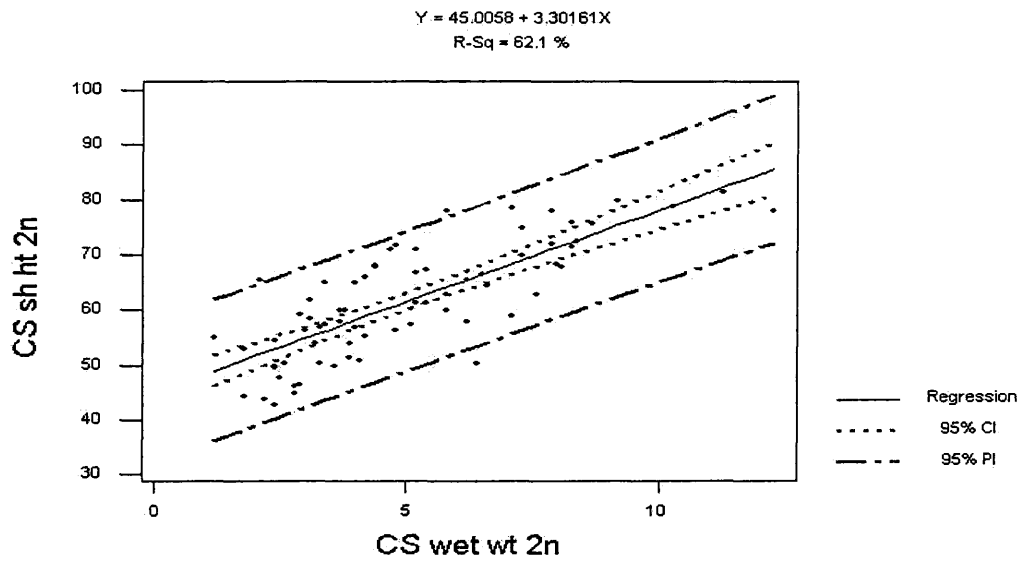
Figure 4. Plot of diploid *C. virginica* growth. X-axis is shell height (mm) and Y-axis is whole wet weight (g). Equation and R-squared are included. CI=95% confidence interval; PI=predicted confidence interval based on linear regression equation. CS=Cherrystone Aquafarms.

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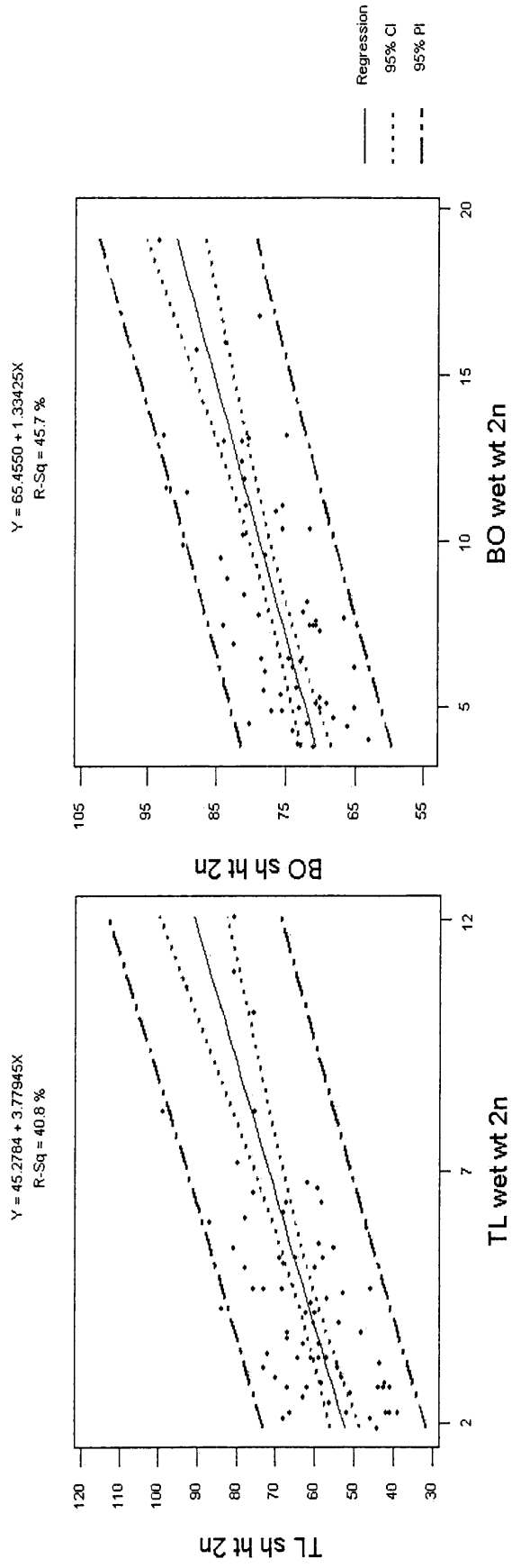
Figure 5. Plots of diploid *C. virginica* growth. X-axis is shell height (mm) and Y-axis is whole wet weight (g). Equation and R-squared values are included. CI=95% confidence interval; PI=predicted confidence interval based on linear regression equation. TL=Tommy Leggett; BO=Bevans Oyster Co.

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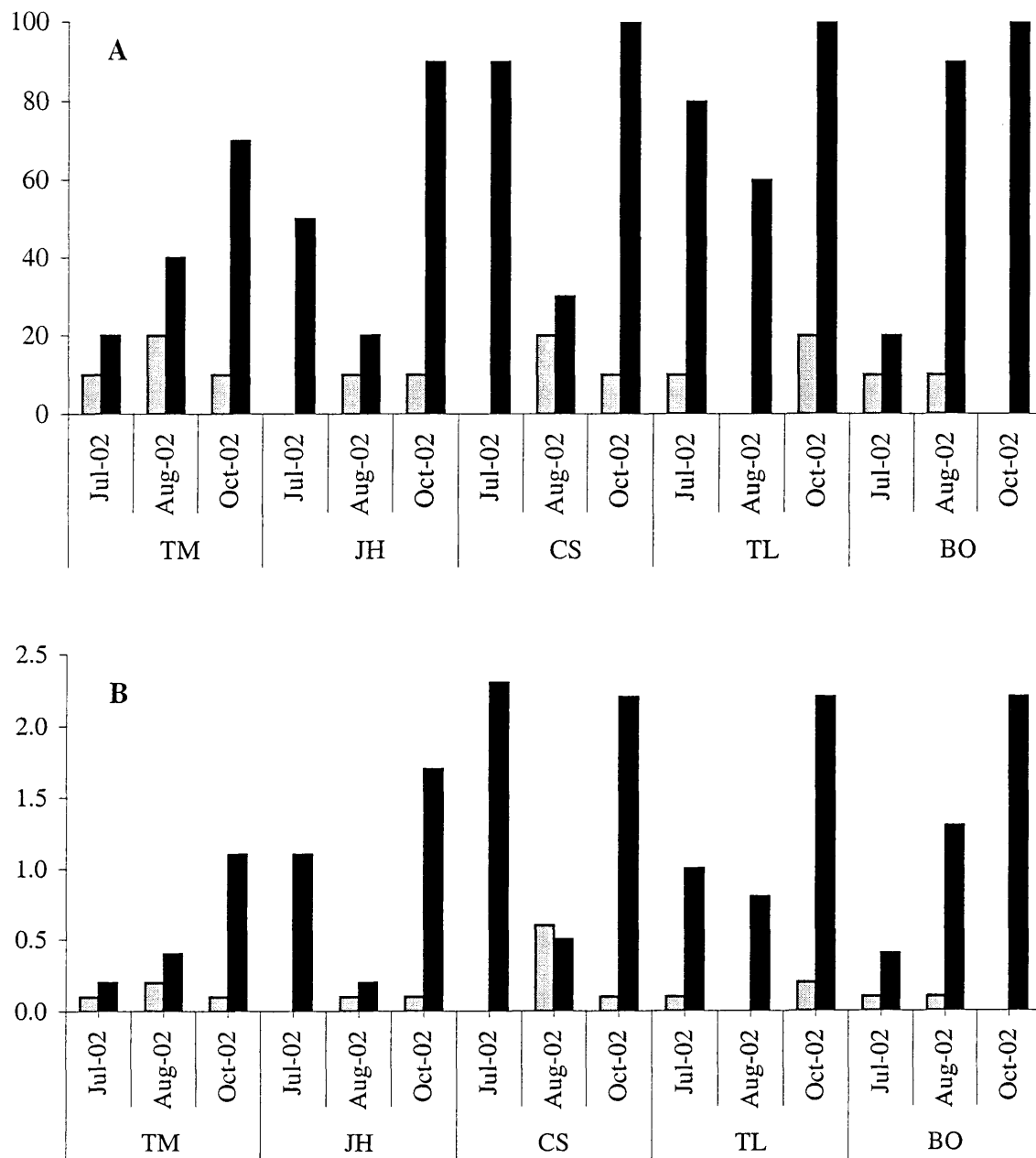
Figure 6. Disease prevalence (A) and weighted prevalence (B) in diploid *C. virginica* across all sites. Solid black bars indicate *Perkinsus marinus* (Dermo) and lightly shaded gray bars indicate *Haplosporidium nelsoni* (MSX). TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; BO=Bevans Oyster.

CS (Figure 4)—62.1%; TL (Figure 5)—40.8%; and BO (Figure 5)—45.7%. All linear

regressions for diploid shell height and wet weight show a positive correlation and were

significant (p<0.000).

Diseases, *H. nelsoni* and *P. marinus*, in *C. virginica* were calculated as prevalence

and weighted prevalence (Figure 6). Overall, *P. marinus* was more prevalent than *H.*

nelsoni in diploid oysters. *P. marinus* followed a general progression of increased

infection through the summer season with the highest prevalence occurring in October.

H. nelsoni, on the other hand, showed consistent low-level prevalence and weighted

prevalence across all sites and no increase with time.

Site mean prevalence for *P. marinus* ranged from 43% at TM to 80% at TL with a

mean of 64% across all sites. Infected animals were more common (93 out of 150

sampled—62%) than uninfected oysters. Site means for weighted prevalence of *P.*

marinus ranged from 0.57 at TM to 1.7 at CS with a mean of 1.2 across all sites.

P. marinus infection intensities, as described by Ray (1952, 1954), ranged from

rare to very heavy especially during October. All sites exhibited a wide range of

infection intensities over the sampling periods.

H. nelsoni prevalence ranged from 7% at JH and BO to 13% at TM with a mean

of 9% across all sites. Uninfected animals were more common than infected animals (14

out of 150—9%). *H. nelsoni* weighted prevalence ranged from 0.07 at JH and BO to 0.23

at CS with a mean of 0.12 across all sites.

C. ariakensis growth and disease

Shell heights and wet weights at first sampling (January) were compared against

the final measures (December) for each site and the differences were recorded in Table 4.

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Mean shell heights ranged from 78.3 mm at TL to 133.7 mm at JH at first sampling and the differences ranged from +5.5 mm (+4%) at JH to +46.4 mm (+59%) at TL. Mean shell height at first sampling was 96.0 mm and mean difference was +23.7 mm.

Mean wet weights ranged from 19.8 g at TL to 35.4 g at TK at first sampling and the differences from –2.7 g (– 8%) at BO to +27.5 g (+141%) at TL. Mean wet weight at first sampling was 27.0 g and mean difference was +12.2 g. Mean whole tissue weights across all sites fluctuated according to sampling (Figure 2). In January triploid oysters weighed 26.0 g, reached a peak in June at 36.8 g, decreased to 28.4 g in August, and subsequently increased to 39.2 g by December.

Five of the six sites (TM, JH, CS, TL, TK) showed positive shell height and wet weight differences, however, one site (BO) exhibited a negative wet weight difference (-2.7) over time.

Mean triploid shell heights versus wet weights were plotted to examine correlations at each site (Figures 7-9). TM (Figure 7)—64.0%; JH (Figure 7)—2.9%; CS (Figure 8)—42.6%; TL (Figure 8)—32.1%; TK (Figure 9)—44.3%; and BO (Figure 9)— 39.2%. All linear regressions for triploid shell height and wet weight were significant ($p<0.000$) with shell height and wet weight positively correlated in each case.

Disease infections in triploid oysters were plotted as prevalence and weighted prevalence (Figure 10) as in diploid oysters. In general, *P. marinus* was detectable at some sites in low prevalence but altogether absent at other sites. *H. nelsoni* was absent at all sites. TM and JH sites were completely disease free throughout sampling. CS maintained low level *P. marinus* prevalence ranging from 4-12% with a mean of 9%.
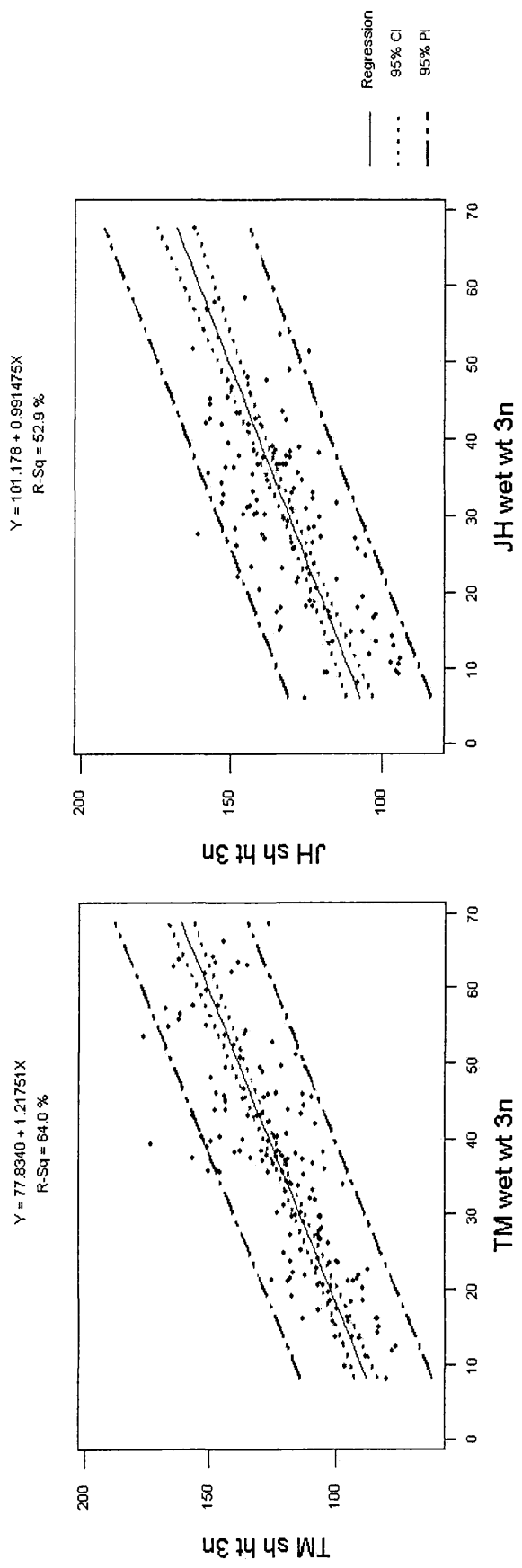
Figure 7. Plots of triploid *C. ariakensis* growth. X-axis is whole wet weight (g) and Y-axis is shell height (mm). Equation and R-squared values are included. CI=95% confidence interval. PI=predicted confidence interval based on linear regression equation. TM=Tommy Mason; JH=Jeff Hammer.

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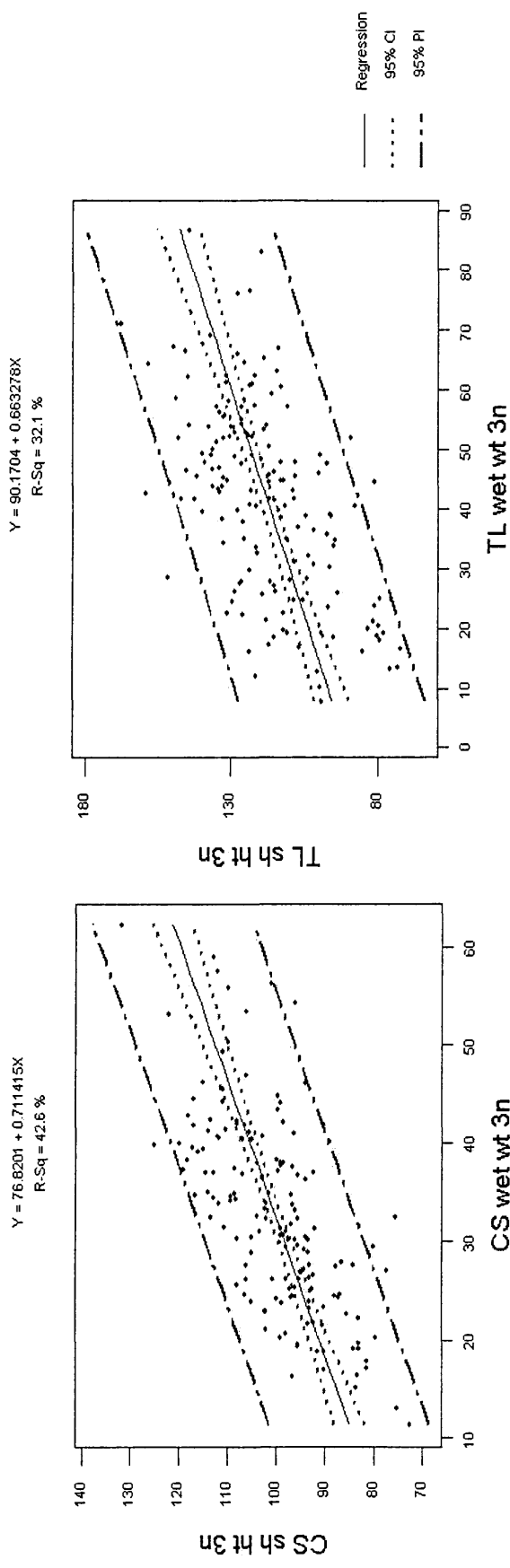
Figure 8. Plots of triploid C. ariakensis growth. X-axis is whole wet weight (g) and Y-axis is shell height (mm). Equation and R-squared values are included. CI=95% confidence interval. PI=predicted confidence interval based on linear regression equation. CS=Cherrystone Aquafarms; TL=Tommy Leggett.

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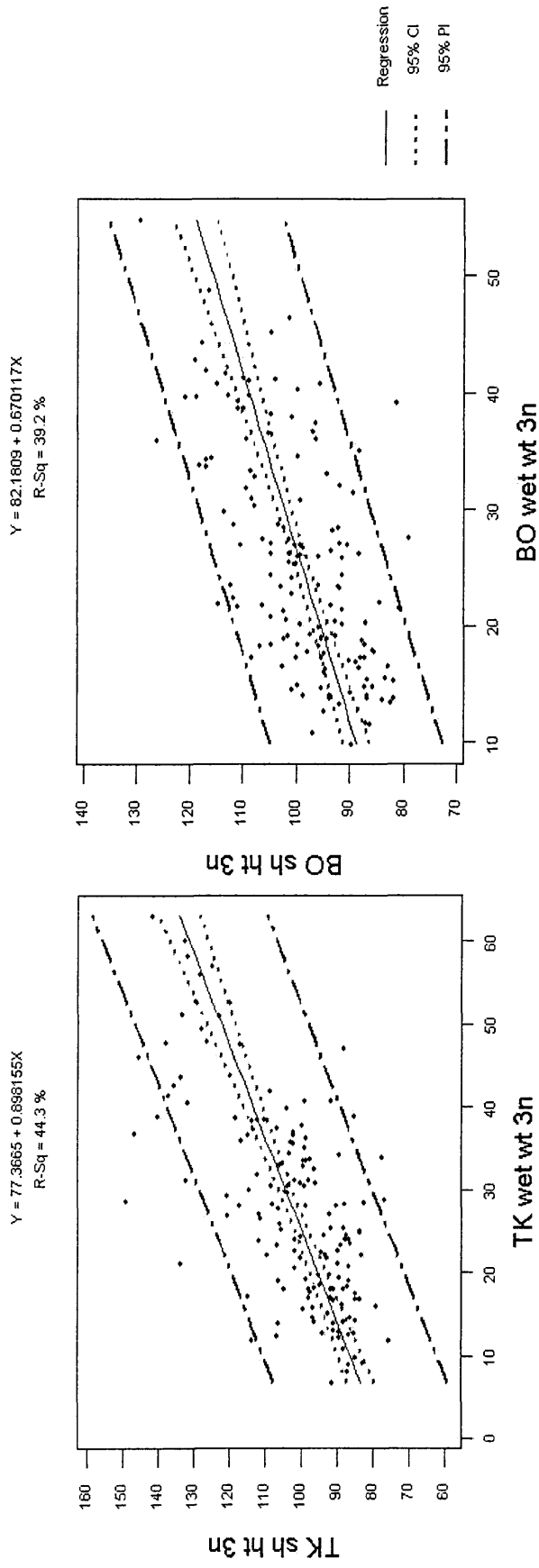
Figure 9. Plots of triploid *C. ariakensis* growth. X-axis is whole wet weight (g) and Y-axis is shell height (mm). Equation and R-squared values are included. CI=95% confidence interval. PI=prediced confidence interval based on linear regression equation. TK=Tommy Kellum; BO=Bevans Oyster Co.

