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The Production of Triploid Oyster Larvae (*Crassostrea Virginica* (Gmelin)) in Louisiana.

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THE PRODUCTION OF TRIPLOID OYSTER LARVAE
(*CRASSOSTREA VIRGINICA* {GMELIN}) IN LOUISIANA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Forestry, Wildlife and Fisheries

by

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ABSTRACT

Gonadal recycling of oyster broodstock, salinity effects during induction, and cytochalasin B (CB) dosage were investigated as factors affecting the production of triploid oyster larvae in Louisiana. These factors were studied at an oyster hatchery on Grand Isle, LA during 1992-1994 to help transfer triploid oyster technology to the Gulf region.

Oyster broodstock were held nearshore in Caminada Bay, Louisiana and histologically analyzed between induced spawnings and during the winter of 1992-1993. Gametogenesis was analyzed qualitatively by developmental staging and quantitatively using mean gonad/body ratios (GBR) per section. The monthly percent occurrence of the developmental stages, as well as histological evidence, showed that gonadal recycling was occurring. Significantly different mean GBR's were found between developmental stages and between successful and unsuccessful spawning attempts ($P < 0.05$).

Experiments were conducted by exposing fertilized oyster eggs to 10 ppt, 20 ppt and 30 ppt seawater after a one week broodstock acclimation period at 13 ppt, 20 ppt and 30 ppt. Resulting polar body extrusion data revealed significant differences between mean synchrony levels of

broodstock salinity*treatment salinity interactions at mean development time ($P < 0.00138$). The rates of change in synchrony at each interaction were also plotted. Both analyses showed the highest levels and rates of synchrony were at broodstock salinity*treatment salinity interactions of 20|30 ppt, 20|20 ppt and 30|30 ppt. Low salinity was detrimental to obtaining synchronous meiotic development.

Survival and ploidy of D-stage oyster larvae were estimated after exposing embryos to CB dosages of 0.5 mg/L , 0.25 mg/L , and 0.125 mg/L for 10-15 minutes, with 0.05% DMSO and ambient seawater as controls. No significant differences were found in survival and triploidy between CB dosages of 0.5 mg/L and 0.25 mg/L and between 0.125 mg/L and the controls ($P < 0.05$).

Recommendations are provided to optimize triploid induction of *Crassostrea virginica* using CB.

CHAPTER I

GENERAL INTRODUCTION

Oyster culture is one of the oldest and most successful forms of mariculture in the world. Its beginnings can be traced back to the Roman Empire and the late 1700s in the United States where simple culture activities were used to increase the yield of wild oyster populations. Today's various forms of oyster culture accounts for some 40% of U.S. production (Korringa 1976; Burrell 1985). The application of intensive oyster culture techniques to commercial farming operations, however, has been limited to a few Northeastern states (Burrell 1985).

Despite the advantages of the various farming techniques used in the oyster industry, nationwide production continues to face problems with disease, habitat degradation, and pollution. Oyster production along the Atlantic coast has suffered major setbacks from two protozoan parasites, namely *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) (Anon. 1990). Similar disease outbreaks in Florida and Texas have significantly reduced production. The Louisiana oyster industry is no exception; severe land loss, saltwater intrusion, and increased harvesting resulting from high unemployment has negatively impacted the state's oyster natural production in the past,

on which the industry is heavily dependent for its source of seed oysters for planting onto private leases.

LOUISIANA OYSTER INDUSTRY

Oyster production in Louisiana has ranged from a low of 2.2 thousand tonnes in 1966 to a high of 6.5 thousand tonnes in 1985 and has averaged 4.7 thousand tonnes annually during the past 20 years. Although Louisiana's oyster production appears relatively stable, not only have there been periodic deviations from the long-term average due to short term environmental factors, but there has also been a gradual decline in the industry's production per unit area. In fact, leased acreage has increased 6 fold since 1960 yet total production on leased acreage has shown sporadic growth (Keithly and Roberts 1988).

Yield is important in the processing of any raw material and oysters are no exception. Seasonal variations in shucked meat yields dramatically affect profitability throughout the year. Such yields are due to the oyster's spawning season, which is controlled by rising and falling seawater temperatures. During spawning season, May through October in the Gulf region, oyster meat yields drop an average of 66%, due to the animals' use of stored glycogen reserves for the production and release of gametes. This loss in meat yield has been an annual economic burden to the

oyster industry since the beginning of commercial utilization. This seasonal economic loss is particularly dramatic during periods of lower oyster landings, when supply and demand forces increase the processing sector's costs for raw material (oysters), and during a static market when low demand forces a reduction in shucked meat prices in order to move the product.