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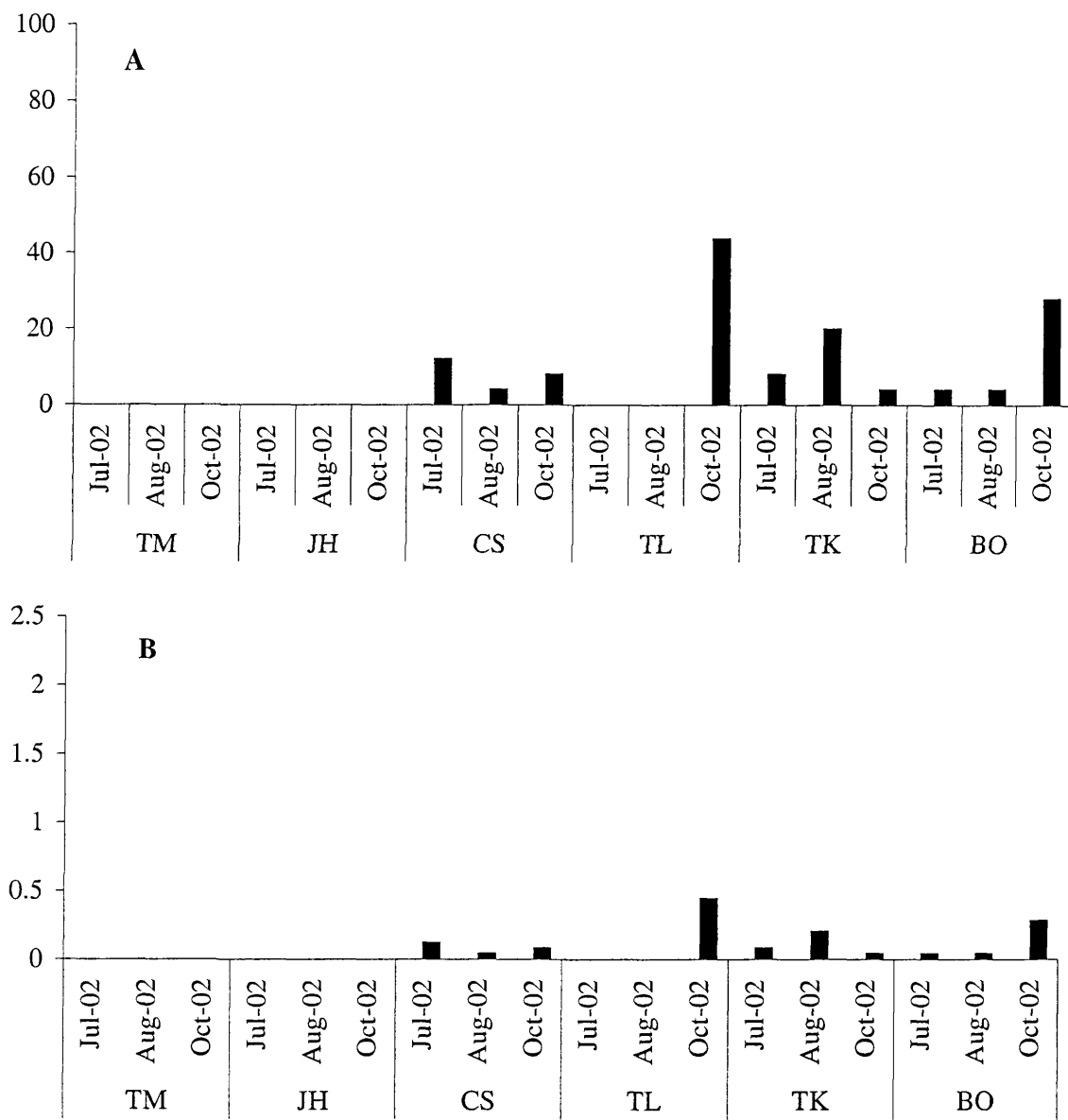
Figure 10. Disease prevalence (A) and weighted prevalence (B) in triploid *C. ariakensis* across all sites. Solid black bars indicate *Perkinsus marinus* and lightly shaded gray bars indicate *Haplosporidium nelsoni*. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; BO=Bevans Oyster.

TL had the highest *P. marinus* infection (44%) during October sampling. The July and

August sampling had 0% infection. TK maintained a low level *P. marinus* infection (4-

20%) with a mean of 11%. BO had low *P. marinus* infections in July and August (4% for

both), but in October the prevalence increased to 28%.

The highest salinity sites TM and JH had no disease infections that were

detectable. The medium and lower salinity sites, CS, TL, TK, and BO, had low level

infections that generally increased during October. All triploid oysters that had infections

exhibited rare to very light infection intensities. Across all sampling periods and sites 34

out of 448 triploids (2.9%) had at least one parasite present.

Overall comparison of *C. ariakensis* and *C. virginica*

Growth

Table 4 shows that *C. virginica* outgrew *C. ariakensis* in terms of relative gain in

whole wet weight and shell height difference across all sites except TL. At TL, the

percent whole wet weight difference in triploid oysters was 141% compared to 108% for

diploid oysters while the percent shell height difference was essentially equal: 59% in

triploid oysters compared to 60% in diploid oysters. The mean percent difference in

whole wet weight from first to last sampling was 58% for *C. ariakensis* and 116% for *C.

virginica*. The mean percent difference in shell height from first to last sampling was

27% in *C. ariakensis* and 42% in *C. virginica*. A Wilcoxon Signed Ranks test revealed

that the percent difference in shell height for diploid oysters was marginally significant

(Z score= -2.023; p=0.043) compared to triploid oysters. Essentially, diploid oysters at

all five sites (TM, JH, CS, TL, BO) were positively ranked higher than triploid oysters.

On the other hand, the percent difference in whole wet weight for diploid oysters was not

significantly higher than triploid oysters at all sites (Z score= -0.674; p=0.50).

Specifically, diploid oysters at three sites (TM, JH, CS) were ranked higher than triploid

oysters and diploid oysters at two sites (TL and BO) were ranked lower than triploids.

Disease prevalence

Because infections were lower than expected, disease categories were combined

to analyze the data. Each oyster was classified as 'infected' or 'uninfected'. Across all

sites, *P. marinus* infections in *C. virginica* were significantly different than *C. ariakensis*

(X^2=183.8, p<0.0001). *H. nelsoni* infections in *C. virginica* were also significantly

different than *C. ariakensis* (X^2=35.7, p<0.0001).

Disease prevalence by site

P. marinus infections in *C. virginica* were significantly different compared to *C.*

ariakensis at TM (X^2=36.6, p<0.0001), JH (X^2=46.1, p<0.0001), CS (X^2=46.8,

p<0.0001), TL (X^2=41.2, p<0.0001), and BO (X^2=25.8, p<0.0001).

H. nelsoni was absent in *C. ariakensis* and very low in *C. virginica*, therefore

differences were marginally significant: TM (X^2=10.3, p=0.0014), JH (X^2=4.9,

p=0.0259), CS (X^2=7.7, p=0.0055), TL (X^2=7.7, p=0.0055), and BO (X^2=5.1, p=0.024).

C. virginica gametogenesis

Gametogenesis was largely descriptive in this research, however categorical

stages were assigned numerical scores for comparisons. Gametogenesis categories

(=maturity state) (Table 3) were transformed to numeric values ranging from 0 to 9

(Inactive to "New" Early Active). A score of 0 indicated inactive, indistinguishable

gonad most commonly observed during January. A score of 9 indicated the gonad had

completed the gametogenic cycle for the reproductive season and "new", undeveloped

and immature follicles had emerged (see Materials and Methods for full category

description). Typically, an oyster with a score of 9 was found in December or January.

Figure 11A shows the mean gametogenesis of diploid (2n) *C. virginica* across all

sites plotted against sampling date. Histology revealed that diploid oysters exhibited

gradual maturation during the summer. *C. virginica* developed a uniform number of

follicles over the reproductive season, each containing a gradient of sex cell types.

January sampling was characterized by inactive or "new" early active gonad in which

gender was generally indistinguishable. Follicles that were visible had little growth and

gonia were undifferentiated. Developing oysters were found throughout May and June

with early, middle, and late active gonads. Males had follicles that were becoming well

branched with spermatids differentiating and spermatozoa radially aligned in the lumen.

Females contained follicles that had developing oocytes at the wall and mature ova free

in the lumen. By August, most diploid oysters had spawned. This was characterized by

complete release of all mature and developed gametes. In October gonads were in the

process of resorbing unspawned gametes. The majority of oysters sampled in December

showed either Indifferent or "New" Early Active gonad. The diploid gametogenesis

cycle is reflected in the whole tissue weight fluctuation (Figure 2). A build-up of gonad

occurs during the early spring, then release of that gonad in late summer, and finally a

rebuilding of somatic tissue, overwintering immature gonad for the following spring.
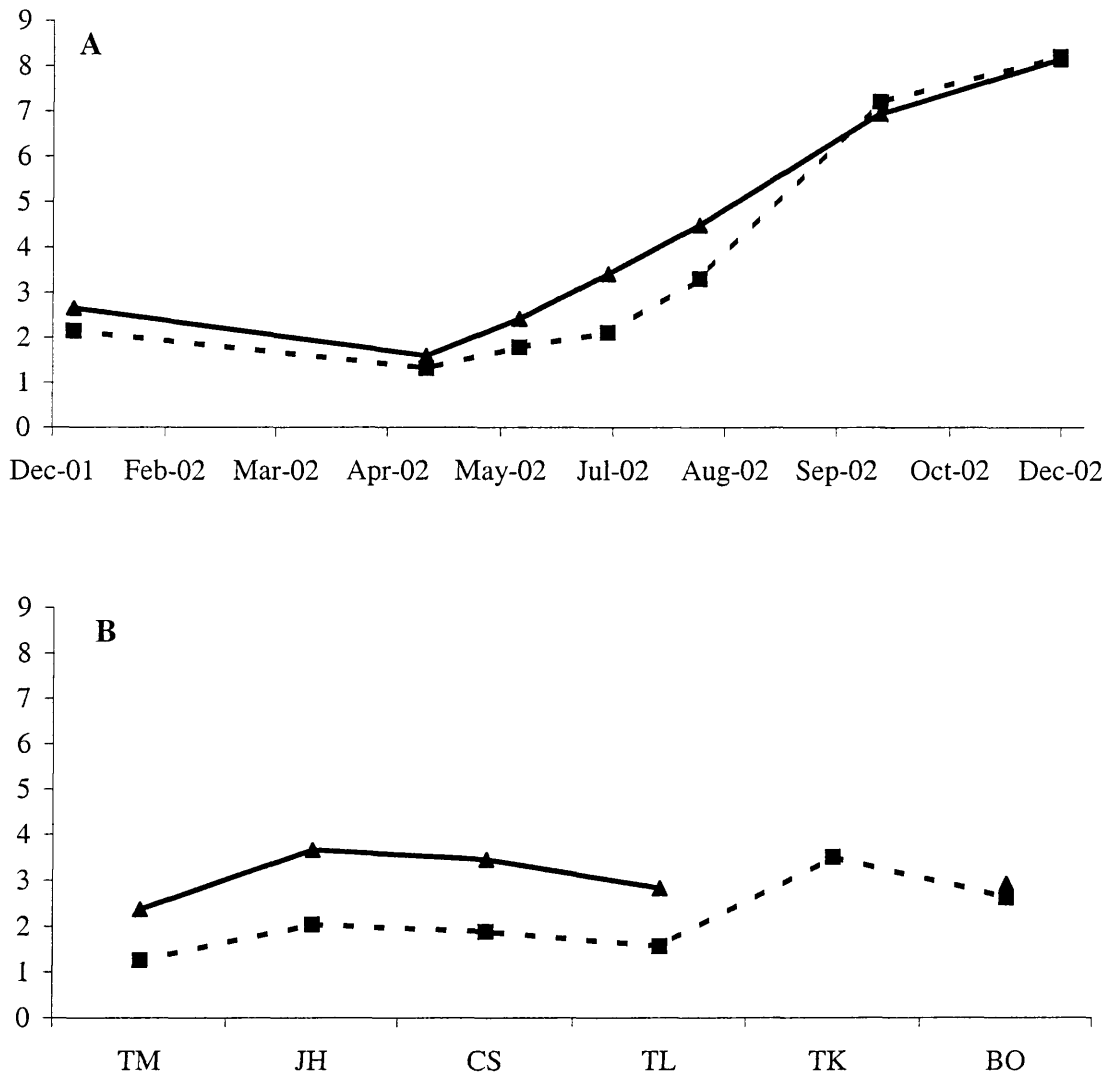
Figure 11. Plots of mean diploid *C. virginica* and triploid *C. ariakensis* gametogenesis across all sites for each sampling date (A), and for the reproductive season, May to August (B) at each site in reverse order of salinity. Solid line=diploid; dashed line=triploid. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co. Gametogenesis categories are as follows: 0=Inactive; 1=Early Active; 2=Middle Active; 3=Late Active; 4=Ripe; 5=Spawn Partially; 6=Spawn Completely; 7=Resorbing; 8=Indifferent; 9="New" Early Active.

Gametogensis at Tommy Mason

Figure 12 shows gametogenesis of diploid *C. virginica* throughout the reproductive season at site TM. In January, all oysters were Inactive. In May, six oysters were Inactive, three were Early Active with immature follicles and spermatogonia beginning to differentiate into spermatocytes. One oyster had matured to Middle Active and contained few spermatids. In June two oysters were Inactive, one was Early Active, three were Middle Active, three were Late Active, and one oyster had matured to Ripe. By July one oyster remained Early Active, two animals were Middle Active, five oysters were Late Active, and two were Ripe. In August, three oysters remained Late Active, three were Ripe, and four exhibited signs of spawning. In October, spawning was apparently complete as six oysters were Spawned, one was Resorbing, and three had Indifferent gonad. The final sampling in December had six oysters that were Resorbing, one was Indifferent, and three had begun redeveloping gonad and were categorized as "New" Early Active.

Gametogenesis at Jeff Hammer

Figure 13 shows gametogenesis in diploid oysters for the reproductive season at site JH. In January, one oyster was still Resorbing gametes, six oysters were Inactive, and three had "New" Early Active gonad. In May, six oysters were still Inactive, and four were Early Active with newly branching follicles. June samples were not collected due to inaccessibility of the site. By July, one animal was Middle Active, three were Late Active, one was Ripe, three had Spawned, and two were Resorbing gametes. In August, one oyster remained Late Active, two were Ripe, five had Spawned, and two were Resorbing. In October, one animal was still Ripe, one had Spawned, six were Resorbing,

Cv 2n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA						UUU	UUU
ID						U	U
RE						MMMFFF	UUUUUUU
SO					M		
SP					MMF		
R		M	M	MF	MMM		
LA		MMM	MMM	MMMFF	MFF		
MA		MFF	MFF	FF			
EA		UUUUU	F	M			
IA	UUUUUUUUU	U	UU				

Ca 3n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA							UUUUUUUUUU
ID						UUUUU	UUUUUUUU
RE						MMMFUUUU	MUUUUUU
SO						MMMMMMMFUU	
SP				FF	MFFFF		
R				FF	FF		
LA					MMMFF		
MA			MFHH	FFFFFHH	FFFFFF		
EA	M	FFFFFFFFFFMM	MMHHHHHHH	MFFFFFHH	MFHH		
IA	UUUUUUUU	UUUUUUFFMM	FFFFFFFFFFFF	FFFFFFF	FFFF		

Figure 12. Progression of diploid *C. virginica* (Cv 2n) and triploid *C. ariakensis* (Ca 3n) gametogenesis throughout this study at Tommy Mason's site. Sex is indicated by M=male; F=female; H=hermaphrodite; U=unknown. Gametogenesis categories are as follows: "N"EA=New Early Active; ID=Indifferent; RE=Resorbing; SO=Spawned Out; SP=Spawn Partially; R=Ripe; LA=Late Active; MA=Middle Active; EA=Early Active; IA=Inactive.

Cv 2n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA	UUU						FFUUU
ID				UU		UU	UUU
RE	M			FU	FU	MUUUU	UU
SO					MMF	MMF	
SP				M	MM	M	
R				R	MM	M	
LA			MMM	MMM	F		
MA		MFFF	M	M			
EA		UUUUU					
IA	UUUUUU	U					

Ca 3n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA	UUUU						MMMMFUUUUU
ID						UUUUUUUUUU	UUUUUUUUUUUU
RE				F	H	MMFUUUUUU	U
SO				F	MM	MM	
SP				MMMMF	MMMMMFH	M	
R				MMFFHHHHH	F		
LA				MMM	MMMMMM		
MA		MMF		FFFF	MMMF		
EA		MMMMMMFF					
IA	UUUUUU	MFFFFFFFUUUU			FFFF		

Figure 13. Progression of diploid *C. virginica* (Cv 2n) and triploid *C. ariakensis* (Ca 3n) gametogenesis throughout this study at Jeff Hammer's site. Sex is indicated by M=male; F=female; H=hermaphrodite; U=unknown. Gametogenesis categories are as follows: "N"EA=New Early Active; ID=Indifferent; RE=Resorbing; SO=Spawned Out; SP=Spawn Partially; R=Ripe; LA=Late Active; MA=Middle Active; EA=Early Active; IA=Inactive.

and two were Indifferent. Lastly, in December two oysters were Resorbing gametes,

three were Indifferent, and five were "New" Early Active.

Gametogenesis at Cherrystone Aquafarms

Refer to Figure 14 for gametogenesis in diploid oysters at site CS. In January,

nine oysters were Inactive, but one had developed "New" Early Active gonad. In May,

one oyster was Early Active, four were Middle Active, and five were Late Active. In

June, three diploid oysters were Middle Active, six were Late Active, and one was Ripe.

In July, one animal remained Middle Active, six were still Late Active, two were Ripe,

and one had Spawned. One oyster was a Ripe hermaphrodite. In the August sampling,

three oysters were Late Active, five had Spawned, and two were Resorbing. By October,

histology showed three oysters had Spawned, four were Resorbing, two were Indifferent,

and one was "New" Early Active. The December sampling showed one oyster had

Spawned, five were Indifferent, and four were "New" Early Active.

Gametogenesis at Tommy Leggett

Figure 15 shows the progression of gametogenesis throughout the reproductive

season at site TL. In the January sampling, six diploid oysters were Inactive, and four

were "New" Early Active. By May, one oyster was Early Active, five were Middle

Active, and four were Late Active. In June, five oysters were Middle Active, two were

Late Active, and three were Ripe. Two oysters were Middle Active hermaphrodites. By

July, two animals were Middle Active, seven were Late Active, and one was Ripe. In the

August sample histology revealed two oysters that remained Early Active, two were

Middle Active, one was Late Active, three were Ripe, one had Spawned, and one was

66

Cv 2n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA	U					M	MFFF
ID						UU	FFUUU
RE					FU	FUUU	
SO				M	MFFF		M
SP					F	FFF	
R			M	MF			
LA		MMFFF	MMMFFF	MMMFFH	MMF		
MA		MMFF	FFF	FFF			
EA		M		H			
IA	UUUUUUUU						

Ca 3n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA	FU					UUUUUUUUUUUU	MMMMMUUUUUUUUUU
ID						UUUUUUUUUUUU	UUUUUUUUUU
RE		FF	FF	F	FFF		
SO						MM	
SP				FFF	FF		
R			F	FFF	FFFFF		
LA		MF	MFFFH	F	FF		
MA		FFH	MFFFFH		FH		
EA		FFFFH	FFFHH	M			
IA	UUUUUUUU	FFFFFFFFFF	FFFFFF	FFFFFFFFFFFFFFHHHH	FFFFFFFHHHH		

Figure 14. Progression of diploid *C. virginica* (Cv 2n) and triploid *C. ariakensis* (Ca 3n) gametogenesis throughout this study at Cherrystone Aquafarms site. Sex is indicated by M=male; F=female; H=hermaphrodite; U=unknown. Gametogenesis categories are as follows: "N"EA=New Early Active; ID=Indifferent; RE=Resorbing; SO=Spawned Out; SP=Spawn Partially; R=Ripe; LA=Late Active; MA=Middle Active; EA=Early Active; IA=Inactive.

Cv 2n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA	FFFF						MMMFU
ID						U	UU
RE					U	MMFFUUUU	FUU
SO							
SP					M	F	
R			MFF	F	MMF		
LA		MFFF	MF	MMFFFFF	M		
MA		MMMFF	MFFHH	MM	MM		
EA		F			MF		
IA	UUUUUU						

Ca 3n

	01/02	05/02	06/02	07/02	08/02	10/02	12/02
"N"EA							MMMMMMMMUUU
ID						UUUUUUUUUU	UU
RE						FUUUU	MMMUUUUU
SO					MF	MMMFFFFFU	MM
SP					M		M
R			FFF	FF	FF		
LA		MMMMMMM	FFH	FF	MFF		
MA		M	MFHH	FFFFH	MMMMHH		
EA		MFHHHHHHH	FFFFFFHHHH	FFFHHHHHH	MFFF		
IA	UUMUUUUUUUU	MFFFFFFF	FFFFFF	FFFFFFFF	FFFFHHU		

Figure 15. Progression of diploid *C. virginica* (Cv 2n) and triploid *C. ariakensis* (Ca 3n) gametogenesis throughout this study at Tommy Leggett's site. Sex is indicated by M=male; F=female; H=hermaphrodite; U=unknown. Gametogenesis categories are as follows: "N"EA=New Early Active; ID=Indifferent; RE=Resorbing; SO=Spawned Out; SP=Spawn Partially; R=Ripe; LA=Late Active; MA=Middle Active; EA=Early Active; IA=Inactive.

Resorbing. By October, one oyster had Spawned, eight were Resorbing, and one was

Indifferent. In the last sampling, December, three animals were Resorbing, two were

Indifferent, and five were "New" Early Active.

Gametogenesis at Tommy Kellum

Refer to Figure 16 for gametogenesis at site TK. No diploid *C. virginica* were

available for sampling during the 2002 reproductive season at this site (refer to Materials

and Methods).

Gametogenesis at Bevans Oyster

Refer to Figure 17 for gametogenesis at site BO. In January, four diploid oysters

were Inactive, and six were "New" Early Active. In the May sample, four animals were

Early Active, four were Middle Active, and one was Late Active. The June sample

showed two oysters were Inactive, one was Early Active, four were Middle Active, one

was Late Active, one was Ripe, and one had Spawned. In the July sample one animal

was still Inactive, one was Middle Active, three were Late Active, two were Ripe, and

three had Spawned. By August, three animals were Middle Active, six had Spawned, and

one was Resorbing gametes. In October, five diploid oysters were Resorbing, three were

Indifferent, and two had developed "New" Early Active gonad. The December sampling

revealed two oysters Resorbing gametes, three were Indifferent, and five developed

"New" Early Active gonad.

		01/02	05/02	06/02	07/02	08/02	10/02	12/02
Cv 2n	"N"EA							
	ID							
	RE							
	SO							
	SP							
	R							
	LA							
	MA							
	EA							
	IA							
Ca 3n	"N"EA	UU						MMMMMMUUUU
	ID						UUUUU	UUUUUU
	RE					FF	UUUUUUUU	MMUUUUU
	SO			M	MMMMMHHHH	MMMMMMFF HHUUU	MMMMMM UUU	
	SP			FHH	MMMMM	MMMFHH		
	R		M	MMMHHHH	MMF	F		
	LA		MMMMMMFHH	MMMMMHH	MF	M		
	MA		MMMMMMHHHH	MMFH	MF			
	EA	MMM	H	F	MH			
	IA	UUUU	FFF	FF	FFHH	FF		

Figure 16. Progression of triploid *C. ariakensis* (Ca 3n) gametogenesis throughout this study at Tommy Kellum's site. No diploid *C. virginica* were available for sampling. Sex is indicated by M=male; F=female; H=hermaphrodite; U=unknown. Gametogenesis categories are as follows: "N"EA=New Early Active; ID=Indifferent; RE=Resorbing; SO=Spawned Out; SP=Spawn Partially; R=Ripe; LA=Late Active; MA=Middle Active; EA=Early Active; IA=Inactive.

70

Group		01/02	05/02	06/02	07/02	08/02	10/02	12/02
Cv 2n	"N"EA	MMFFF					MF	MFFUU
	ID	F					UUU	MUU
	RE					U	UUUUU	MU
	SO			F	MMF	U		
	SP				FF	MMMMF		
	R			M	FFF			
	LA			F	M			
	MA		FFFFF	MMFF		MMF		
	EA		FFFH	F	U			
	IA	UUUU		FF				
Ca 3n	"N"EA	MMMMMF						MMMUUUU
	ID						MMUUUUUU UUUUUU	MUUUUUUUUUUU UUU
	RE					FFU	UUUUUUUUUU	UU
	SO				HHH	MU	M	
	SP				HHH	MMMMHHHH		
	R			MFF	MMFF			
	LA			MM	MHH	MM		
	MA	M	MMMMMMMMMM MMFFHHHHHHHHHH	MMMFFFH HHHHHHH	MMMM HHHHH	MHHHH		
	EA			FF				
	IA	UUU	FFH	FFFFH	FHU	FFFFUUH		

Figure 17. Progression of diploid *C. virginica* (Cv 2n) and triploid *C. ariakensis* (Ca 3n) gametogenesis throughout this study at Bevans Oyster site. Sex is indicated by M=male; F=female; H=hermaphrodite; U=unknown. Gametogenesis categories are as follows: "N"EA=New Early Active; ID=Indifferent; RE=Resorbing; SO=Spawned Out; SP=Spawn Partially; R=Ripe; LA=Late Active; MA=Middle Active; EA=Early Active; IA=Inactive.

71

C. *ariakensis* gametogenesis

In general, triploid (3n) gametogenesis was delayed compared to diploid *C. virginica* (Figure 11A), favoring the earlier stages of maturity. These triploid oysters exaggerated the early stages of gametogenesis because 65% were categorized as Inactive, Early Active, or Middle Active compared to 60% in diploid oysters in the same categories. Triploid oysters rarely exhibited Late Active and Ripe stages of maturity (7%), although in diploid oysters it was more common (22%).

Triploid *C. ariakensis* developed irregularly compared to diploid *C. virginica*. Few animals produced "normal" gonad with a gradient of sex cell types. The majority developed inconsistent pockets of follicles with mature gametes and undifferentiated gonia. Males were generally more capable of producing intermediate sex cell types, i.e., primary and secondary spermatocytes, however, these stages persisted later into the reproductive season compared to diploid oysters. By August, triploid male oysters had spawned either developed (and undeveloped gametes) or were in the active stage of maturity. By October, all triploid male oysters exhibited signs of having spawned and were resorbing leftover gametes or the gonad had already become Indifferent.

Triploid female oysters typically did not develop a gradient of sex cell types. Characteristic was a lack of developing oocytes throughout the reproductive season. Occasionally follicles contained developing oocytes, but the majority was categorized as Inactive or Late Active/Ripe. Few triploid female oysters (27 out of 930 triploid oysters—2.9%) developed a ripe condition with mature ova free in the lumen and gonoduct. Like triploid male oysters, females showed clear signs of spawning in August and October. In December, gonad was either Indifferent or "New" Early Active and generally indistinguishable by sex.

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Gametogenesis at Tommy Mason

Refer to Figure 12 for triploid gametogenesis at site TM. In January, nine oysters were Inactive and one was Early Active. The May sample showed ten oysters were Inactive, twelve were Early Active, and three were Middle Active. By June, thirteen oysters remained Inactive, eight were Early Active, and four were Middle Active. Also in June, eight hermaphrodites were observed: six were Early Active and two were Middle Active. In July, seven oysters remained Inactive, eight were Early Active, six were Middle Active (Plate XXIV), two were Late Active, and two were Ripe. Three hermaphrodites were found: two were Early Active and one was Middle Active. The August sample showed four oysters were Inactive, four were Early Active, six were Middle Active, five were Late Active, and five had Spawned. Two hermaphrodites were found in August: one was Early Active and one was Middle Active (Plate XXX). By October, eleven triploid oysters showed signs of having Spawned, eight were Resorbing, and six were Indifferent. The December sample had seven oysters that were Resorbing, eight were Indifferent, and ten were "New" Early Active.

Gametogenesis at Jeff Hammer

Figure 13 shows gametogenesis in triploid oysters at site JH. The January sample had six triploid oysters that were Inactive and four that were "New" Early Active. In May, thirteen oysters were Inactive (Plate XV), nine were Early Active, and three were Middle Active. The June sample was not collected due to inaccessibility of the site. By July, four oysters were Inactive, three were Early Active, nine were Middle Active, five were Late Active, one was Ripe, and one had Spawned. Five of the individuals that were

Plate XV. Inactive triploid *C. ariakensis* male at site JH in May 2002. Follicles are rudimentary and spermatogonia produced are arrested (arrow). Large amounts of interfollicular space can be observed. 100x
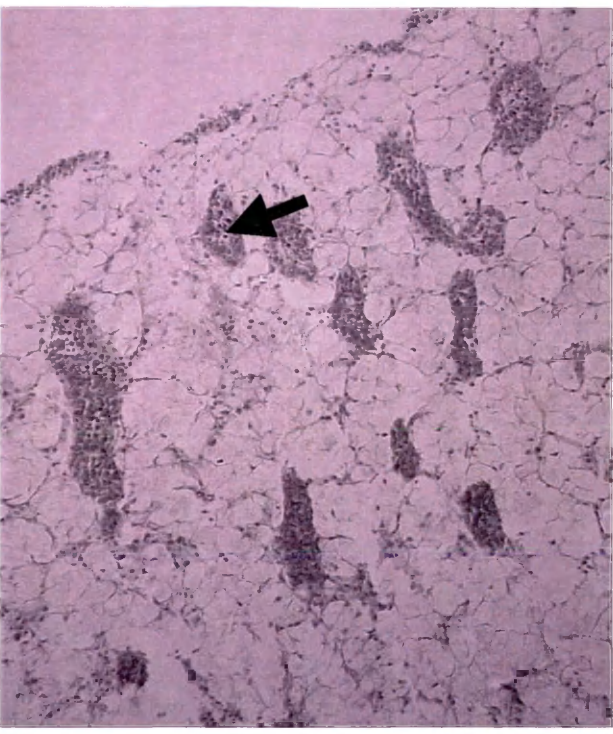
Plate XVI. Early Active triploid *C. ariakensis* male at site TL in May 2002. Follicles are beginning to branch and grow. Spermatogonia line the walls of follicles (arrow). Some spermatocytes can be observed toward the lumen of follicles. 200x

74
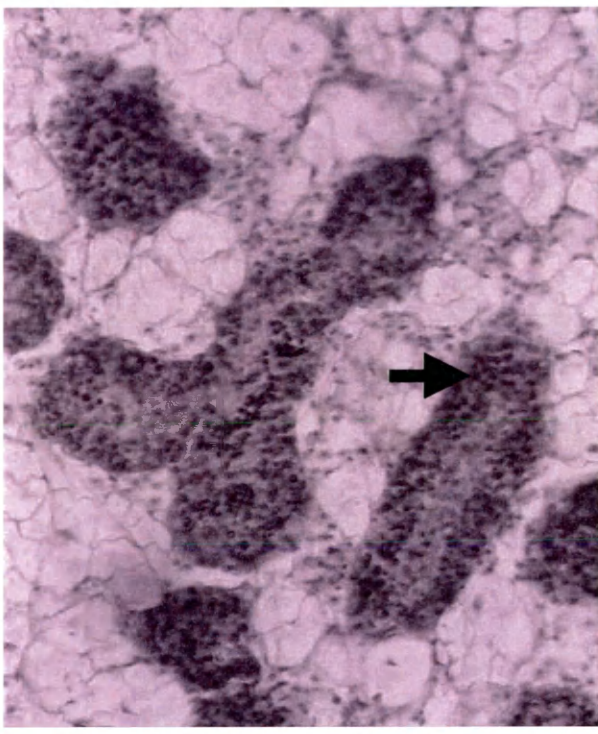
Plate XVII. Middle Active triploid *C. ariakensis* male at site TL in June 2002. Follicles are well developed with abundant spermatids (large arrow), but they have not differentiated into spermatozoa. Some spermatogonia remain along the follicle wall (small arrow). 200x
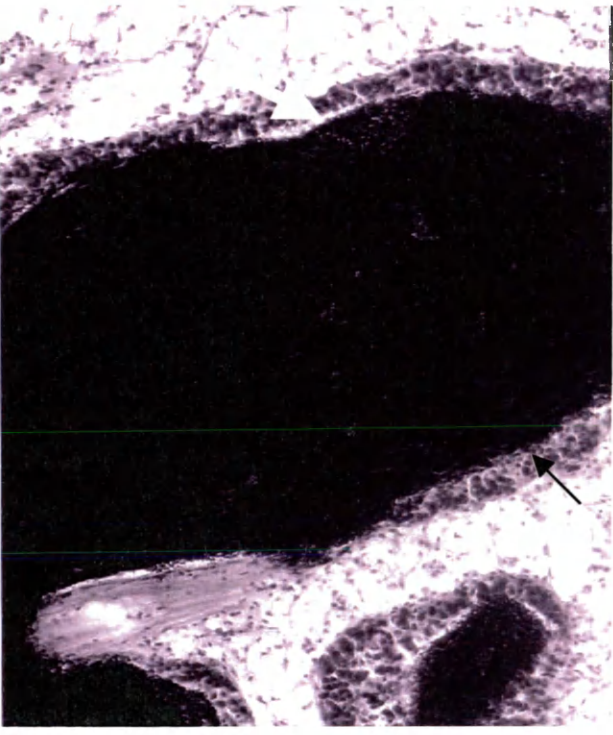
Plate XVIII. Late Active triploid *C. ariakensis* male at site CS in June 2002. Swirling spermatozoa are present in the lumen of mature follicles (large arrow) and spermatids (small arrow) are closer to the follicle wall developing follicles. 200x

75
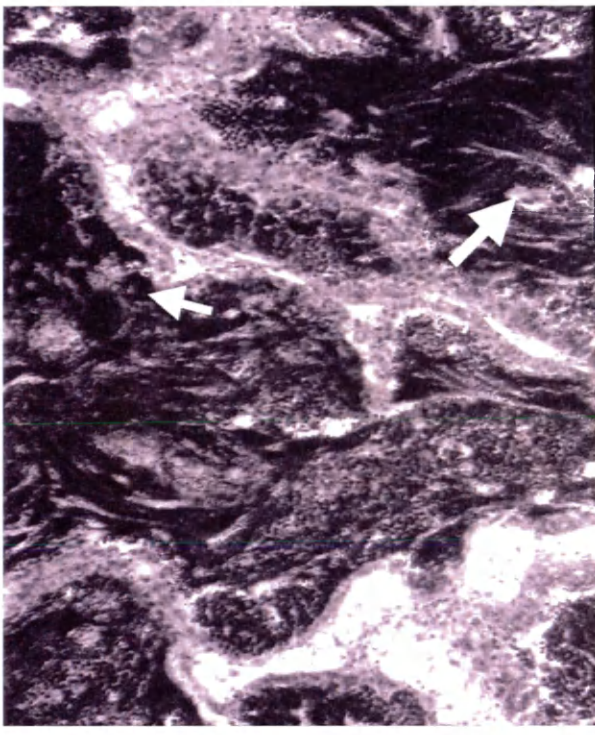
Middle Active were hermaphrodites. In August, four oysters were Inactive, four were

Middle Active, six were Late Active, one was Ripe, nine had Spawned, and one was

Resorbing. One Spawned and one Resorbing hermaphrodite was observed in August.

By October, three triploids had Spawned, ten were Resorbing, and twelve were

Indifferent. Lastly, the December sample revealed one oyster was Resorbing, thirteen

were Indifferent, and eleven were "New" Early Active.

Gametogenesis at Cherrystone Aquafarms

Refer to Figure 14 for gametogenesis in triploid oysters. In January, eight triploid

oysters were Inactive and two were "New" Early Active. The May sample showed

twelve oysters were Inactive (Plate XXIII), five were Early Active, three were Middle

Active, two were Late Active, and two were Resorbing. Two hermaphrodites were

observed in May: one was Early Active and the other was Middle Active. By June, six

animals were Inactive, five were Early Active, six were Middle Active, five were Late

Active (Plate XVIII), one was Ripe, and two were Resorbing. Also in June, four

hermaphrodites were found: two were Early Active, one was Middle Active, and one was

Late Active. The July sample had sixteen oysters that were Inactive, one was Early

Active, one was Middle Active, three were Ripe (Plate XXVI), three had Spawned, and

one was Resorbing. July sample also showed three hermaphrodites: all three were

Inactive. By August, eleven triploid oysters were still Inactive, two were Middle Active,

two were Late Active, five were Ripe, two had Spawned (Plate XXVII), and three were

Resorbing. Five hermaphrodites were observed in August: four were Inactive and one

was Middle Active. In October, two triploid oysters had Spawned, eleven were

Plate XIX. Ripe triploid *C. ariakensis* male at site TK in June 2002. Swirling spermatozoa now dominate all follicles (large arrow). Spermatids may be observed in some follicles. Follicles well branched and gonoduct is full of mature gametes. Spermatocytes are not present. 200x

Plate XX. Spawned triploid *C. ariakensis* male at site BO in July 2002. Only residual sperm are left in empty follicles (arrow) or swollen gonoducts. 100x

77
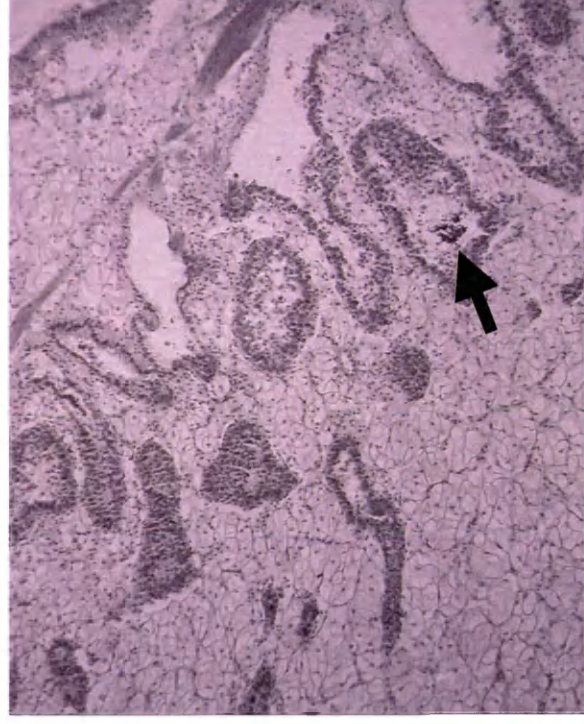
Resorbing, and twelve were Indifferent. The last sample in December had ten oysters that were Indifferent and fifteen were "New" Early Active.

Gametogenesis at Tommy Leggett

Figure 15 shows gametogenesis at site TL. In January, all ten triploid oysters were Inactive. By May, nine oysters were Inactive, seven were Early Active (Plate XVI), and nine were Middle Active. Five Inactive hermaphrodites were observed in May. In June, seven animals were Inactive, nine were Early Active, four were Middle Active, three were Late Active (Plate XXV), and three were Ripe. Six hermaphrodites were observed in June: three were Early Active, two were Middle Active (Plate XXXI), and one was Late Active. The July sample showed eight oysters were Inactive, eight were Early Active, five were Middle Active, two were Late Active, and two were Ripe. Six hermaphrodites were also found in July: five were Early Active and one was Middle Active. By August, seven triploid oysters were Inactive, four were Early Active, six were Middle Active, three were Late Active, two were Ripe, and three had Spawned. Four hermaphrodites were found in August: two were Inactive and two were Middle Active. In October, nine oysters had Spawned, five were Resorbing, and eleven were Indifferent. Lastly, in December, three triploid oysters had Spawned, eight were Resorbing, two were Indifferent, and twelve were "New" Early Active.

Gametogenesis at Tommy Kellum

Refer to Figure 16 for triploid gametogenesis at site TK. In January, four triploid oysters were Inactive, one was Early Active, three were Middle Active, and two were "New" Early Active. By May, three oysters were Inactive, one was Early Active, eleven

Plate XXI. Resorbing triploid *C. ariakensis* male at site BO in October 2002. Sperm left in gonoduct (large arrow) or empty follicles are now accompanied by hemocytes (small arrow) to digest residual gametes. 100x
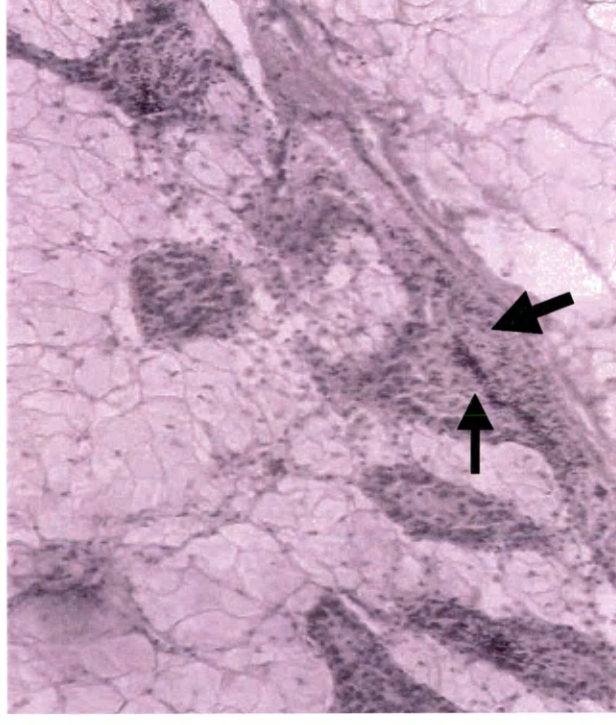
Plate XXII. "New" Early Active triploid *C. ariakensis* male at site BO in January 2002. Newly formed immature follicles are beginning to show developing spermatogonia (arrow). 200x
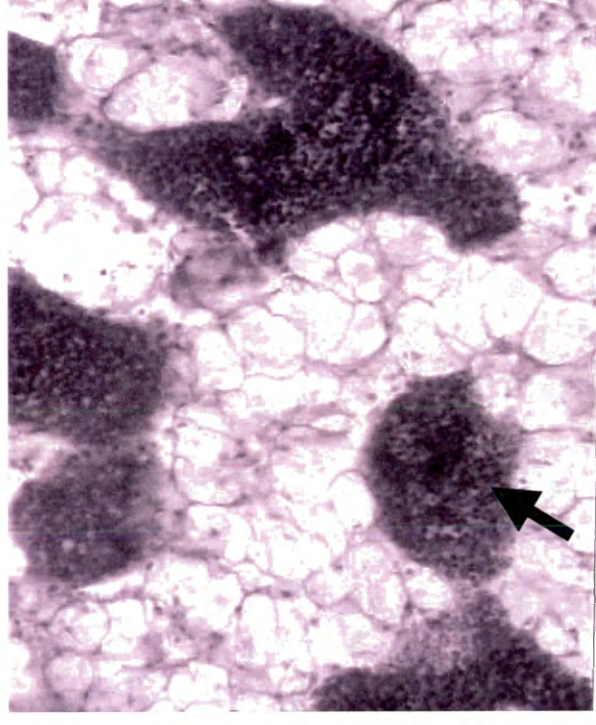
Plate XXIII. Inactive triploid *C. ariakensis* female at site CS in May 2002. Mature ova are infrequent and abnormal (arrow) among arrested gonia. Some ova may be in the process of being resorbed. 200x
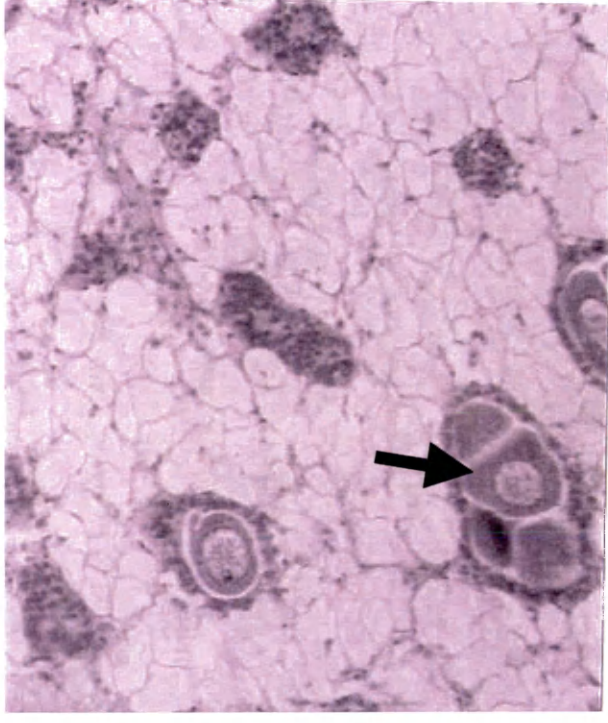
Plate XXIV. Middle Active triploid *C. ariakensis* female at site TM in July 2002. Oocytes are developing along the follicle walls (arrow). A range of mature and immature oocytes can be observed. 100x
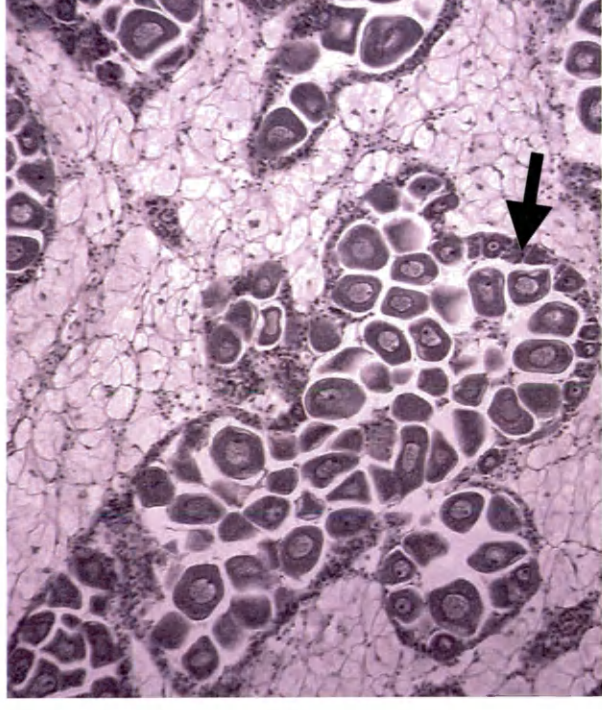
Plate XXV. Late Active triploid *C. ariakensis* female at site TL in June 2002. Almost all interfollicular space is occupied by mature follicles with fully grown oocytes (arrow).100x
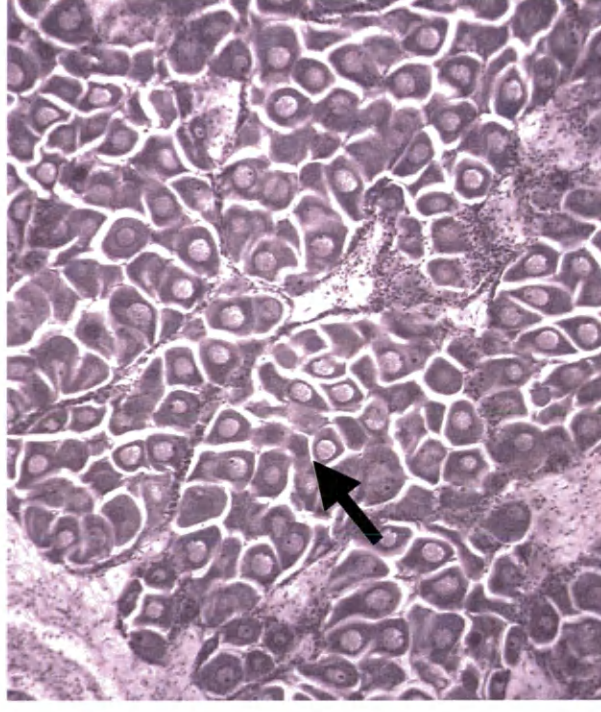
Plate XXVI. Ripe triploid *C. ariakensis* female at site CS in July 2002. Mature ova are free in the lumen of mature follicles (arrow). It is rare to observe developing ooyctes at this stage. 200x

81
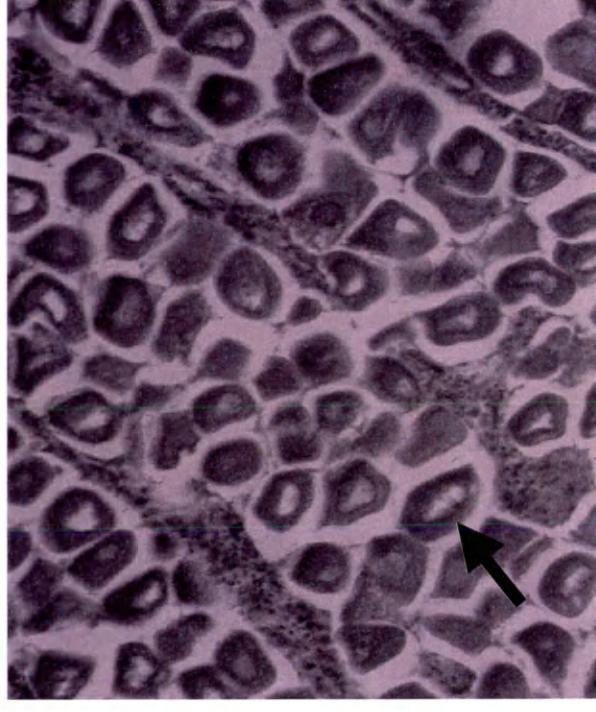
were Middle Active, nine were Late Active, and one was Ripe. Also in May, eight hermaphrodites were found: one was Early Active, five were Middle Active, and two were Late Active. In June, two oysters were Inactive, one was Early Active, four were Middle Active (Plate XVII), six were Late Active, seven were Ripe (Plate XIX), and four had Spawned. Nine hermaphrodites were observed in June: one was Middle Active, two were Late Active, four were Ripe, and two were Spawned. The July sample showed four triploid oysters were Inactive, two were Early Active, two were Middle Active, three were Late Active, five were Ripe, and nine had Spawned. Seven hermaphrodites were found in July: two were Inactive, one was Early Active, and four were Spawned. In August, two oysters were Inactive, one was Late Active, one was Ripe, nineteen had Spawned, and two were Resorbing. Also in August, four Spawned hermaphrodites were observed. By October, ten oysters had Spawned, nine were Resorbing, and six were Indifferent. In the last sample, December, six triploid oysters were Resorbing, seven were Indifferent, and twelve were "New" Early Active.