When processing oysters, shucked meat yields determine profitability. A 1.5 bushel sack (commercial measure) typically yields 1.4 to 2.1 L (3 to 4.5 pints [pounds]) at their lowest and 4.2 to 5.7 L (9 to 12 pints) at their highest meat yield (personal communication, Al Sunseri, P & J Oyster Co., New Orleans, LA.). If sold by the gallon (3.785 L [8 pints]), it takes twice as many oysters to make a gallon during low meat yield periods. Oyster yields are higher from November through April, with the highest yields from January through March. Oysters typically yield less meat from May through October with the lowest yield during mid-July through the end of September (personal communication, Al Sunseri, P & J Oyster Co., New Orleans, LA.). The bottom line is, if a sack of oysters yields ≥ 2.7 kg (6 pounds [1 pt.=1 lb.]) of meat (November through April), selling shucked meat is profitable; if a sack yields

< 2.7 kg (6 pounds), it's not (personal communication, Mike Voisin, Motivatit Seafood, Houma, LA.).

A static market during the past 2 years has increased economic losses for some oyster processors in Louisiana. Oyster meat prices can usually be raised by the processor to about \$6.75 to \$7.00/0.45 kg (pound) during the summer to make up for losses in meat yield. Market conditions over the past two years, however, have not permitted such a price increase. Losses on shucked meat were reported to be \$1,600/day from June through October, based on 200 sacks (1.5 bushels) of oysters shucked per day and an \$8/sack loss due to lower meat yield (personal communication, Mr. Mike Voisin).

In this case, the operator broke even in June, 1992, lost \$6,000 to \$7,000 during July, \$10,000 during August and \$2,000 during September. Monetary loss can occur as late as October if warm weather prevails, extending the spawning season (personal communication, Mr. Al Sunseri).

Culture methodologies which stabilize production and improve yields per unit would boost the economic viability of this industry both in Louisiana and other states. Oyster culture involves a variety of techniques which range from the simple management of wild populations to intensive hatchery and raceway culture (Bardach 1986; Burrell 1985). For generations, oystermen have been enhancing settlement

substrate to increase recruitment and/or transporting young oysters to more favorable growing conditions to increase yield (Pausina 1988). Hatchery production has been used to enhance wild populations and supply oyster farmers for intensive grow-out (Burrell 1985). Most of these techniques are unique to oyster species and strains in the region where they were developed. At the state level, however, there is much work to be done. If any proposed research is to provide a rejuvenation of the oyster resource for private or public use, there must be a significant culture component to any activity (Anon. 1990). One of the immediate ways to improve lease production is through hatchery-based seed production.

HATCHERIES

Modern hatchery techniques have been described for the Pacific and American oysters (Breese and Malouf 1975; Dupuy et al. 1977). Since hatcheries are site specific, consistent, suitable water quality is the most important criterion. Algal culture techniques used for rearing larval food range from the brown water method using coarsely-filtered ambient seawater to bloom naturally occurring phytoplankton (Ogle 1982), to pure, mass cultures of specific algal species (Loosanoff and Davis 1963).

Previous hatchery efforts centered around producing seed and market-size oysters. The advent of remote setting techniques provided a division of labor between the hatchery operator and the oyster farmer, improving the efficiency of both. By freeing hatchery operators of cultch handling and grow-out, and by utilizing very large larval rearing tanks (e.g., 30,000 to 60,000 L), West coast oyster hatcheries evolved into the higher volume production facilities necessary for profitability.

REMOTE SETTING

Remote setting is the setting (attachment) of pediveligers onto cultch in a remote location from the hatchery. It was first investigated with the Pacific oyster (*Crassostrea gigas* [Thunberg]) during 1972 in laboratory studies at Oregon State University (OSU) (Lund 1972). Larval handling and remote setting of this species was further refined by West coast oyster growers (Budge 1973) and by B. Henderson at OSU (Henderson 1983).