Gametogenesis at Bevans Oyster Co.

Figure 17 shows gametogenesis in triploid oysters. In January, three oysters were Inactive, one was Middle active, and six were "New" Early Active (Plate XXII; Plate XXIX). In May, three animals were Inactive, and twenty-two were Middle Active. Nine hermaphrodites were found in May: one was Inactive and eight were Middle Active. The June sample showed five animals were Inactive, two were Early Active, thirteen were Middle Active, two were Late Active, and three were Ripe. Also in June, eight hermaphrodites were observed: one was Inactive and seven were Middle Active. In July, three triploid oysters were Inactive, nine were Middle Active, three were Late Active,

Plate XXVII. Spawned triploid *C. ariakensis* female at site CS in August 2002. Only mature ova remain in empty follicles (arrow). Gonoducts may contain residual oocytes. 100x

Plate XXVIII. Resorbing triploid *C. ariakensis* female at site BO in August 2002. Oocytes (large arrow) are being consumed by hemocytes in the gonoduct (small arrow). Follicles are being broken down and any remaining gametes are being consumed by hemocytes. 100x

83
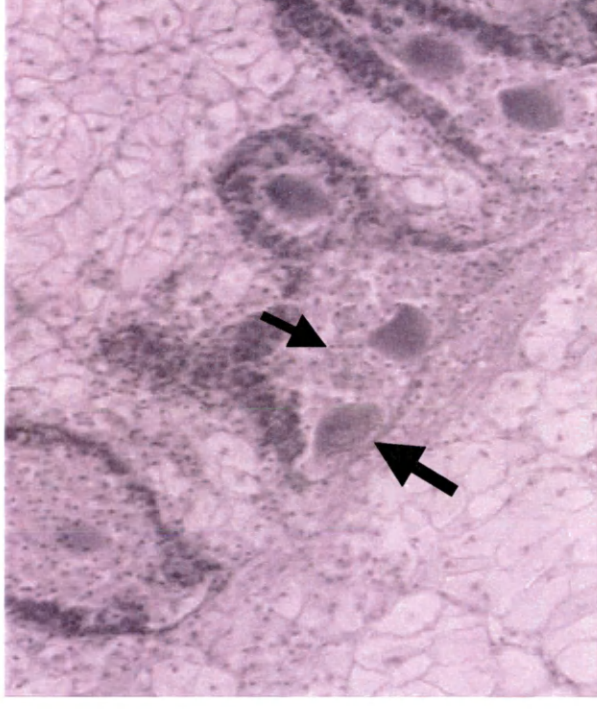
Plate XXIX. "New" Early Active triploid *C. ariakensis* female at site BO in January 2002. Oogonia begin to line the walls of immature follicles (arrow). Interfollicular space is abundant. 100x
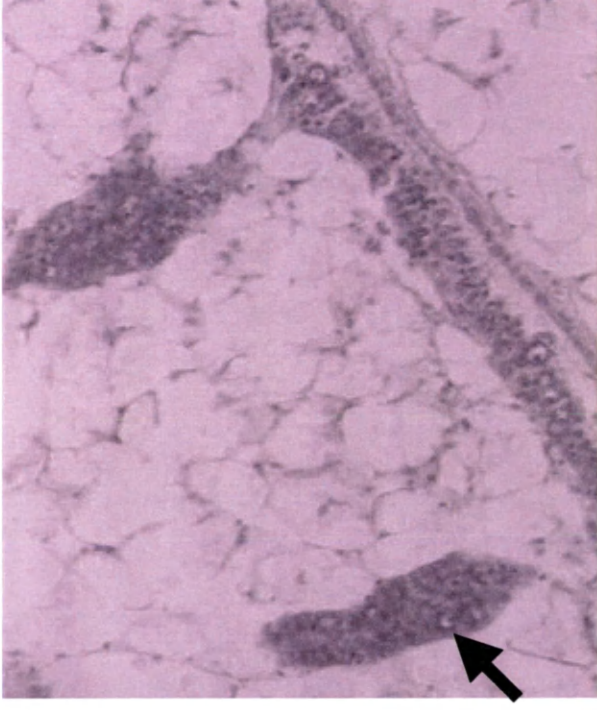
Plate XXX. Middle Active triploid *C. ariakensis* hermaphrodite at site TM in August 2002. Spermatids (small arrow) fill the lumen of young follicles. The occasional oocyte (large arrow) appeared in the same follicle. 200x
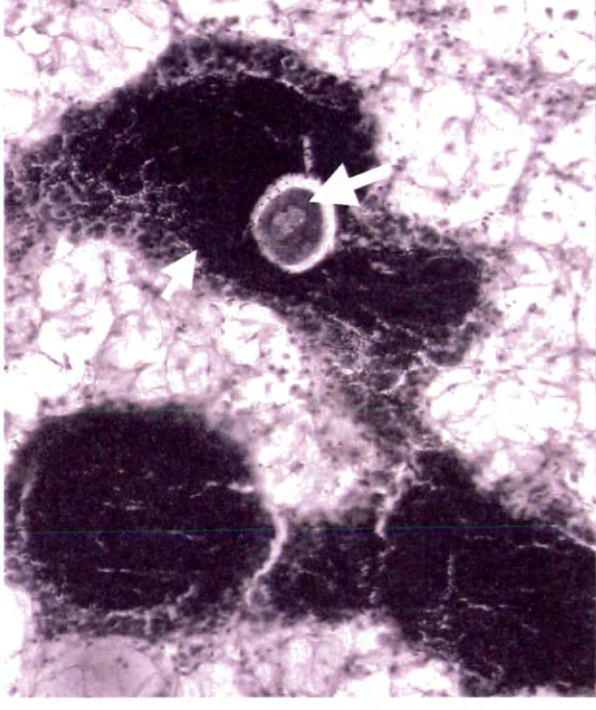
four were Ripe, and six were Spawned (Plate XX). Fourteen hermaphrodites were found in July: one was Inactive, five were Middle Active, two were Late Active, and six were Spawned (Plate XXXII; Plate XXXIII). By August, seven oysters were Inactive, four were Middle Active, two were Late Active, nine had Spawned, and three were Resorbing (Plate XXVIII). Also in August, eight hermaphrodites were observed: one was Inactive, three were Middle Active, three were Spawned (Plate XXXIV), and one was Resorbing. In October histology showed one triploid oyster had Spawned, nine were Resorbing (Plate XXI), and fifteen were Indifferent. In the last sample, December, two oysters were Resorbing, fifteen were Indifferent, and eight were "New" Early Active.

Table 5 summarizes the state of maturity at each site for diploid and triploid oysters throughout the sampling year. The maturity states of Inactive, Early Active, Active, Ripe, Spawned, and Cleaning were used to be concise. Similar categories were combined: Early Active included "New" Early Active and Early Active stages, Active included Middle Active and Late Active stages, Spawned included Spawned Partial and Spawned Completely stages, and Cleaning included Resorbing and Indifferent stages. It is clear that both diploid and triploid oysters completed gametogenesis by the December sampling. In fact, no developing (Active or Ripe stage) diploid or triploid oysters were observed after August at any site, except one Ripe diploid oyster at JH in October.

Comparison of C. virginica *and* C. ariakensis *gametogenesis*

Graphically, diploid *C. virginica* exhibited a consistently more rapid rate of mean development compared to triploid *C. ariakensis* (Figure 11A). Examining the reproductive season (May to August) at all sites (Figure 11B) excluding TK, diploid oysters were found to achieve a more advanced mean state of maturity compared to

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Plate XXXI. Middle Active triploid *C. ariakensis* hermaphrodite at site TL in June 2002. Spermatogonia (small arrow) line some of the follicle walls. Mature oocytes are more common (large arrow) in the same or separate follicles. 200x
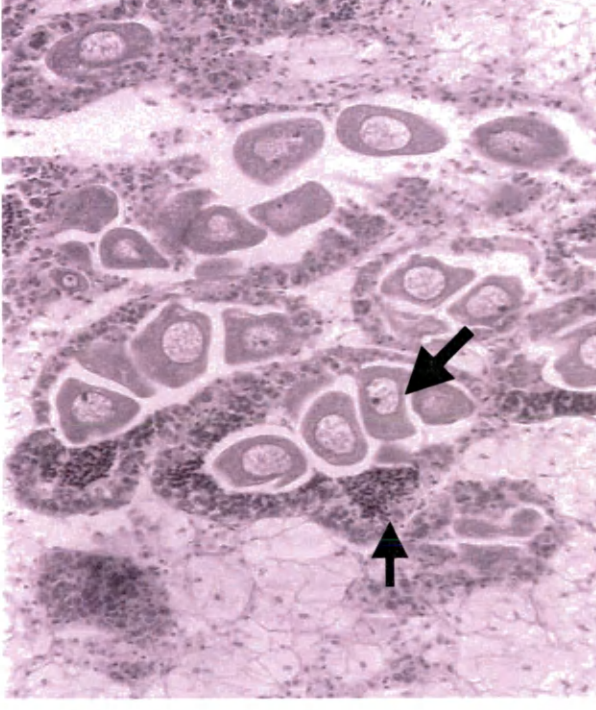
Plate XXXII. Spawned triploid *C. ariakensis* hermaphrodite at site BO in July 2002. Spermatozoa and spermatids fill the gonoduct (small arrow). A few oocytes are left in an otherwise empty follicle (large arrow). 100x
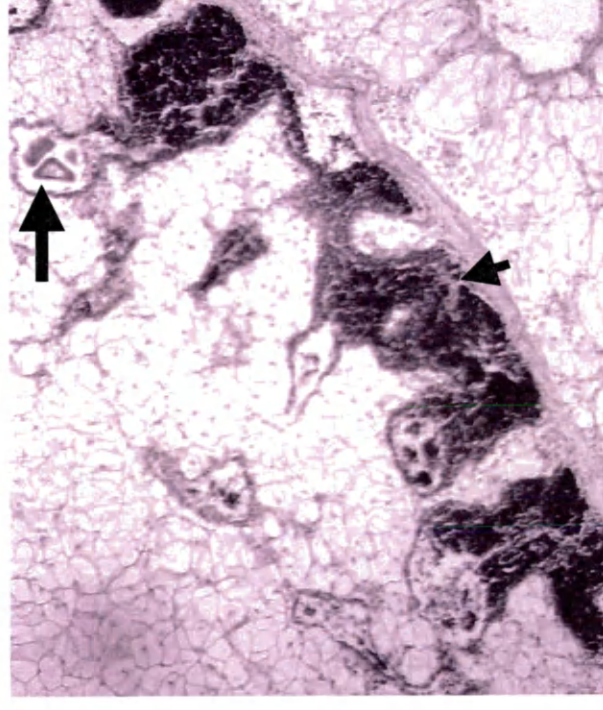
Plate XXXIII. Spawned triploid *C. ariakensis* hermaphrodite at site BO in June 2002. Oocytes (large arrow) are left in an empty follicle post spawning. Spermatids are the residual male gametes (small arrow). Follicles are being broken down by invading hemocytes. 200x
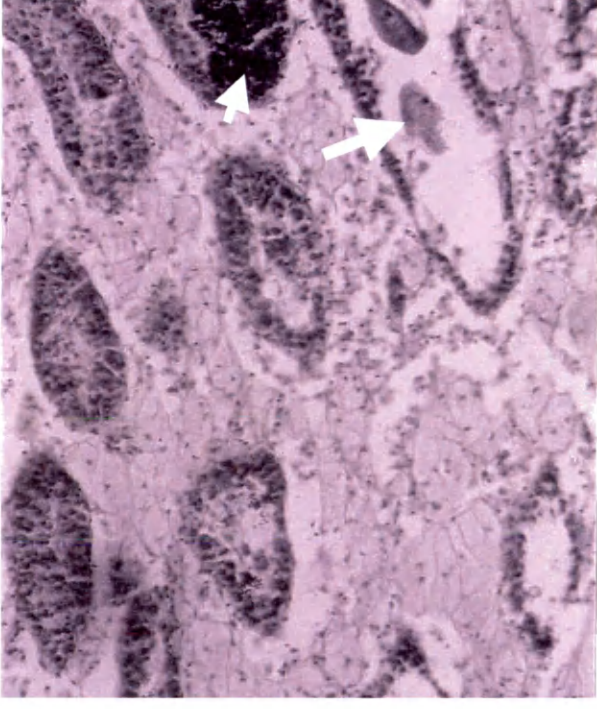
Plate XXXIV. Spawned triploid *C. ariakensis* hermaphrodite at site BO in August 2002. Several spermatozoa and spermatids are left in the swollen gonoducts (small arrow). Mature oocytes (large arrow) are randomly dispersed among the male sex cells. Mostly likely both male and female sex cells were spawned. 100x
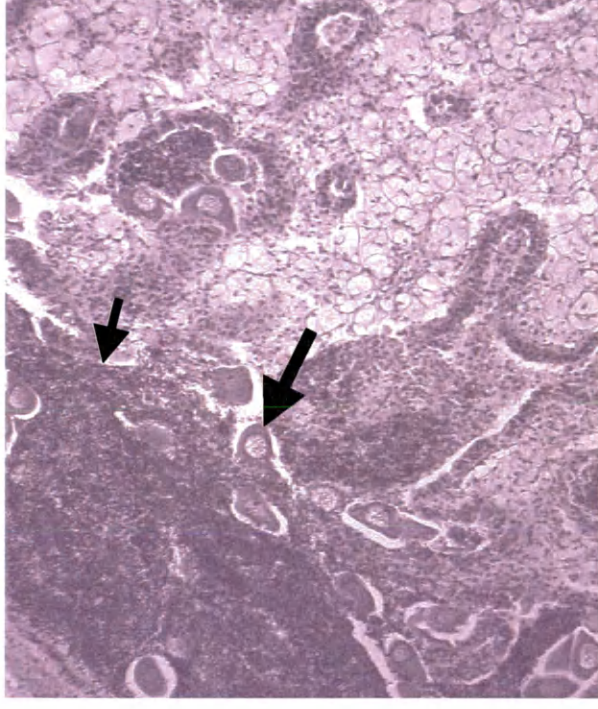
Table 5. Numbers of oysters in each maturity state for diploid *C. virginica* (2n) and triploid *C. ariakensis* (3n) across all sampling dates at each site. na=not available. Gametogenesis categories were collapsed into general states of maturity accordingly: Inactive=Inactive only; Early Active="New" Early Active and Early Active; Active=Middle and Late Active; Ripe=Ripe only; Spawned=Spawn Partially and Completely; Cleaning=Resorbing and Indifferent. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

TM Site

Maturity state	Species	January	May	June	July	August	October	December
Inactive	2n	10	6	2	0	0	0	0
	3n	9	10	13	7	4	0	0
Early active	2n	0	3	1	1	0	0	3
	3n	1	12	8	8	4	0	10
Active	2n	0	1	6	7	3	0	0
	3n	0	3	4	8	11	0	0
Ripe	2n	0	0	1	2	3	0	0
	3n	0	0	0	2	0	0	0
Spawned	2n	0	0	0	0	4	6	0
	3n	0	0	0	0	5	11	0
Cleaning	2n	0	0	0	0	0	4	7
	3n	0	0	0	0	0	14	15

JH Site

Maturity state	Species	January	May	June	July	August	October	December
Inactive	2n	6	6	na	0	0	0	0
	3n	6	13	na	4	4	0	0
Early active	2n	3	4	na	0	0	0	5
	3n	4	9	na	3	0	0	11
Active	2n	0	0	na	4	1	0	0
	3n	0	3	na	14	10	0	0
Ripe	2n	0	0	na	1	2	1	0
	3n	0	0	na	1	1	0	0
Spawned	2n	0	0	na	3	5	1	0
	3n	0	0	na	1	9	3	0
Cleaning	2n	1	0	na	2	2	8	5
	3n	0	0	na	0	1	22	14

Table 5 continued. Numbers of oysters in each maturity state for diploid *C. virginica* (2n) and triploid *C. ariakensis* (3n) throughout the 2002 sampling season.

CS Site

Maturity state	Species	January	May	June	July	August	October	December
Inactive	2n	9	0	0	0	0	0	0
	3n	8	12	6	16	11	0	0
Early active	2n	1	1	0	0	0	1	4
	3n	2	5	5	1	0	0	15
Active	2n	0	9	9	7	3	0	0
	3n	0	5	11	1	4	0	0
Ripe	2n	0	0	1	2	0	0	0
	3n	0	0	1	3	5	0	0
Spawned	2n	0	0	0	1	5	3	1
	3n	0	0	0	3	2	2	0
Cleaning	2n	0	0	0	0	2	6	5
	3n	0	2	2	1	3	23	10

TL Site

Maturity state	Species	January	May	June	July	August	October	December
Inactive	2n	6	0	0	0	0	0	0
	3n	10	9	6	8	7	0	0
Early active	2n	4	1	0	0	2	0	5
	3n	0	7	9	8	4	0	12
Active	2n	0	9	7	9	3	0	0
	3n	0	9	7	7	9	0	0
Ripe	2n	0	0	3	1	3	0	0
	3n	0	0	3	2	2	0	0
Spawned	2n	0	0	0	0	1	1	0
	3n	0	0	0	0	3	9	3
Cleaning	2n	0	0	0	0	1	9	5
	3n	0	0	0	0	0	16	10

Table 5 continued. Numbers of oysters in each maturity state for diploid *C. virginica* (2n) and triploid *C. ariakensis* (3n) throughout the 2002 sampling season.

TK Site

Maturity state	Species	January	May	June	July	August	October	December
Inactive	2n	na	na	na	na	na	na	na
	3n	4	3	2	4	2	0	0
Early active	2n	na	na	na	na	na	na	na
	3n	3	1	1	2	0	0	12
Active	2n	na	na	na	na	na	na	na
	3n	3	20	10	5	1	0	0
Ripe	2n	na	na	na	na	na	na	na
	3n	0	1	7	5	1	0	0
Spawned	2n	na	na	na	na	na	na	na
	3n	0	0	4	9	19	10	0
Cleaning	2n	na	na	na	na	na	na	na
	3n	0	0	0	0	2	15	13

BO Site

Maturity state	Species	January	May	June	July	August	October	December
Inactive	2n	4	0	2	1	0	0	0
	3n	3	3	5	3	7	0	0
Early active	2n	6	4	1	0	0	2	5
	3n	6	0	2	0	0	0	8
Active	2n	0	6	5	4	3	0	0
	3n	1	22	15	12	6	0	0
Ripe	2n	0	0	1	2	0	0	0
	3n	0	0	3	4	0	0	0
Spawned	2n	0	0	1	3	6	0	0
	3n	0	0	0	6	9	1	0
Cleaning	2n	0	0	0	0	1	8	5
	3n	0	0	0	0	3	24	17

triploid oysters. Figures 18-20 show the discrepancy or lag between mean diploid and triploid gametogenesis for each site. This phenomenon has been termed gametogenesis lag.

Figure 18A shows the gametogenesis lag for TM. Diploid and triploid oysters are developing similarly during the beginning (Inactive stage) and toward the end (Cleaning stage) of the gametogenic cycle, however the lag in triploid gametogenesis was evident during the warmer, reproductive season. The maximum gametogenesis lag was observed during August. Figure 18B shows the gametogenesis for JH. Since the June sample was not collected it is difficult to piece together the early part of the summer, however the maximum gametogenesis lag according to this data occurs during July.

Figure 19A shows the gametogenesis lag for CS. A similar trend seen in TM and JH was observed during the middle of the reproductive season. The maximum gametogenesis lag occurs during August. Figure 19B shows the gametogenesis lag for TL. The high diploid value in January was due to the presence of four oysters that were in "New" Early Active stages (category 9). The maximum gametogenesis lag at TL was observed during July.

Figure 20A shows triploid gametogenesis at TK. Diploid C. virginica were not available for comparison, however triploid oysters developed remarkably early in the season and only briefly prolonged gametogenesis from June to July. Figure 20B shows the gametogenesis lag for BO. Interestingly, this lower salinity site showed very little gametogenesis lag compared to TM, JH, CS, and TL. In fact, triploid gametogenesis resembled diploid gametogenesis during June and July, and only a slight gametogenesis lag was observed in August.
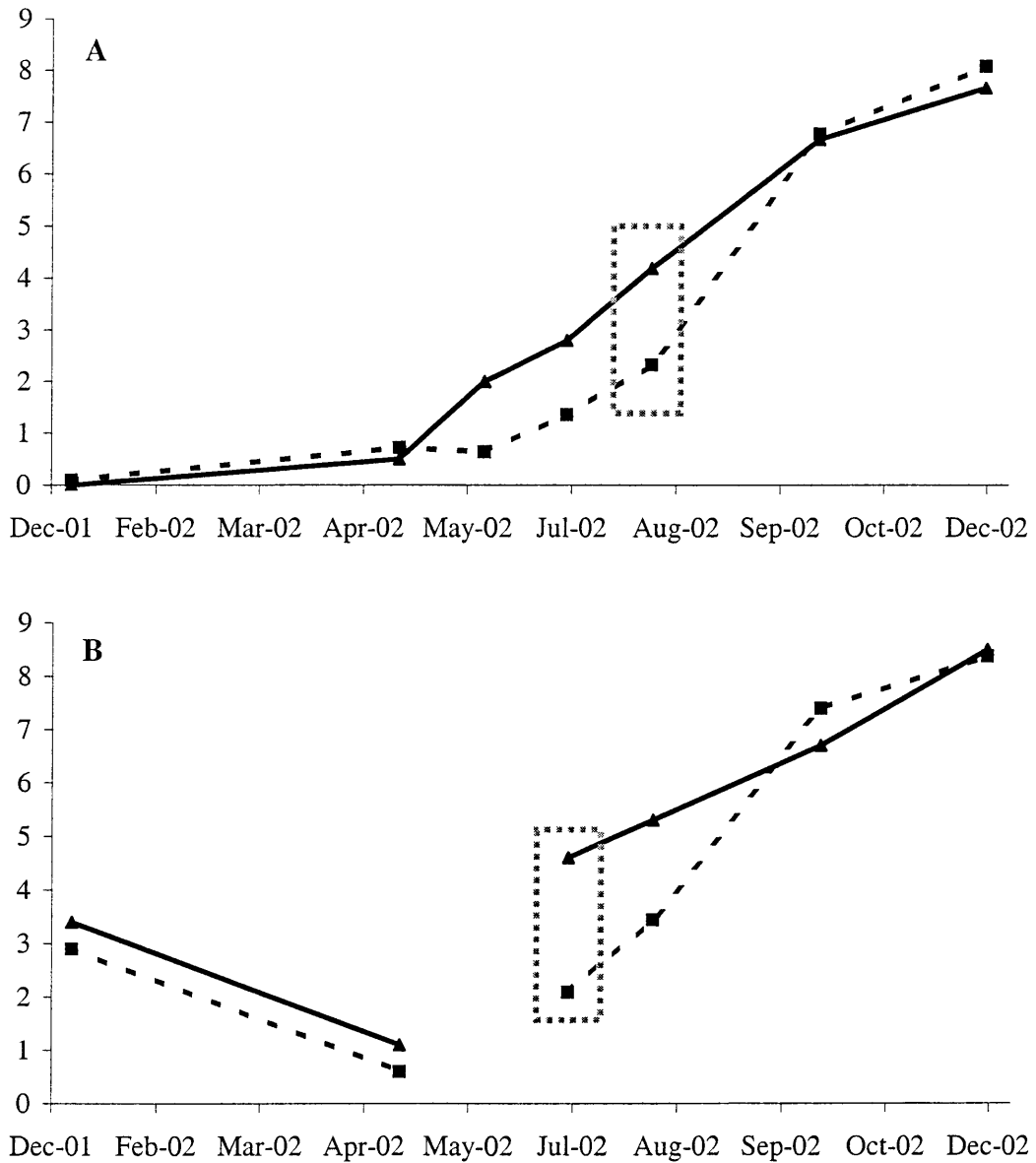
Figure 18. Gametogenesis lag of triploid *C. ariakensis* compared to diploid *C. virginica* in 2002 at high salinity sites, Tommy Mason (A) and Jeff Hammer (B). June sample was not collected at Jeff Hammer's due to inacessibility of the site at that time. Solid line=diploid; dashed line=triploid. Boxed region indicates maximum difference. Gametogenesis categories are as follows: 0=Inactive; 1=Early Active; 2=Middle Active; 3=Late Active; 4=Ripe; 5=Spawn Partially; 6=Spawn Completely; 7=Resorbing; 8=Indifferent; 9="New" Early Active.

A

9
8
7
6
5
4
3
2
1
0

Dec-01 Feb-02 Mar-02 Apr-02 May-02 Jul-02 Aug-02 Sep-02 Oct-02 Dec-02

B

9
8
7
6
5
4
3
2
1
0

Dec-01 Feb-02 Mar-02 Apr-02 May-02 Jul-02 Aug-02 Sep-02 Oct-02 Dec-02

Figure 19. Gametogenesis lag of triploid *C. ariakensis* compared to diploid *C. virginica* in 2002 at medium salinity sites, Cherrystone Aquafarms (A) and Tommy Leggett (B). Solid line=diploid; dashed line=triploid. Boxed region indicates maximum difference. Gametogenesis categories are as follows: 0=Inactive; 1=Early Active; 2=Middle Active; 3=Late Active; 4=Ripe; 5=Spawn Partially; 6=Spawn Completely; 7=Resorbing; 8=Indifferent; 9="New" Early Active.
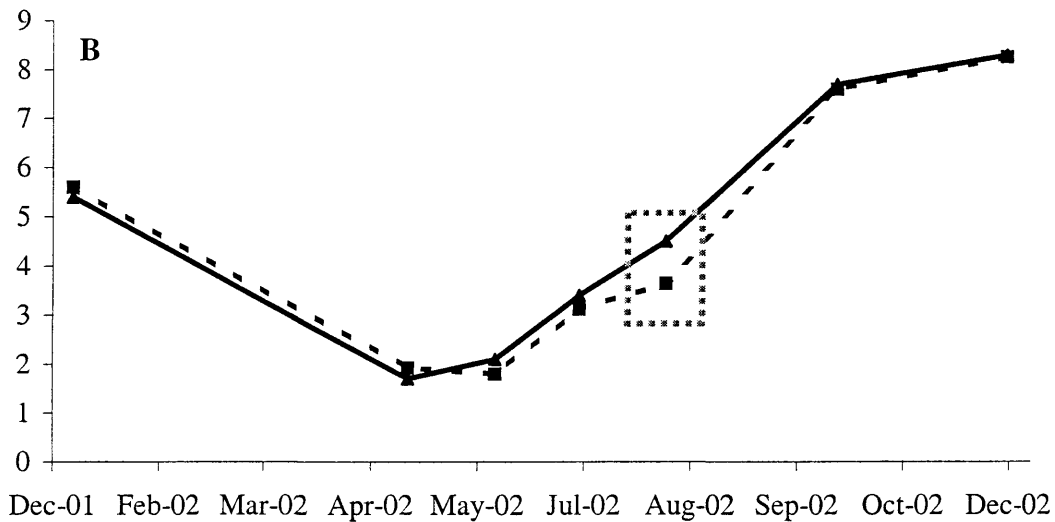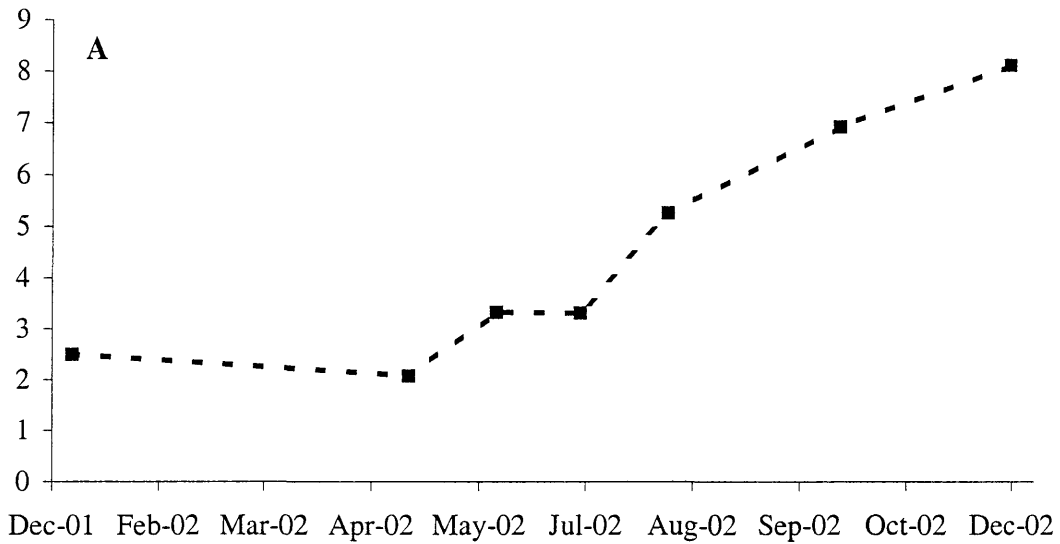
Figure 20. Gametogenesis lag of triploid *C. ariakensis* compared to diploid *C. virginica* in 2002 at low salinity site Bevans Oyster Co (B). Note that the other low salinity site, Tommy Kellum (A), only shows triploid gametogenesis because diploid *C. virginica* samples were not available. Solid line=diploid; dashed line=triploid. Boxed region indicates maximum difference. Gametogenesis categories are as follows: 0=Inactive; 1=Early Active; 2=Middle Active; 3=Late Active; 4=Ripe; 5=Spawn Partially; 6=Spawn Completely; 7=Resorbing; 8=Indifferent; 9="New" Early Active.

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Table 6 summarizes the gametogenesis lag difference at each site according to sample date. Consistently, the greatest difference occurs in either July or August at each site. The difference in the January sample at TL (3.6) was a result of four *C. virginica* that had "New" Early Active gonad compared to triploid oysters that were all Inactive. The largest mean discrepancy or lag between diploid and triploid gametogenesis occurred at site TL. The smallest mean lag was observed at site BO.

Table 7 shows the overall relative fecundity of mated triploid *C. ariakensis* at all six sites from May through August. A "fecund" individual was defined as being in Late Active and Ripe stages of maturity. BO had the highest proportion (62%) of fecund animals compared to the other sites. For this time period of gametogenesis, "male sites" (JH, TK, BO) contained more fecund animals compared to "female sites" (TM, CS). Site TL had 37% fecund triploid oysters at this time and the sex ratio was male 15: female 17. Several triploid oysters produced active gametes beginning in May and continuing through August. Generally, Active males (84) were more common than Active females (55) however Ripe females (22) were more common than Ripe males (11) during this time period.

Sex ratio and hermaphroditism

Sex was determined by examining histological sections. Diploid *C. virginica* and triploid *C. ariakensis* were categorized according to male, female, hermaphrodite, or unknown. Table 8 follows the diploid and triploid oyster sex ratios at each site from January through December. Sex ratio could not be followed in 2n at TK and are represented by "na" (not available) (Table 8).

Table 6. A quantitative examination of the lag of mean gametogenesis in triploid *C. ariakensis* from that of diploid *C. virginica* (mean diploid minus mean triploid gametogenesis) at each site by sampling date. na=not available. These numbers are the difference between diploid and triploid oysters, and are based on the transformed variables (0-9) for gametogenesis. 0=Inactive; 1=Early Active; 2=Middle Active; 3=Late Active; 4=Ripe; 5=Spawn Partially; 6=Spawn Compl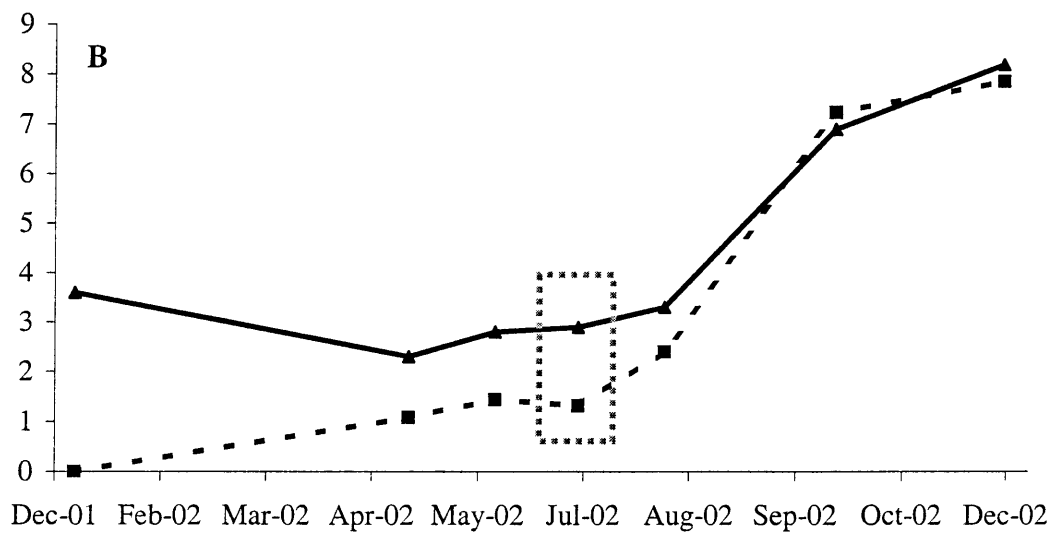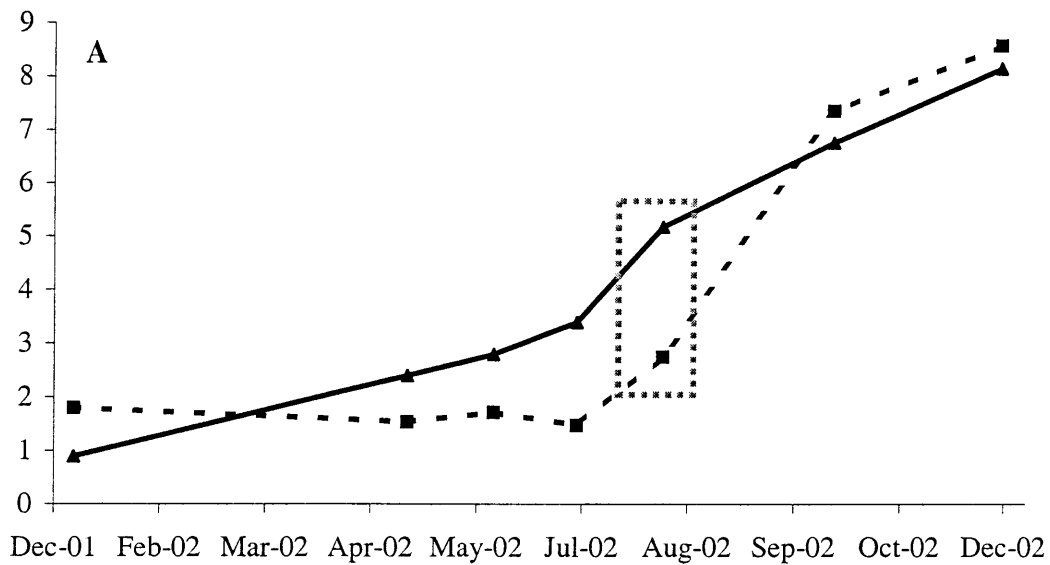etely; 7=Resorbing; 8=Indifferent; 9="New" Early Active.TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; BO=Bevans Oyster Co.

Site	Jan-02	May-02	Jun-02	Jul-02	Aug-02	Oct-02	Dec-02	Mean
TM	-0.1	-0.2	1.4	1.4	1.9	-0.1	-0.4	0.6
JH	-0.2	-0.2	na	2.6	1.9	-0.6	-0.1	0.6
CS	-0.9	1.1	0.8	1.9	2.8	-0.6	-0.4	0.7
TL	3.6	1.3	1.3	1.6	1.3	-0.2	0.3	1.3
BO	-0.2	-0.1	0.3	0.4	1.2	-0.1	0.0	0.2
Mean	0.4	0.4	1.0	1.6	1.8	-0.4	-0.1	

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Table 7. Summary of the number of fecund mated triploid *C. ariakensis* from May to
August 2002. Ripe category includes Late Active individuals. Fecund=triploids that
developed to advanced states of maturity (% Fecund = Ripe/N*100).
Herm.=hermaphrodite. N=total number of triploid oysters sampled from May to
August. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms;
TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

Site	Ripe herm.	Ripe female	Ripe male	N	% Fecund
TM	0	6	3	100	9
JH	0	2	4	72	8
CS	1	15	2	99	18
TL	1	12	1	100	14
TK	8	2	21	99	31
BO	2	4	7	100	13

January sample

Overall the diploid and triploid oysters were undeveloped during January and several sites predominantly had indistinguishable sex oysters.

Diploid oysters at TM and CS could not be sexed. One diploid male oyster was observed at JH. Four diploid female oysters were found at TL. Two diploid male oysters and four diploid female oysters were observed at BO. No hermaphrodites were found in January.

Triploid oysters at JH and TL could not be sexed. One triploid male oyster was observed at TM and one triploid female oyster was found at CS. Four triploid male oysters were observed at TK, while six triploid male oysters and one triploid female were found at BO. No triploid hermaphrodites were observed.

May sample

In May, only two sites had oysters that could not be sexed. Generally, the proportion of males and females were equal for diploid oysters and became skewed for triploid oysters.

Diploid oyster sex ratio at TM showed four males and six oysters with unknown sex. Diploid oysters at JH had one male, three females, and six oysters with unknown sex. CS and TL exhibited equal sex ratios, male 5: female 5 and male 4: female 6, respectively. Diploid oyster sex ratio at BO was skewed in May, male 1: female 8: 1 hermaphrodite.

Triploid oyster sex ratio at TM was skewed, favoring females, male 6: female 13: unknown 6. JH had male 10: female 11: unknown 4. CS exhibited male 1: female 21: hermaphrodite 2. TL showed male 11: female 9: hermaphrodite 5. TK had male 13:

Table 8. Occurrence of sexes for diploid *C. virginica* (2n) and triploid *C. ariakensis*
(3n-shaded area) at all sites for January and May sampling. na=not available.
Herm.=hermaphrodite. TM=Tommy Mason; JH=Jeff Hammer;
CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum;
BO=Bevans Oyster Co.

January

Site	Species	Male	Female	Herm.	Unknown
TM	2n	0	0	0	10
	3n	1	0	0	9
JH	2n	1	0	0	9
	3n	0	0	0	10
CS	2n	0	0	0	10
	3n	0	1	0	9
TL	2n	0	4	0	6
	3n	0	0	0	10
TK	2n	na	na	na	na
	3n	4	0	0	6
BO	2n	2	4	0	4
	3n	6	1	0	3

May

Site	Species	Male	Female	Herm.	Unknown
TM	2n	4	0	0	6
	3n	6	13	0	6
JH	2n	1	3	0	6
	3n	10	11	0	4
CS	2n	5	5	0	0
	3n	1	21	2	0
TL	2n	4	6	0	0
	3n	11	9	5	0
TK	2n	na	na	na	na
	3n	13	4	8	0
BO	2n	1	8	1	0
	3n	12	4	9	0

female 4: hermaphrodite 8. Lastly, triploid oyster sex ratio at BO was male 12: female 4: hermaphrodite 9.

June sample

Diploid oyster sex ratio at TM was male 5: female 3: unknown 2. The JH site was inaccessible during June so oysters were not collected (Table 8). Diploid oyster sex ratio at CS showed male 4: female 6, TL was male 3: female 5: hermaphrodite 2, and BO was male 3: female 7.

Triploid oyster sex ratios were more heavily skewed during June sampling. Triploid oyster hermaphrodites were found at all six sites. TM had male 3: female 14: hermaphrodite 8, CS had male 2: female 19: hermaphrodite 4, TL had male 1: female 18: hermaphrodite 6, TK was male 10: female 5: hermaphrodite 9, and BO was male 6: female 11: hermaphrodite 8.

July sample

In general, diploid oyster sex ratio remained equal while triploid oyster sex ratio remained skewed.

Diploid oyster sex ratio at TM was male 5: female 5, JH was male 6: female 1, CS had male 5: female 3: hermaphrodite 2, TL was male 4: female 6, and BO was male 3: female 6: unknown

Triploid oyster sex ratio was variable during July: TM was male 1: female 21: hermaphrodite 3, JH had male 9: female 9: hermaphrodite 5, CS was male 1: female 21: hermaphrodite 3, TL was female 19: hermaphrodite 6, TK was male 14: female 4: hermaphrodite 7, BO was male 7: female 3: hermaphrodite 14: unknown 1.

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Table 8 continued. Occurrence of sexes for diploid *C. virginica* (2n) and triploid *C. ariakensis* (3n-shaded area) at all sites for June and July sampling. na=not available. Herm.=hermaphrodite. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

June

Site	Species	Male	Female	Herm.	Unknown
TM	2n	5	3	0	2
	3n	3	14	8	0
JH	2n	na	na	na	na
	3n	na	na	na	na
CS	2n	4	6	0	0
	3n	2	19	4	0
TL	2n	3	5	2	0
	3n	1	18	6	0
TK	2n	na	na	na	na
	3n	10	5	9	0
BO	2n	3	7	0	0
	3n	6	11	8	0

July

Site	Species	Male	Female	Herm.	Unknown
TM	2n	5	5	0	0
	3n	1	21	3	0
JH	2n	6	1	0	3
	3n	9	9	5	0
CS	2n	5	3	2	0
	3n	1	21	3	0
TL	2n	4	6	0	0
	3n	0	19	6	0
TK	2n	na	na	na	na
	3n	14	4	7	0
BO	2n	3	6	0	1
	3n	7	3	14	1

August sample

Surprisingly, diploid oyster sex ratio was more skewed than other sampling periods: TM had male 7: female 3, JH had male 6: female 3: unknown 1, CS was male 3: female 6: unknown 1, TL had male 7: female 2: unknown 1, and BO was male 6: female 2: unknown 2.

Triploid oyster sex ratio was skewed similar to other sampling periods: TM had male 5: female 17: hermaphrodite 2, JH was male 16: female 7: hermaphrodite 2, CS had female 20: hermaphrodite 5, TL was male 8: female 12: hermaphrodite 4: unknown 1, TK had male 10: female 8: hermaphrodite 4: unknown 3, and BO was male 8: female 6: hermaphrodite 8: unknown 3.