Successful commercial remote setting methods of the Pacific oyster along the Pacific coast have been well documented (Jones and Jones 1983, 1988; Roland and Broadley 1990). Remote setting has also been demonstrated along the East coast with the American oyster (*C. virginica*) (Gibbons

1988; Bohn 1989). The process has begun to be utilized in the Gulf region as well (Supan 1991).

HATCHERY AND REMOTE SETTING EFFORTS IN LOUISIANA

Demonstration of hatchery and remote setting of larvae for private oyster seed production began in Louisiana during 1989 as part of the Molluscan Shellfish Technology Transfer Program funded by the Louisiana Sea Grant College Program (Supan and Wilson 1993). Over the past 5 years, hatchery techniques by the author and remote setting efforts by industry have been relatively successful. Recent advances in triploidy induction of oysters in a hatchery setting have afforded us new opportunities for the research and development of improved farming yields. However, there are two questions that have prevailed in the field.

- (1) How does broodstock gonadal cycling affect egg availability at the hatchery?

Broodstock maintenance is fundamental to consistent production of larvae and successful triploid induction. Documentation of broodstock gonadal development of *Crassostrea virginica* in the Gulf region is limited. The relationship between cyclical gametogenesis and induced spawning needs study.

- (2) Can triploid oysters be produced and lead to improved product yields in Louisiana?

The equipment and labor associated with cultch preparation and handling for remote setting (e.g., washing and grading of cultch, loading and unloading the setting tank and nursery area) are major costs associated with remote setting (Supan et al. 1994). The technology is not competitive during times of high natural oyster seed production, as is currently the case. For hatchery and remote setting operations to be competitive during such periods, the end-product must be more valuable.

The production of triploid oysters involves chromosomal manipulation of newly fertilized eggs, under controlled conditions at a specific time and temperature. The oysters would then have three sets of chromosomes (triploid), as opposed to the normal two sets (diploid) (Figures 1.1, and 1.2), resulting in oysters which do not undergo gonadal maturation and, therefore, do not spawn. Since spawning does not occur, the oyster industry would benefit in processing and marketing oysters that do not have substantial, spawning-related meat yield losses from May through October.

Ploidy manipulation (Figure 1.2) is best accomplished with oysters using cytochalasin B (CB), a metabolite of certain fungi, which inhibits cytokinesis (e.g., the formation of polar bodies). Meiotic synchrony, measured by the observation of polar body I (PBI) formation, is most