October sample

Both diploid and triploid oysters of unknown sex were more abundant than the previous sampling periods. No hermaphrodites were observed.

Diploid oysters were largely unidentifiable: TM had male 3: female 3: unknown 4, JH had male 3: unknown 7, CS had male 1: female 4: unknown 5, TL was female 1: unknown 9, and BO was male 1: female 1: unknown 8.

Generally, triploid male oysters (31) were more abundant than triploid female oysters (8). Sex ratios were as follows: TM male 11: female 2: unknown 12, JH male 5: female 1: unknown 19, CS male 2: unknown 23, TL male 3: female 5: unknown 17, TK male 7: unknown 18, and BO male 3: unknown 22.

Table 8 continued. Occurrence of sexes for diploid *C. virginica* (2n) and triploid *C. ariakensis* (3n-shaded area) at all sites for August and October sampling. na=not available. Herm.=hermaphrodite. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

| | | **August** | | | |
Site	Species	Male	Female	Herm.	Unknown
TM	2n	7	3	0	0
	3n	5	17	2	0
JH	2n	6	3	0	1
	3n	16	7	2	0
CS	2n	3	6	0	1
	3n	0	20	5	0
TL	2n	7	2	0	1
	3n	8	12	4	1
TK	2n	na	na	na	na
	3n	10	8	4	3
BO	2n	6	2	0	2
	3n	8	6	8	3

| | | **October** | | | |
Site	Species	Male	Female	Herm.	Unknown
TM	2n	3	3	0	4
	3n	11	2	0	12
JH	2n	3	0	0	7
	3n	5	1	0	19
CS	2n	1	4	0	5
	3n	2	0	0	23
TL	2n	0	1	0	9
	3n	3	5	0	17
TK	2n	na	na	na	na
	3n	7	0	0	18
BO	2n	1	1	0	8
	3n	3	0	0	22

December sample

Overall, the sex of oysters was unidentifiable during December. No hermaphrodites were observed.

Diploid oyster sex ratios were as follows: TM unknown 10, JH female 2: unknown 8, CS male 2: female 5: unknown 3, TL male 3: female 2: unknown 5, BO male 3: female 2: unknown 5.

Triploid oyster sex ratios were as follows: TM male 1: unknown 24, JH male 4: female 1: unknown 20, CS male 5: unknown 20, TL male 15: unknown 10, TK male 9: unknown 16, BO male 4: unknown 21.

Table 9 shows the overall percent occurrence of sexes across all sites for diploid and triploid oysters throughout the study. For triploid oysters, three sites were female (TM, CS, TL) and three were male (JH, TK, BO). The diploid cohort of *C. virginica* did not follow this same trend.

Site by site contrast comparisons of male:female sex ratio in diploid and triploid oysters reveals these differences. Site TK is not included in the comparison contrasts because diploid oyster sex ratios were unavailable. Site contrast comparison at JH and CS revealed little significant differences between diploid oysters (X^2=4.1; p=0.04) and significant differences between triploid oysters (X^2=43.3; p<0.0001). Site contrast comparisons at JH and TL revealed no significant differences between diploid oysters (X^2=2.8; p=0.09) yet significant differences were observed between triploid oysters (X^2=9.4; p=0.002). Site contrast comparisons at JH and TM showed no significant differences between diploid oysters (X^2=0.01; p=0.92) but significant differences were observed between triploid oysters (X^2=16.7; p<0.001). Site contrast

Table 8 continued. Occurrence of sexes for diploid *C. virginica* (2n) and triploid *C. ariakensis* (3n-shaded area) at all sites for December sampling. na=not available. Herm.=hermaphrodite. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

| Site | Species | December | | | |
		Male	Female	Herm.	Unknown
TM	2n	0	0	0	10
	3n	1	0	0	24
JH	2n	0	2	0	8
	3n	4	1	0	20
CS	2n	2	5	0	3
	3n	5	0	0	20
TL	2n	3	2	0	5
	3n	15	0	0	10
TK	2n	na	na	na	na
	3n	9	0	0	16
BO	2n	3	2	0	5
	3n	4	0	0	21

Table 9. Overall percent occurrence of sexes in diploid *C. virginica* and triploid *C. ariakensis* across all sites. Absolute numbers are in parentheses. na=not available. Mean is given for the percents. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

Site	*C. ariakensis*				*C. virginica*			
	Male	Female	Herm.	Unknown	Male	Female	Herm.	Unknown
TM	18 (28)	42 (67)	8 (13)	32 (51)	34 (24)	24 (14)	0 (0)	46 (32)
JH	33 (44)	22 (29)	5 (7)	40 (53)	28 (17)	15 (9)	0 (0)	57 (34)
CS	7 (11)	52 (82)	8 (14)	33 (52)	29 (20)	41 (29)	3 (2)	27 (19)
TL	24 (38)	39 (63)	13 (21)	24 (38)	30 (21)	37 (26)	3 (2)	30 (21)
TK	42 (67)	13 (21)	18 (28)	27 (43)	na	na	na	na
BO	29 (46)	16 (25)	24 (39)	31 (50)	27 (19)	43 (30)	1 (1)	29 (20)
Mean	26	31	13	31	30	32	1	38

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comparisons at BO and CS found no significant differences between diploid oysters (X^2=0.04; p=0.84) yet significant differences were observed between triploid oysters (X^2=49.8; p<0.001). Site contrast comparisons at BO and TL revealed no significant differences between diploid oysters (X^2=0.41; p=0.52) and significant differences were observed between triploid oysters (X^2=13.1; p=0.0003). Site contrast comparisons at BO and TM showed significant differences between diploid oysters (X^2=5.6; p=0.02) yet highly significant differences were found between triploid oysters (X^2=21.3; p<0.0001). These site contrast comparisons show that triploid male:female sex ratio was significantly different indicating skewed sex ratio. On the other hand, the differences were not significant for diploid oyster cohorts, except at sites BO and TM.

At all sites, male:female sex ratio revealed a marginally significant difference in diploid oysters (X^2=10; p=0.04) and a highly significant difference in triploid oysters (X^2=106.9; p<0.0001). A comparison of diploid and triploid oyster male:female sex ratio at each site showed significant differences at TM (X^2=12.9; p<0.000), CS (X^2=15.8; p<0.000), and BO (X^2=7.9; p=0.005) and no differences at JH (X^2=0.21; p=0.65) and TL (X^2=0.67; p=0.41).

The obvious difference between diploid and triploid oyster sex ratios was the prevalence of triploid hermaphrodites. BO had the highest proportion of triploid hermaphrodites (n=39, 24%) and JH had the lowest (n=7, 5%). Across all sites, the male:female:hermaphrodite sex ratio revealed significant differences between diploid and triploid oysters (X^2=35.9; p<0.000). The number of hermaphrodites at each site was inversely proportional to the salinity (Figure 21, R^2=-0.94; p=0.005). Although JH had fewer hermaphrodites (7) at a higher salinity (33) compared to a site such as CS, the general trend was lower salinity, more hermaphrodites.
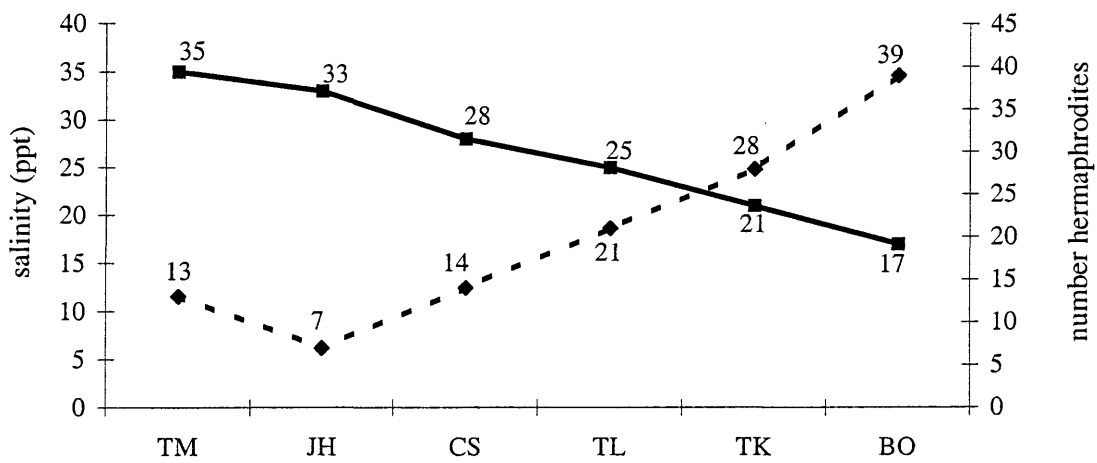
Figure 21. Mean salinity and the absolute number of triploid *C. ariakensis* hermaphrodites observed at each site throughout the study. Solid line=salinity; dashed line=number of triploid hermaphrodites. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy Kellum; BO=Bevans Oyster Co.

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Reversion in *C. ariakensis*

All triploid oysters were tested for reversion. Hemolymph biopsy was used as an initial indicator of reversion (refer to Materials and Methods). If flow cytometry detected diploid cells in the hemolymph, then additional tissues were sampled including heart, adductor, gill, and gonad.

Mosaicism in mated triploid *C. ariakensis* was uncommon: 11 mosaic oysters out of 930 triploid oysters (1.2%). Table 10 shows the number of mosaic oysters found at each site according to the sampling period. No mosaic oysters were detected in January. In May one mosaic oyster was found at TM. The June sampling had two mosaic oysters, one at CS and one at TK. In July two mosaic oysters were found, one at TM and one at CS. In August two mosaic oysters were found at BO. No mosaic oysters were found in October. In December four mosaic oysters were found, one at JH, one at CS, and two at BO. Across all sampling periods, two mosaic oysters were found at TM, one at JH, three at CS, zero at TL, one at TK, and four at BO. Figure 22 shows the number of mosaic oysters through time. The number of mosaic oysters generally increases with time, however, zero mosaic oysters were found in October resulting in a low correlation coefficient ($R^2 = 0.3634$; p=0.199).

Figure 23 shows reversion in individual triploid oysters by tissue type. The 11 mosaic oysters were plotted as percent diploid cells found in each tissue type according to site and sampling date. The most sensitive indicator of reversion was hemolymph tissue. Gill tissue detected reversion in 10 of the 11 mosaic oysters and heart tissue detected reversion in 9 of the 11 mosaic oysters. Adductor and gonad tissue samples were less reliable indicators of reversion. Figure 23 also demonstrates that if a high percentage of diploid cells are found in hemolymph tissue it will generally translate to heart, adductor,

Table 10. Number of mated triploid *C. ariakensis* mosaics by site and
sampling date. A mosaic is defined as a triploid individual that
contains both diploid and triploid cells. Hemolymph tissue was
used as the initial indicator. TM=Tommy Mason; JH=Jeff Hammer;
CS=Cherrystone Aquafarms; TL=Tommy Leggett; TK=Tommy
Kellum; BO=Bevans Oyster Co.

Sampled	TM	JH	CS	TL	TK	BO	Totals
Jan-02	0	0	0	0	0	0	0
May-02	1	0	0	0	0	0	1
Jun-02	0	0	1	0	1	0	2
Jul-02	1	0	1	0	0	0	2
Aug-02	0	0	0	0	0	2	2
Oct-02	0	0	0	0	0	0	0
Dec-02	0	1	1	0	0	2	4
Totals	2	1	3	0	1	4	
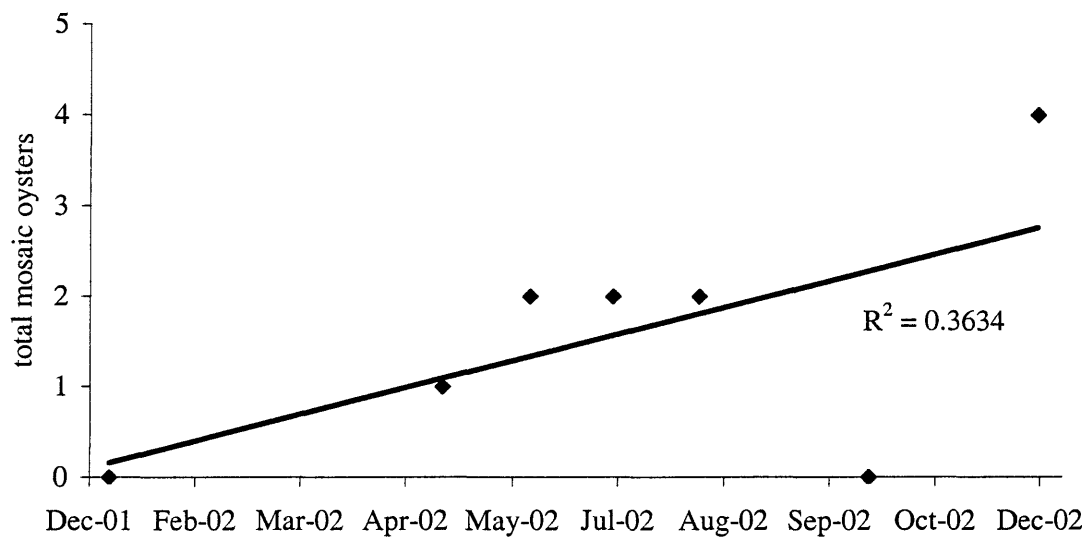
Figure 22. The occurrence of mosaic oysters throughout this study versus time. R^2 is displayed for reference.

	Aug-02 BO	Aug-02 BO	Jun-02 TK	Jun-02 CS	Jul-02 TM	Jul-02 CS	May-02 TM	Dec-02 BO	Dec-02 CS	Dec-02 JH	Dec-02 BO
hemolymph	65	44	18	16	15	6	6	6	5	5	4
gill	40	25	13	12	11	2	2	na	2	2	2
adductor	63	43	19	0	17	na	na	na	na	na	na
heart	55	34	18	19	7	4	na	na	3	2	3
gonad	46	na	na	0	11	na	3	na	na	na	na

Figure 23. Percent diploid cells in each mated triploid *C. ariakensis* mosaic by tissue type. Site and the sampling month are indicated on the X-axis. Data table included for reference. na=not available. TM=Tommy Mason; JH=Jeff Hammer; CS=Cherrystone Aquafarms; TK=Tommy Kellum; BO=Bevans Oyster Co.

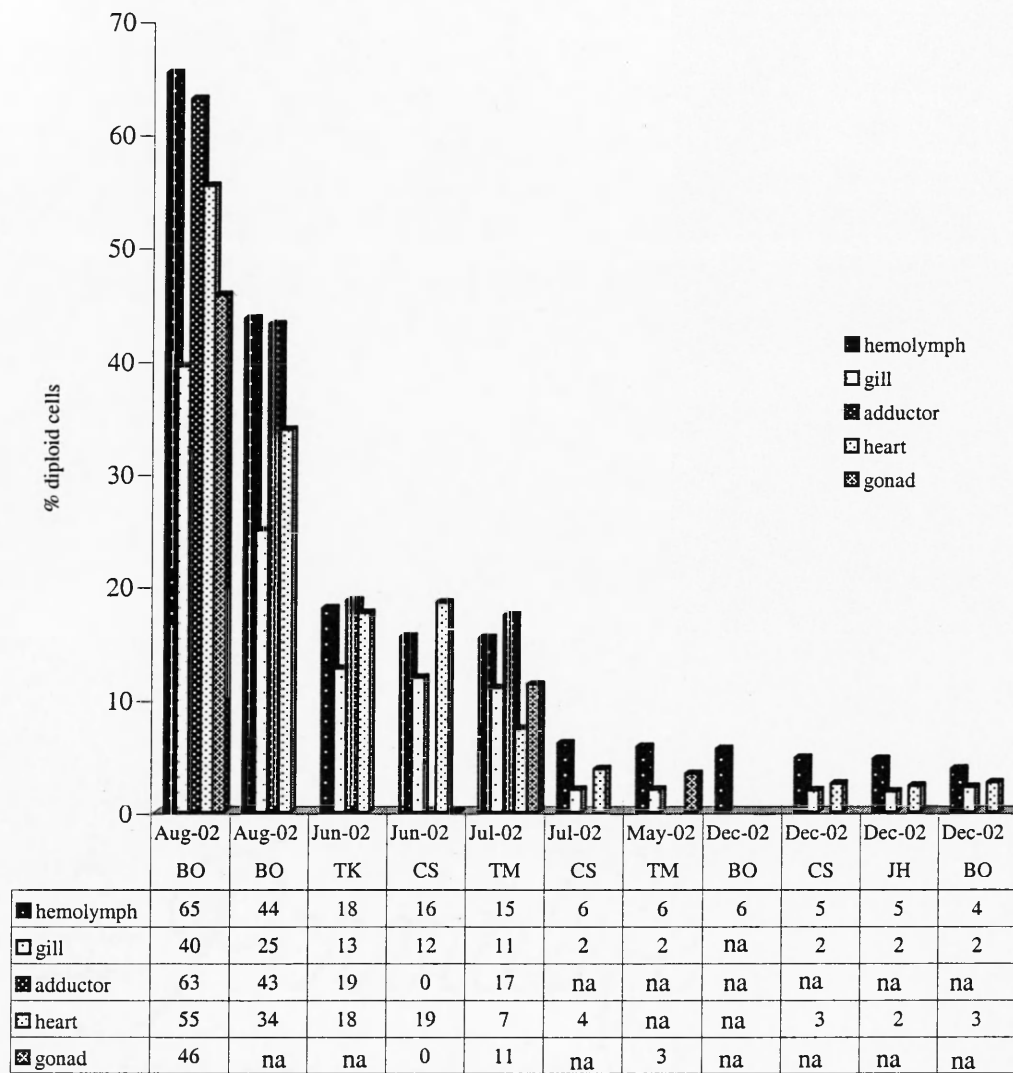gill and gonad tissues (i.e., BO #10). Figure 24 shows the correlation (R^2=0.938; p=0.032) between reversion in hemolymph and gonad tissue samples. Only four gonad samples were used in the correlation because this tissue was difficult to determine discrete peaks due to noise. Essentially, a high percentage of diploid cells in the hemolymph tissue would correlate to a similar percent in the gonad tissue.

Flow cytometry produced a histogram for each triploid oyster sampled for reversion. Figure 25 shows a mated triploid *C. ariakensis*. The one narrow peak at approximately 90 standard units indicates all triploid cells in the somatic tissue. Figure 26 shows a mosaic oyster determined by hemolymph tissue sample. The smaller peak to the left indicates the presence of diploid cells in the somatic tissue. Figure 27 shows a mosaic oyster determined by the gonad tissue sample. The smaller peak to the right indicates meiotic cells that have gone through chromosome doubling. The smaller peak to the left indicates diploid somatic cells present in the gonad. If these cells were meiotic then a tetraploid peak would be expected at approximately 120 standard units due to chromosome doubling in preparation for the equational second meiotic division. Mitotic cells would not have a tetraploid peak because only one division occurs in mitosis.
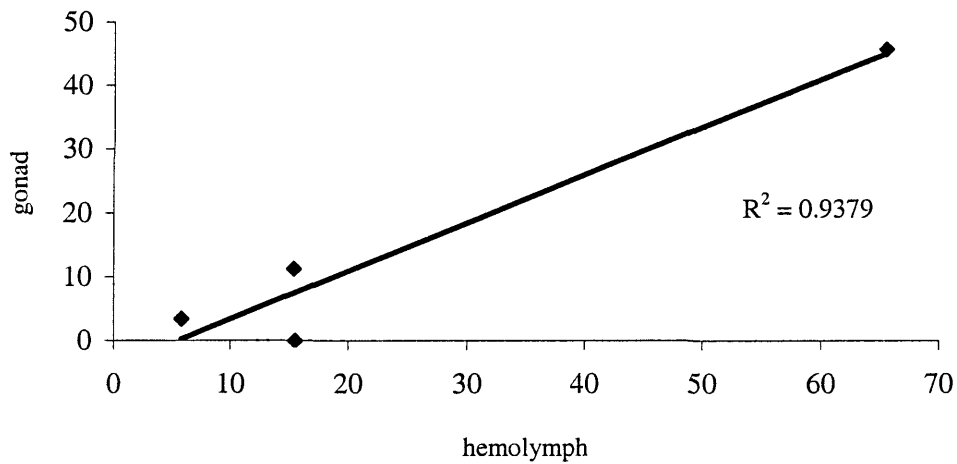
Figure 24. Four mated triploid mosaic *C. ariakensis* showing the correlation
between percent diploid cells in hemolymph and gonad tissues. R^2 included
for reference. Only four of the eleven mosaics found in this study produced
flow cytometry readings clean enough to determine the percent diploid cells
in gonad tissue.

Figure 25. Example histogram of flow cytometry on hemolymph tissue of a
mated triploid *C. ariakensis*. Peak 1=3n triploid cells at ~90
standard units. Peak 2=doublets (two cells going through the flow
cytometer at one time) of peak 1 at ~180 standard units.
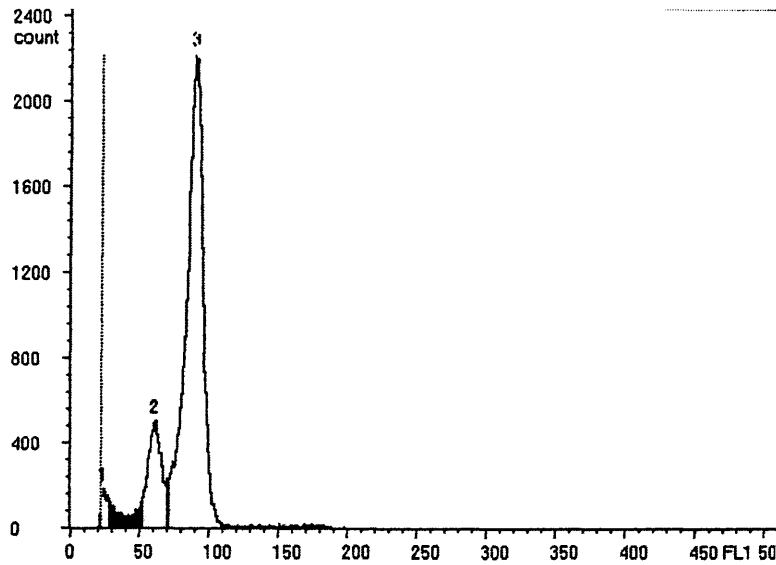
Figure 26. Example histogram of flow cytometry on hemolymph tissue of a
mated triploid *C. ariakensis* mosaic. Peak 1=standard
determined by the flow cytometer (~20 standard units). Peak
2=2n diploid cells at ~60 standard units. Peak 3=3n triploid cells
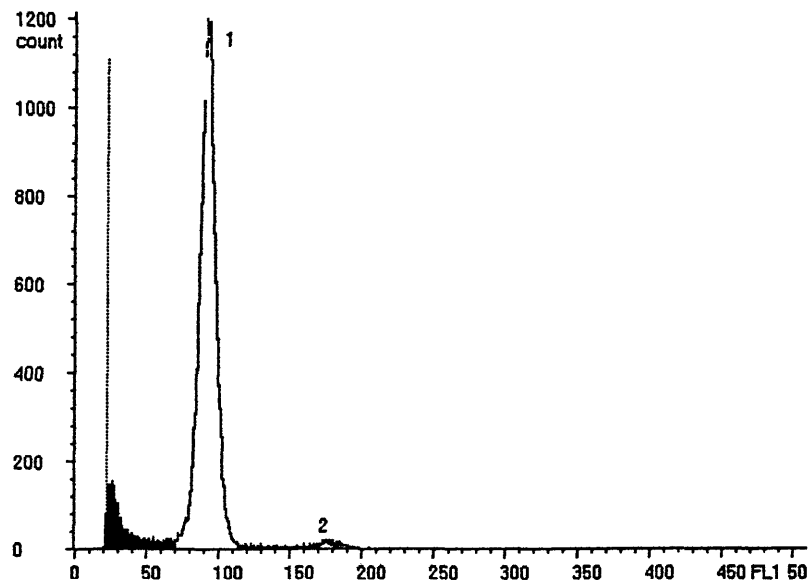at ~90 standard units. This mosaic triploid was sampled in July
2002 from site TM.
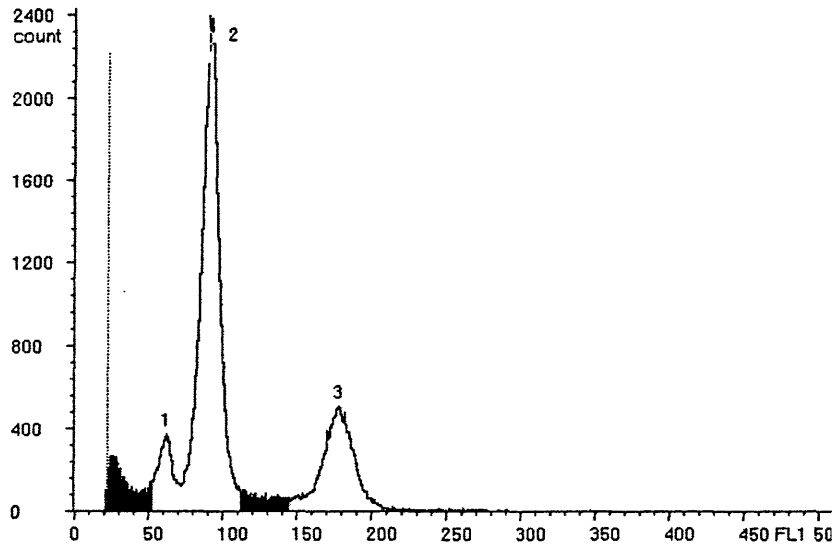
Figure 27. Example histogram of flow cytometry on gonad tissue of a
mated triploid *C. ariakensis* mosaic. Peak 1=2n diploid cells
at ~60 standard units. Most likely these cells are somatic
because a tetraploid peak was not observed at ~120 standard
units. Peak 2=3n triploid cells at ~90 standard units. Peak
3=6n hexaploid cells at ~180 standard units. The majority of
these cells may be meiotic since they represent pre-meiotic
doubling of the triploid cells. This mosaic triploid was a Late
Active female sampled in July 2002 from site TM.

DISCUSSION

Growth

The results from this study showed that *C. virginica* and *C. ariakensis* exhibited an overall positive correlation between shell height and wet weight. All correlations were statistically significant. This indicates that shell height and wet weight are dependent variables and are correlated for both species, although only about half of the variance in the data is explained by these correlations.

In my study *C. ariakensis* did not outgrow *C. virginica* across all sites (Table 4). Diploid *C. virginica* had a higher percent increase in shell height across all sites compared to triploid *C. ariakensis*. Percent increase in whole wet weight was higher at three of the five sites in *C. virginica* compared to *C. ariakensis*. The fact that *C. ariakensis* did not outgrow *C. virignica* may be a result of different age class oysters; *C. virginica* was age 1-2 and *C. ariakensis* was age 2-3.

The two species used in my study may have specific environmental or genetic factors that favor a higher growth rate. As an example of environmental factors on growth rate, Zhou (2002) showed that one-year old triploid *C. ariakensis* growth was superior to that of one-year old triploid *C. virginica* in high and medium salinity regimes. She found that the low salinity environment favored fouling by barnacles, sponges, and seaweed and reduced *C. virginica* growth rate. In another study, Calvo *et al.* (2001) demonstrated that two-year old triploid *C. ariakensis* outperformed two-year old diploid *C. virginca* in high and medium salinity environments. Once again the low salinity environment seemed subject to a lower growth rate. These two studies clearly

demonstrate that a significant difference in growth rate exists between the two species at medium and higher salinity regimes. The results from my study suggest a similar trend to that seen in Zhou (2002) and Calvo *et al.* (2001) that high and medium salinity regimes provided a more stable environment for growth.

A genetic factor that may influence growth rate is the tendency for oysters to utilize energy reserves for growth in the first year and then reallocate that energy for gametogenesis in the second year (Barber and Mann 1991; Allen and Downing 1990). It could be that the age 1-2 *C. virginica* used in this study were in the process of redistributing their energy reserves and maintained some somatic growth into their second year. This may account for comparable growth rates for both species. Whereas the other two studies, Zhou (2002) and Calvo *et al.* (2001), generally found higher growth rates in *C. ariakensis* than *C. virginica*, my study revealed equal and higher percent increases in growth in *C. virginica*.

The results from this study need to be interpreted carefully. A true comparison of growth rate would not have involved diploid *C. virginica* and triploid *C. ariakensis*. However, diploid *C. ariakensis* cannot be deployed in Chesapeake Bay, so this was the best alternative. In terms of growth, choosing a comparison between triploid *C. virginica* and triploid *C. ariakensis* would have been more advantageous, but this was a study primarily concerned with *C. ariakensis* gametogenesis.

Disease

Findings from this research support previous work (Calvo *et al.* 2001) that *C. ariakensis* are tolerant to *P. marinus* and *H. nelsoni* infections. In *C. ariakensis*, *H. nelsoni* was absent and *P. marinus* was found in low prevalence and intensity. In *C.*

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virginica, *H. nelsoni* was observed in low prevalence and *P. marinus* was observed in higher prevalence and intensity. Similarly, Calvo *et al.* (2001) found *H. nelsoni* was absent and *P. marinus* prevalence was 0-28% in *C. ariakensis*, whereas in *C. virginica H. nelsoni* was 4-8% and *P. marinus* was 100% at high salinity regimes. An important distinction between these two studies was my study used the disease tolerant DEBY strain of *C. virginica* instead of the wild Virginia strain used in Calvo *et al.* (2001). The DEBY strain has been selectively bred for five generations at VIMS to resist both *H. nelsoni* and *P. marinus* (Ragone Calvo *et al.* 1997). Disease tolerance combined with only one season of disease exposure could explain why *H. nelsoni* infections in *C. virginica* were low in this study. *P. marinus* was still present in heavy to moderate infections. Apparently, the DEBY strain seems more tolerant to *H. nelsoni* and less tolerant to *P. marinus* infections. Generally, this research found low prevalence and light intensity *P. marinus* infections in *C. ariakensis* and high prevalence and moderate to heavy intensity infections in *C. virginica*. Across all sites, *H. nelsoni* infections were low in *C. virginica* and absent in *C. ariakensis*.

It is apparent that *C. ariakensis* filters and retains some *P. marinus* parasites however moderate or heavy infections have not been observed in Virginia waters. Why is this non-native more disease tolerant than native oysters? A possible explanation may be higher concentration of hemocytes or other defense mechanism in *C. ariakensis* compared to *C. virginica*. La Peyre (1993) found that *C. gigas* increased the total number of circulating hemocytes when exposed to *P. marinus*, whereas no change was evident in *C. virginica*. Hemocyte concentration at the time of parasite exposure may control the ability of an oyster to resist infection (Fisher and Newell 1986). Hemocyte concentration and circulation in *C. gigas* and *C. ariakensis* may be similar as a result of these oysters

sharing a native range and environment. It seems that oysters originating from Japan and China are more tolerant to the local parasites *H. nelsoni* and *P. marinus* in Chesapeake Bay.

As a different mechanism against parasite infections, Oliver *et al.* (1999) suggested that *C. gigas* contains a different protein target of *Perkinsus marinus* proteases than *C. virginica*. It was shown that the *P. marinus* proteases readily attacked the protein target in *C. virginica* thus exerting virulence factors that compromises the host. The protein target in *C. gigas* does not seem to compromise the host and *P. marinus* virulence factors are either suppressed or the parasite is rapidly cleared before proliferation can occur. No studies have determined if different target proteins or suppressor mechanisms exist for *C. ariakensis*, which may translate into higher disease tolerance than *C. virginica*. However, in a preliminary study conducted at VIMS it was demonstrated that virulence factors of *P. marinus* were expressed more often in *C. virginica* than *C. ariakensis* (G. Brown, VIMS, personal communication). This may suggest that *C. ariakensis* does not have the correct protein composition to initiate and foster parasite proliferation. It seems reasonable that *C. ariakensis* may possess one or more of these defense mechanisms similar to *C. gigas* because of coexistence in their native ranges. If "disease tolerant genes" exist for *C. gigas*, a similar genetic makeup may be present in *C. ariakensis*.

Reversion

Reversion is an innate feature of *Crassostrea* species that increases at the individual and population level (Allen *et al.* 1999; Allen *et al.* 1996). This study examined the number of mosaic oysters in the population over time (Figure 22). Since

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tetraploid production is a relatively new technology for *C. ariakensis*, most studies of

reversion have focused on chemically induced triploid oysters. For example, Zhou

(2002) examined 919 age 0 to 1 chemical triploid oysters and found 23 mosaic oysters

(frequency = 2.5%). Another study (Calvo *et al.* 2001) examined 1164 age 2 to 3

chemical triploid oysters and found 62 mosaic oysters (frequency = 5.3%). In this study,

I examined 930 age 2 to 3 mated triploid oysters and found 11 mosaic oysters (frequency

= 1.2%). Furthermore, as part of a larger examination of reversion in mated triploid

oysters, VIMS/ABC sampled 2747 animals and found a total of 19 mosaic oysters

(frequency = 0.7%). It seems that mosaic oysters are more commonly found in chemical

triploid oyster populations than mated triploid oyster populations. In a direct comparison

of mated and chemical triploid *C. gigas*, mean percent mosaicism was 3.0 and 5.6,

respectively (S.K. Allen, Jr., unpublished data).

Why are mated triploid oysters apparently more chromosomally stable than

chemical triploid oysters? At this time a definitive answer is not available. To date, no

study is capable of explaining this difference.

One possible explanation for reversion in triploid oysters involves chromosome

segregation errors. This model suggests the unusual phenomenon of chromosome

clumping and subsequent elimination of multivalents during mitotic metaphase in *C.*

gigas and *C. ariakensis* (Allen *et al.* 1999; Zhang *et al.* unpublished data). The

hypothesis put forward was that mosaic oysters with a higher percentage of clumped

chromosomes resulted in more hypotriploid cells. Therefore, chromosomes in the clumps

may be lost from daughter cells as they are unable to take part in normal segregation

(Zhang *et al.* unpublished data). Since cells are constantly dividing, the number of errors

(i.e., chromosomes lost) is going to accumulate. Potentially this explains the increase in mosaic oysters over time.

Another explanation may involve the mechanism by which mated triploid oysters are produced. Specifically, the "ripe" triploid female oysters identified to be used as parents to produce tetraploid oysters (Guo and Allen 1994a) must have segregated chromosomes correctly. It may be that a regulatory mechanism exists, such as a "segregation gene" facilitates successful synapsis in some triploid oysters. Clearly, segregation of the extra set of chromosomes in triploid oysters is problematic, typically resulting in abnormalities or multivalent formation of chromosomes. However, some organisms have overcome this by the use of genetic mechanisms. Common wheat, *Triticum vulgare*, is an allohexaploid that contains three homoeolgous genomes (Riley and Chapman 1958). During prophase chromosomes typically pair in groups of up to six, however, by metaphase I only homologous bivalents are formed (Riley and Chapman 1958). It seems that there is an elimination mechanism that ensures normal bivalent formation by removing the multivalents formed during prophase. This phenomenon has been reported to largely be under the control of the *Ph* (Pairing homoeologous) locus in wheat (Swanson *et al.* 1981). It has been shown that this gene can suppress or enhance its stabilizing capabilities and maintain homologous pairing. For example, Swanson *et al.* (1981) recognized that the monosomic state of this gene compensates for the lack of chromosomes and maintains diploid-like behavior. Furthermore, when this gene is in a trisomic or tetrasomic state, it becomes depressant by a progressive reduction in chiasmata (crossing over) frequency. A specific example involved the hybrid *T. boeticum* x *Aegilops mutica* in which homoeologous pairing is typically present,

however, the presence of this gene lowered the chiasmata frequency from an average of 4.5 per cell to below 1.0 (Swanson *et al.* 1981).

Could a *Ph*-like gene be present in triploid oysters? The "segregation gene" hypothesized previously may act as the *Ph* gene in wheat does to properly segregate homologous chromosomes. It is possible to envision this gene working irregularly in triploid oysters because this is an unnatural condition that has not evolutionarily evolved over time like some naturally occurring polyploid organisms (i.e., ferns and angiosperm plants). This irregular "segregation gene" could act on triploid cells within an oyster to produce some diploid cells yielding a mosaic oyster.

An important aspect of this study was to follow reversion in different tissue types. As it pertains to this study, flow cytometry was used as a tool that allowed rapid quantification of DNA content in individual cells from a heterogeneous population. Chandler *et al.* (1999b) examined two-year old *C. ariakensis* mosaics and determined that hemolymph was the most sensitive indicator of reversion. My study used hemolymph tissue as the indicator of reversion and subsequently sampled gill, gonad, heart, and adductor tissue when reversion was detected. Reversion was quantified according to the percent diploid cells contained in specific tissue samples within a triploid individual. This study found two mosaic oysters that had an unusually high percentage of diploid cells (65 and 44%), however the other nine individuals had less than 20% diploid cells. Similarly, Zhou (2002) observed generally less than 10% diploid cells in mosaic oysters, but two individuals produced 50 and 70% diploid cells. The results from this study indicate if a high percentage of diploid cells were found in hemolymph tissue, then similar proportions were observed in other tissues. Of particular interest were the gonad tissue samples. The presence of diploid cells in the gonad tissue could indicate the

potential for production of haploid gametes. To date, no haploid gametes have been observed in mated triploid *C. ariakensis* in this or other studies.

Comparing both *C. gigas* and *C. ariakensis*, it is apparent that reversion in mated triploid oysters is lower than that of chemical triploid oysters (S.K. Allen, Jr., unpublished data; Zhou 2002). My study found 1.2% mosaicism in the mated triploid oyster population whereas Zhou (2002) found 2.5% mosaicism within the chemical triploid oyster population of *C. ariakensis*. This suggests that the preferred method is the mated production of triploid oysters for use aquaculture. My study was not capable of tracking reversion in age 0-1 mated triploid oysters because they were not available, however, Zhou (2002) followed reversion in age 0-1 chemical triploid oysters (essentially from seed to market size). It is reasonable to extrapolate that mosaicism may be lower than 2.5% in mated triploid oysters age 0-1. From an aquaculture perspective of growing oysters from seed to market size, the risk associated with reversion in mated triploid oysters is nearly zero. Aquaculture practices rely on complete harvesting. Therefore, only "lost" or "unharvested" product may eventually undergo reversion.

Gametogenesis

Histology of diploid gametogenesis revealed normal development in concordance with literature descriptions (Kennedy and Krantz 1982; Kennedy and Battle 1963). Variation was observed from site to site. In May, most diploid *C. virginica* were actively developing oocytes and spermatocytes. By June and July, well-developed follicles contained either mature ova or spermatozoa. By August, some diploid oysters showed signs of spawning, and in October, all diploid oysters were either spawned completely or were resorbing.

Gametogenesis in triploid oysters was somewhat reduced and abnormal. Most triploid oysters exhibited relatively fewer gametes and far less follicle development compared to the baseline diploid *C. virginica*. The animals that produced mature gametes appeared similar to diploid oysters. Triploid male *C. ariakensis* contained developing spermatocytes and numerous spermatids. Differentiated spermatozoa were common. Triploid female *C. ariakensis* contained ripe ova free in the lumen of follicles and gonoducts. At this stage, "ripe" triploid *C. ariakensis* were indistinguishable from diploid *C. virginica*. Surprisingly, at least one "ripe" triploid *C. ariakensis* out of 25 sampled at each site was found during July; at TK five "ripe" triploid male *C. ariakensis* were observed (frequency = 20%). Actively developing triploid *C. ariakensis* (including Early, Middle, and Late Active stages) were common during July at 46.6%. Across all sites in July, 17 out of 148 triploid *C. ariakensis* (frequency = 11.5%) produced fully mature sex cells. This study found that triploid male *C. ariakensis* are more capable of producing intermediate cell types (i.e., spermatocytes) than triploid female *C. ariakensis*. Typically, triploid male oysters can produce significant spermatocytes, spermatids, and spermatozoa, however, triploid female *C. ariakensis* either produce sparse amounts of mature oocytes and arrested oogonia, or an abundance of mature oocytes and no oogonia. These findings corroborate the results from Allen (1987) that demonstrated triploid male *C. gigas* develop uniformly compared to triploid female oysters. In fact, several studies from the literature have focused on triploid gametogenesis in shellfish.

Triploid *Mercenaria mercenaria, Mya arenaria, Chlamys nobilis, Argopectin irradians, A. ventricosus*, and *Saccostrea commercialis* all exhibit functional and morphological reduction of gametogenesis (Eversole *et al.* 1996; Allen *et al.* 1986; Komaru and Wada 1989; Tabarini 1984; Ruiz-Verdugo *et al.* 2000; Cox *et al.* 1996). In

general, the authors found some male and female triploid shellfish were capable of very early gametogenesis. Triploid male shellfish could produce some spermatocytes and females occasionally produced oocytes, but arrested gonia were more common. In sharp contrast, *C. gigas*, *C. virginica*, and *Pinctada fucata* were capable of producing spermatozoa and mature oocytes (Allen 1987; Allen *et al.* 1986; Komaru and Wada 1990). My study revealed triploid *C. ariakensis* could attain active stages and readily exhibited mature stages of development during the reproductive season. What mechanism might be responsible for the varying degrees of sterility in triploid oysters?

First, it seems reasonable to accept that triploid shellfish are sterile to varying degrees because they contain an extra set of chromosomes that acts differently depending on the species. This addition could disrupt meiosis in several ways. Allen *et al.* (1986) suggested that triploid *M. arenaria* with a chromosome complement of 2N=34 and 3N=51 either asynapsis or multivalent formation would result in abortive or abnormal gametogenesis. The odd number of chromosomes in triploid *M. arenaria* would frequently result in aberrant meiosis. However, the few gametes formed may indicate a mechanism to overcome this sterility. The authors hypothesized that the few oocytes observed in triploid *M. arenaria* could have arisen from multivalent formation/aberrant segregation. The oocytes appeared to have developed into auxocytes suggesting that gametogenesis is arrested, rather than completely abortive, in triploid *M. arenaria*. In another example of triploid sterility, Grey and Mackiewicz (1980) found that the triploid 'race' (3N=30) of trematode, *Glaridacris catastomi*, forms mulitvalents in the spermatocytes disrupting further meiotic divisions. Specifically, multivalent formation resulted in nondisjunction during anaphase I yielding abnormally large spermatids that simply differentiated instead of undergoing further divisions. Rarely do the latter stages

of meiosis, anaphase and telophase II, show isolated fragments or whole chromosomes. This resulted in variations within chromosome number and size, confirming that occasionally a large sperm underwent "one-division meiosis". It is clear that spermatogenesis in triploid *G. catastomi*, if "completed", contains an irregular assortment of chromosomes and genes, which could account for observed sterility (Grey and Mackiewicz 1980).

A second mechanism for triploid sterility may be failure of homologous chromosomes to synapse (Allen *et al.* 1986). Sakaguchi (1980) reported asynapsis occurring in spermatocytes and oocytes in the triploid form of the common liver fluke, *Fascioloa* sp., in Japan. This resulted in reproductive sterility. Both oocytes and spermatocytes contained 30 univalent chromosomes, clearly suggesting sterility via asynapsis because no synaptic mate was available. Lack of homologues pairing would cease meiosis and abort gametogenesis. Spermatogenesis was aberrant and no sperm were found, however, it was suggested that oocytes divided mitotically to produce triploid eggs (Sakaguchi and Tada 1980). Different species may be more successful than others at polyploid synapsis.

Clearly, certain triploid species avert the problem of sterility to some degree, but how? One explanation may be that "clumping" of extra chromosomes in just the right number or configuration during mitotic divisions could restore certain aspects of gametogenesis (Allen *et al.* 1999; Zhang *et al.* unpublished data). Those cells destined to enter meiosis would then have the appropriate chromosome number to at least initiate gamete development. This phenomenon may happen variously within individuals, populations, or species, accounting for variation in fecundity. For example, a triploid cell undergoing mitosis would conceivably replicate chromosomes and initiate segregation

into daughter cells. Of course this process is irregular and coalescence of uni-, bi-, and trivalents would aid in the elimination of some chromosomes. Mitotic cells that have gone through DNA synthesis (replication phase) align chromosomes along the metaphase plate in preparation for anaphase segregation. However, it has been proposed that tripolar spindle fibers may not have proper anaphase tension thereby releasing pieces or whole chromosomes (Allen *et al*. 1996). This mechanism could result in a relaxation of complete triploid sterility and explain the observed fecundity in triploid oysters.

Another possible explanation for triploid sterility is meiotic checkpoints. It is feasible to have repair mechanisms prior to meiotic configurations or divisions that would allow for some "normal" gametogenesis. For example, it was documented that male and female triploid catarina scallops, *A. ventricosus*, arrest gametogenesis at the prophase I stage of meiosis (Maldonado-Amparo and Ibarra 2002). The authors hypothesized that a pachytene checkpoint had recognized errors or problems, corrected them, and allowed certain triploid females to produce some gametes. Oliveria *et al*. (1995) found that in male triploid rainbow trout multivalents formed in zygotene were eliminated by the end of pachytene. The authors hypothesized that a repair mechanism may be responsible for correctly forming the synaptonemal complex in spermatocytes. These meiotic repair checkpoints or mechanisms largely are species specific, but variation can also exist within a species. For example, Gui *et al*. (1992) demonstrated that repair mechanisms were not used to correct multivalent formation during metaphase I in triploid male colored crucian carp, *Carassius auratus*, and males remained sterile. Some females, on the other hand, were able to produce oocytes. The females that contained primary oocytes had mostly bivalent and univalent pachytene chromosomes along with a few trivalent and multivalent chromosomes (Gui *et al*. 1995). This suggests a meiotic

checkpoint or repair mechanism is present in female triploid colored crucian carp yet is apparently absent in triploid males. Triploid sterility seems to vary among and within certain triploid species. Corrective mechanisms may account for overcoming obstacles in triploid gametogenesis. Is it possible that triploid *C. ariakensis* exhibits within species variation and some individuals are more capable of circumventing sterility?

An important distinction between studies from the literature and this research is that I used mated triploids (refer to Materials and Methods). As mentioned above, I observed an unusual number of triploid male and female oysters that produced "ripe" or mature gametes. It seems that gametogenesis in mated triploid *C. ariakensis* was reduced and abnormal compared to diploid *C. virginica*, but apparently more mature individuals were observed in mated triploid oysters than triploid oysters from other studies. Are mated triploid oysters more capable of completing gametogenesis than chemical triploid oysters?

A study examining gonadogenesis in crossbred and chemical triploid *C. gigas* demonstrated at one site, 33 of 39 (85%) mated triploid *C. gigas* had a higher cross-sectional gonad (>50%) compared to 18 of 29 (62%) chemical triploid *C. gigas* (Eudeline and Allen 2000). The authors also found that gametogenesis revealed mated triploid *C. gigas* females had a higher tendency to produce oocytes than chemical triploid *C. gigas* females and one exceptionally fecund mated triploid *C. gigas* male was observed. Aspects of gametogenesis may be different among mated and chemical triploid *C. gigas* as well as *C. ariakensis*. My study did not examine gametogenesis in chemical triploid *C. ariakensis*, although, it is apparent that mated triploid oysters seem to have a propensity to produce mature gametes.

My study shows that high fecundity in two-year old mated triploid *C. ariakensis* is possible. Across all sites from May to August, 91 out of 570 (15.5%) triploid oysters were fecund. Similarly, Eudeline and Allen (2000) showed mated triploid *C. gigas* had the capability of producing "exceptionally" fecund males and females. At one site, 2 females out of 19 (11%) and 15 males out of 39 (39%) were fecund, and at another site, 0 females out of 26 (0%) and 4 males out of 17 (24%) were fecund. It seems that these triploids are overcoming difficulties associated with the extra set of chromosomes, by elimination or repair mechanisms.

An alternative explanation for higher fecundity observed in mated *C. ariakensis* derives from the method used to produce these animals. By implementing tetraploid technology (Guo and Allen 1994a), a very "ripe" triploid female oyster is identified and used to produce tetraploids, which in turn are used to produce mated (tetraploid x diploid) triploid oysters. I hypothesize that the maternal contribution from triploid eggs includes one or several "fecund genes" that are passed onto the tetraploid progeny and are subsequently responsible for the increased fecundity observed in mated triploid oysters. It is only by chance that these "fecund genes" will be expressed and not all mated triploid oysters will produce mature gametes. But it is not hard to envision future generations of mated triploid oysters increasing their potential to produce numerous gametes if this "fecund gene" is passed on to progeny. It would follow that subsequent mating of a tetraploid male with a diploid female could in fact pass on "fecund gene(s)" to the triploid progeny. Although specific examples from the literature do not exist to corroborate this hypothesis, it is feasible that fecundity is genetically controlled to some degree.

Certainly not all mated triploid oysters would receive the gene that codes for fecundity, so what controls this variability? It is known that eukaryotic species contain an essential set of chromosomes, the A-chromosomes, which function as the basic genome. However, a secondary set of chromosomes, the B-chromosomes, has been identified in some species of molluscs, insects, and flatworms (Swanson *et al.* 1981). For example, in a diploid oyster (2N=20) both the A and B set of chromosomes would be involved in gene expression, possibly one set more than the other. The B-chromosomes are therefore as much a part of the crossbred make-up as the A-chromosomes. It may be hypothesized that these B-chromosomes, if present in oysters, controls the "fecund gene". Interestingly, the *Ph* gene discussed earlier, which ensures bivalent formation in hexaploid *T. vulagare*, is contained on the B5 chromosome (Swanson *et al.* 1981). Possibly a *Ph*-like gene contained in the B-chromosomes of triploid oysters would assist in pairing two of the three homologues, leaving the extra chromosome to essentially disintegrate. My results indicate that some triploid oysters are capable of producing numerous gonia cells and undergoing gametogenesis. In some individuals this is a complete process and in others it is completely abortive. There exists a gradation of sexually mature and immature triploid oysters that produce a gradient of sex cells occurring throughout the reproductive season. Remarkably, the majority of these triploid oysters showed signs of spawning. In contrast to diploid *C. virginica* that spawned predominantly fully mature sex cells, triploid *C. ariakensis* spawned both immature and mature gametes.

In my study triploid male and female oysters spawned. From an aquaculture standpoint this doesn't necessarily seem relevant however ecologically it seems important for estimating the reproductive likelihood of mated triploid *C. ariakensis* in Chesapeake

Bay. Obviously reproductive potential of these non-natives has implications on biological control and colonization of a sustainable non-native population. There are several fates for spawned triploid gametes: triploid sperm could fertilize triploid eggs from another individual, triploid sperm could fertilize triploid eggs from itself, triploid sperm could fertilize diploid *C. virginica* eggs, or diploid *C. virginica* sperm could fertilize triploid eggs. Ahmed (1973) demonstrated that hybridization between *C. virginica* and *C. ariakensis* produced viable progeny however a more recent study (Allen *et al.* 1993) determined that this specific hybridization did not produce viable larvae after 8-10 days. Allen *et al.* (1993) also demonstrated that fertilization between these two species had relatively few barriers. It may be hypothesized that spawned sex cells from triploid *C. ariakensis* may act as a sink for *C. virginica* gametes and that hybridization would not occur.

To estimate the reproductive capacity of mated triploid *C. ariakensis* I have taken some values from the literature and employed the results from my study. For example in my study, out of 570 triploid oysters examined from May to August 41 ripe females (7%), 38 ripe males (6%), and 11 ripe hermaphrodites (2%) were identified. Fully reduced 1.5N aneuploid sperm was observed and confirmed by FCM, and mature and unreduced triploid eggs probably occupied follicles, both gametes capable of fertilization. From the literature, Guo and Allen (1994b) reported that survival (3 months post fertilization) in *C. gigas* triploid x triploid (TT) crosses was 0.0085%. Since this research has not been conducted for *C. ariakensis*, I will assume the survival parameter in *C. gigas* TT crosses is the same. The authors also determined that diploid x diploid (DD) survival was 20.6% and triploid female fecundity was 2% that of diploid females. Based on the TT survival parameters, an estimate of DD *C. ariakensis* survival to be 20%, the mated

triploid fecundity of 7% from my study it may be reasonable to estimate reproductive capacity for TT crosses at 0.003% (0.000085/0.20*0.07). For example, when diploid oysters have 1 million chances to survive and reproduce, mated triploid *C. ariakensis* would have 30. This estimate is unlikely to change if I do the reciprocal cross using 6% fecundity that represents mated triploid male oysters. The reproductive capacity of hermaphrodites (assuming both sperm and egg are functional) would be 0.0008% (or 8 in 1 million).

These estimates of reproductive capacity in mated triploid *C. ariakensis* are preliminary and require further research. Of course several environmental aspects including gamete retention, estuarine circulation, and temperature and salinity effects have been overlooked in this calculation. In terms of risks associated with triploid *C. ariakensis* field experiments, the reproductive capacity lies in TT crosses since apparently hybridization is not an issue. It has been shown that the ploidy of progeny from TT crosses in *C. gigas* is approximately 90% triploid oysters (Guo and Allen 1994b). The possibility of producing a diploid oyster is therefore not zero (Guo and Allen 1994b). Presumably, these estimates for TT progeny would hold for *C. ariakensis* as well.

Further study on the reproductive capacity of mated triploid *C. ariakensis* and diploid-triploid mosaic *C. ariakensis* may yield more concrete results to assess non-native introductions.

Hermaphrodites

One interesting feature of triploid *C. ariakensis* was the occurrence of hermaphrodites. The number of hermaphrodite oysters varied from site to site. For example, only five percent of triploid *C. ariakensis* were hermaphrodites at JH yet 24%

were hermaphrodites at BO. In the July sample (n = 25), 14 hermaphrodites were

observed at BO (frequency = 56%). In my study, masculine hermaphrodites were most

common (74 out of 122; frequency = 71%), then hermaphrodites with equal amounts of

gametes from each sex (31 out of 122; frequency = 25%), and finally feminine

hermaphrodites (17 out of 122; frequency = 14%). Allen (1987) suggested that

spermatogenesis is predominantly a function of the ability of sister chromatids to

segregate during meiosis II. He went further to hypothesize that nondisjunction or other

pairing abnormalities would not necessarily prevent spermatogenesis. Oogenesis, on the

other hand, requires a large amount of RNA synthesis and subsequent transcription and

storage in the oocytes (Allen 1987) increasing the energetic demand compared to

spermatogenesis. In hermaphrodites, this may indicate that when both sex cells are

developing and the oyster has a "decision" to make, spermatogenesis prevails.

Hermaphrodites were rare in diploid *C. virginica*, ranging from 0-3% across all

sites. Not surprisingly, hermaphrodites were also rare in diploid *C. gigas*, 0-1% (Allen

1987).

In its native range, *C. ariakensis* is classified as an oviparous oyster, as sex is

usually stable during the spawning season, and the occurrence of hermaphrodites is rare.

Generally, *Crassostrea* species are termed protandric hermaphrodites meaning they have

the ability to change sex, but sex begins as male. Asif (1979) reported that

hermaphrodites in *C. rivularis* (*=ariakensis*) were rare, 7 out of 955 (0.81%).

Why then do triploid oysters exhibit such a higher number of hermaphrodites

during the reproductive season? One possible explanation may be environmental

conditions. Vaschenko *et al.* (1997) suggested that a polluted grow-out area could

increase hermaphroditism in a population of oysters. In this study, the number of

hermaphrodites at each site was generally inversely proportional to salinity. This could indicate that a harsher environment (i.e., low salinity environment) may produce more hermaphrodites. The results presented here cannot provide a definitive answer because diploid *C. ariakensis* could not be deployed and environmental assessments were not done for each site. Zhou (2002) suggested that adverse environmental conditions might increase the number of mosaic oysters observed at that site. It is possible, however, to relate the number of mosaic oysters with the number of hermaphrodites, both possibly arising from unstable environmental conditions. BO site had the highest number of mosaic oysters and hermaphrodites, but the lowest salinity. Furthermore, in the comparison of triploid and diploid gametogenesis, triploid oysters at this site were most like diploid oysters (Figure 20B). Lower salinity environments, such as TK and BO, exhibited a higher number of hermaphrodites and the triploid gametogenesis lag was less pronounced compared with other sites. Could this be an indication that environment suppresses certain mechanisms in triploid oysters such as sex determination and sterility?

Environment and Fecundity

A major finding of this research was the exceptional fecundity observed in some mated triploid *C. ariakensis*. These individuals had the ability to produce numerous mature sperm and ova. Interestingly, the vast majority of fecund males were found at sites BO and TK and most of the fecund females were found at sites TL and CS. The lower salinity environments, BO and TK, apparently favored spermatogenesis and the occurrence of masculine hermaphrodites. On the other hand, moderate salinity environments, such as TL and CS, favored oogenesis and contained fewer hermaphrodites. Identifying fecund triploid female oysters is paramount for the

production of tetraploid oysters. The results from this study suggest that moderate salinity environments provide the most favorable conditions to produce fecund triploid female oysters. From an aquaculture perspective this piece of information is valuable for the continued production of mated triploid oysters. Brood stock may be obtained from various sites in moderate salinity, thereby maintaining genetic diversity.

The results from lower salinity environments indicate that one may expect higher triploid fecundity, hermaphroditism, and mosaicism when culturing mated triploid *C. ariakensis*. An important caveat to this hypothesis is that all other sites, where higher and moderate salinities prevailed, also produced fecund triploid oysters, some hermaphrodites, and a few mosaic oysters.

Sex ratio

Diploid *C. virginica* generally had equal numbers of males and females across all sites, with little variation. In sharp contrast, triploid *C. ariakensis* distinctly had three "male sites" and three "female sites". The incidence of masculine hermaphrodites was 4.5 times greater than feminine hermaphrodites. This may indicate that a sex determining mechanism in mated triploid *C. ariakensis* is paternally mediated. A few hypotheses have been suggested for a sex determination mechanism in oysters.

Haley (1977) proposed a multiple allele sex determining mechanism for *C. virginica*. This model contained three loci (a, b, c) and two alleles (male and female). Essentially, an oyster with three male alleles or more and one fixed loci was considered male. Conversely, a female would have five or six female alleles and at least two fixed loci. Working through the various genotype combinations suggests that increased heterozygosity would favor maleness, which is significant for age 0-1 oysters. Haley's

model provides some evidence of genetic control for determining sex in oysters, but was not well accepted at the time.

A more refined approach to sex determination in *C. gigas* was done by Guo *et al.* (1998). The authors proposed a single locus model with alleles 'M' for dominant maleness and 'F' for protandric femaleness. They indicated that there is significant paternal yet little maternal influence on sex determination. The Guo *et al.* model would give two genotypes for males, however, only one genotype for females. This, in part, comes from the single locus model but also from Coe (1932) that hypothesized two types of males: true males that contained very few and small oocytes, and protandric males that contained mature larger oocytes. Guo *et al.* (1998) concluded that multiple genotypes for males to account for all variation (i.e., Haley's model) was unnecessary because some of the variation in sex ratio is probably dictated by secondary genes and environmental factors.

Both models fit their respective data sets well. Hermaphrodites are rare in nature so these models essentially need not explain that subtle incidence. However, the Guo *et al.* (1998) model suggested that the occurrence of functional hermaphrodites could be caused by genetic abnormalities. This would certainly account for the increased number of hermaphrodites observed in triploid oysters. I have hypothesized that genes control such factors as fecundity and reversion in mated triploid *C. ariakensis*. Genetic control could determine the synapses, potential for crossing over, and whole or partial chromosome loss. Even though it may not be necessary to account for hermaphroditism in diploid oysters, it seems important to understand this variation for triploid oysters. My data suggests that in a large enough sample size, sex ratios will tend to be equal in triploid *C. ariakensis* (overall, 234 males to 287 females), but sex ratios at individual

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sites are heavily skewed (for example at TK, 67 males to 21 females). Recall, Guo *et al.*

(1998) found sex determination to be under paternal control, therefore in mated triploid

oysters the paternal contribution is greatest, possibly increasing the probability of passing

on "sex chromosomes". The tetraploid father, with diploid (instead of haploid) sperm,

contributes a "double dose" of paternal genes to the mated triploid progeny. I

hypothesize that the sex determining mechanism in mated triploid oysters would still

contain two alleles 'M' for male and 'F' for female, with 'M' being dominant. The

addition of another set of chromosomes would give an extra 'M' or 'F' allele to the

genome, resulting in some oysters having a 2:1 M or F allele ratio. For example, a

triploid oyster may be MMF or MFF. This intermediate genotype may give rise to

varying degrees of hermaphrodites because mitotic divisions could become "confused"

and begin to produce the opposite sex gametes. As shown by Allen (1987) and Haley

(1977), increased heterozygosity favors males. This may corroborate the results from my

study, which found a tendency for masculine hermaphrodites. A departure from the

MMM genotype for triploid male oysters and FFF genotypes for triploid female oysters,

such as MMF, could result in spermatogenesis occurring to some degree. Clearly, the

extra set of chromosomes in triploid oysters disrupts several aspects of meiosis; therefore

it is reasonable to expect some triploid oyster genomes to include all or parts of the "sex

chromosomes". Loss or retention of these chromosomes may involve a meiotic

checkpoint protein that ensures chromosome pairing or an elimination mechanism for the

third chromosome. This undoubtedly reflects an incomplete process in triploid oysters

that may account for skewed sex ratios in triploid oysters. This system would favor a

skewed ratio of males or females (all males or all females) and a propensity for masculine

hermaphrodites as the histology of gametogenesis in mated triploid *C. ariakensis* has suggested.

Summary and Conclusions

1.) *H. nelsoni* was absent and *P. marinus* occurred at a very low prevalence in triploid *C. ariakensis*.

2.) The proportion of mosaic oysters in this population was 1.2%. Nine individuals contained less than 20% diploid cells in reverted tissue, but two animals had 40 and 70%.

3.) Across all six sites and overall, triploid oyster sex ratios were nearly equal. On a site by site basis, three sites were distinctly male and three were distinctly female. Hermaphrodites were found at each site. Masculine hermaphrodites were most common.

4.) Triploid gametogenesis was reduced and abnormal compared to diploid *C. virginica*. Generally, triploid follicles were underdeveloped and occurred sporadically. Intermediate gametes were observed mostly in triploid male oysters. Fecund triploid males, females, and hermaphrodites were observed at each site. Spawning occurred in triploid oysters. Apparently, timing of this event coincided with spawning of diploid oysters which presumably released all mature gametes; however, histology revealed that triploid oysters released both immature and mature gametes.

The main goal of this research was to assess gametogenesis. Two objectives were associated with this goal. First was to determine the abundance of "ripe" triploid females for use in production of tetraploid oysters for commercial scale production of 100%

mated triploid oysters. This is clearly an economic objective that is important for aquaculture and the resurrection of a dying oyster industry in Virginia. Second was to determine the extent of sterility in triploid oysters. Ecologically this has far-reaching implications for introducing a non-native oyster into Chesapeake Bay, Virginia.

This study has shown that triploid *C. ariakensis* are "fecund-enough" to produce the occasional "ripe" female necessary to produce tetraploid oysters. The rate-limiting step of commercial-scale triploid production is ability to obtain triploid females. If mated triploid oysters were gametically sterile (Chevassus 1983), like triploid *M. mercenaria* (Eversole *et al.* 1996), then producing tetraploid oysters would be impossible. Such is not the case. "Ripe" triploid female oysters were found at all six sites in at least one sampling period. Skewed sex ratio at certain sites was observed, for example, CS had 11 males and 82 females, and TK had 67 males and 21 females. But this result could be vital for producing tetraploid oysters. The oyster industry in Virginia has been supportive of research and development for techniques that would kick start non-native research and ultimately lead to a new oyster fishery. They have committed time, money, and manpower toward this goal. The successful production of tetraploid *C. ariakensis* (S.K. Allen, Jr., unpublished data) has allowed field deployments of mated triploid oysters to be scaled up. Currently, there is a plan to deploy one million mated triploid oysters in the fall of 2003. This is a golden opportunity to study further the biology and economics of this non-native oyster candidate in Chesapeake Bay. Researchers and industry members are working together to assess market acceptance, analyze cost benefits, quantify disease, document reversion, follow gametogenesis, and develop molecular markers. The National Academy of Sciences has been investigating the literature on risks and benefits of *C. ariakensis* in Chesapeake Bay and their recommendations are due in summer 2003.

However, it seems apparent that stakeholders in Virginia (i.e., researchers and the seafood industry) are already prepared and eager to determine the advantages and disadvantages of *C. ariakensis*.

If approved in Virginia's portion of the Chesapeake Bay, aquaculture of mated triploid oysters could flourish. It is possible to envision a revitalized oyster industry in Virginia, at the beginning consisting primarily of crossbred triploid oyster seed produced in a research hatchery and disseminated to aquafarms for grow-out. Then, tetraploid oyster brood stock may be distributed to appropriate hatcheries in the Bay for triploid oyster production at several sites, thus maintaining a robust gene pool. Further genetic manipulations, back at the research hatchery, may lead to successful tetraploid x tetraploid crosses in both *C. ariakensis* and *C. virginica* which may allow for hybridization. Although hybridization between these two species has been unsuccessful at the diploid level it could be successful at higher ploidy levels. All of these non-native applications would be proceeding along side native research on disease tolerance and restoration. Possibly, in the future, a "super oyster" may be available which contains genes from *C. virginica* that have evolved over millions of years to survive harsh Chesapeake Bay conditions and genes from *C. ariakensis* that promote fast growth and disease tolerance.

The results from this study from an ecological standpoint demonstrate mated triploid oysters are capable of producing gonia cells that can undergo gametogenesis and achieve fully mature sex cells. Clearly, however, both mature and immature sex cells were released. The implication for Chesapeake Bay is unknown. Judging from the data so far, mature gametes are aneuploid (abnormal 1.5N) gametes. This study found no true haploid gametes, only aneuploid in the sense that they were fully reduced "triploid

gametes", yielding 1.5N. It is not possible to rule out that haploid gametes may be observed in a future study.

The reproductive potential of these triploid oysters needs to be examined further, but the estimates from this study suggest a very small chance (0.003%) of triploid x triploid survival and reproduction.

Further study is required to gain a better understanding of mated triploid *C. ariakensis* for both aquaculture and research. A reproductive assessment to determine the viability of gametes, fertilization rate, development, and viability of larvae in triploid x triploid crosses is necessary. Research to elucidate control mechanisms, such as "segregation genes", "fecund genes", or "sex genes" in triploid *C. ariakensis* is necessary.

APPENDIX I

Definitions of terms

ANEUPLOID – A cell or individual that lacks a multiple of the haploid set of chromosomes (i.e. a fully reduced triploid sperm has a chromosome complement of 1.5n).

AQUACULTURE – The culture or growing of an aquatic organism in a semi-controlled environment.

AUXOCYTE – A cell, such as a primary oocyte, that is destined to enter the meiotic cycle.

DIPLOID – The natural complement of two chromosome sets (20 chromosomes in an oyster=2n).

FECUND (or Ripe) – An oyster that produces numerous mature follicles and gametes.

FLOW CYTOMETRY – Stained DNA from the nucleus of individual cells is quantified yielding a histogram of ploidy types.

GAMETOGENESIS – The maturation of undifferentiated sex cells (oogonia or spermatogonia), which become mature through meioses producing oocytes or sperm.

HERMAPHRODITE – An individual that contains both male and female sex cells.

HOMEOLOGOUS – Chromosomes that are only partially homologous and only pair with other homeologues.

HOMOLOGOUS – Chromosomes that contain the same genetic information and pair during meiosis; one homologue is from the mother and the other from the father.

HYPOTRIPLOID – Cells that contain one or more chromosomes less than the characteristic triploid number (i.e. 3n-1, 3n-2, etc.).

INTERSEX – An oyster that is not male or female, but exhibits sexual characteristics between males and females.

MATED – Hatchery mediated spawn between tetraploid male and diploid female oysters.

MOSAIC – An individual that contains more than one ploidy type.

RESISTANT – No disease infection present.

REVERSION – In oysters the gradual transition from triploid to diploid cells.

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STERILITY – Incomplete or complete loss of gamete production.

TETRAPLOID – An oyster that contains four chromosome sets (40 chromosomes in an oyster=4n).

TOLERANT – Disease infection observed but does not cause mortality.

TRIPLOID – An oyster that contains three chromosome sets (30 chromosomes in an oyster=3n).

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