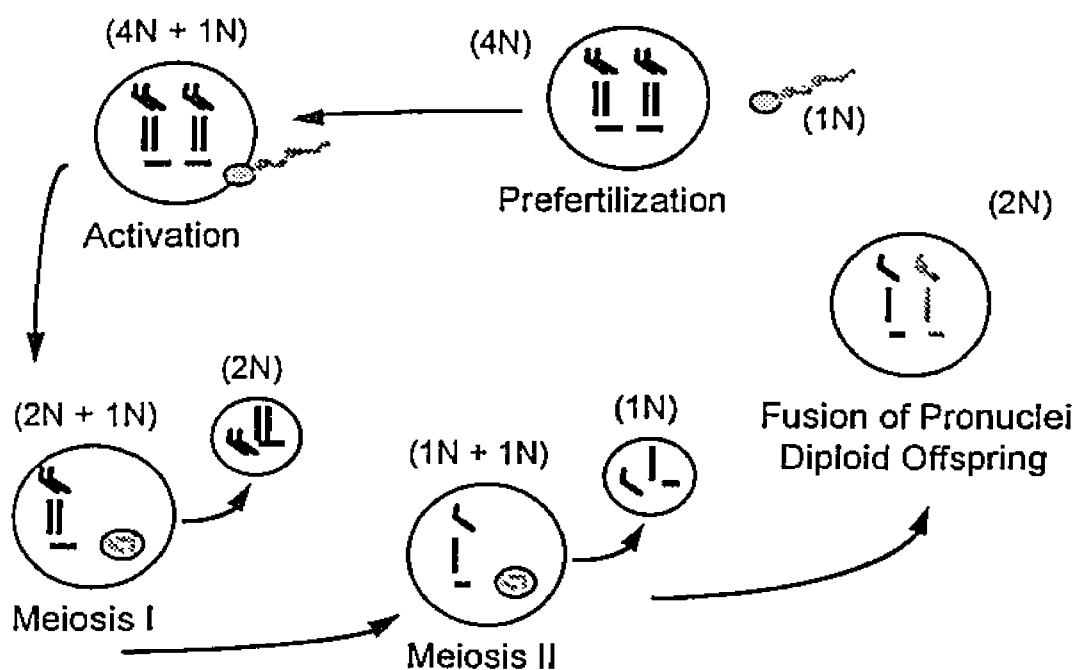


Figure 1.1. Normal meiosis in oyster eggs. The sperm is haploid ($1N$) but the egg is tetraploid ($4N$) when spawned. Chromosomal reduction of the egg continues after activation by insemination. A polar body containing two sets of chromosomes is released during meiosis I; another with one set during meiosis II. Diploidy ($2N$) results after paternal and maternal chromosomes combine. From Allen et al. (1989).

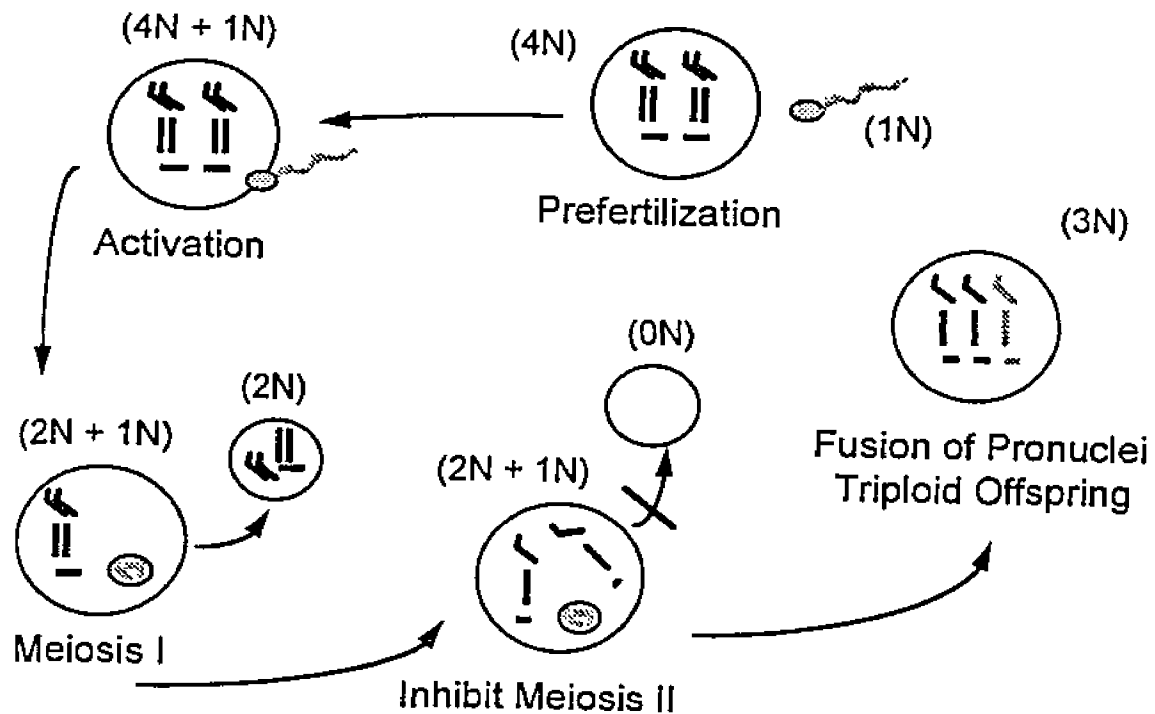


Figure 1.2. Interruption of meiosis to yield triploids. Activation and meiosis I proceed as in figure 1.1, but meiosis II is inhibited. The egg retains two sets of chromosomes, which combine with the paternal set, producing triploid ($3N$) progeny. From Allen et al. (1989).

important in order to get the highest number of eggs within the "treatment window" (e.g., 50% at PBI). Cytochalasin B is used to inhibit polar body II formation, thus causing a retention of a second maternal set of chromosomes. Nearly 100% triploidy can be obtained with this technique if good meiotic synchrony is achieved, as well as the use of proper CB dosage, and induction timing (Allen et al. 1989).

Although induction techniques have been documented for *Crassostrea gigas* in the Northwest region and *Crassostrea virginica* in the Northeast region of the U. S., triploid production in the South has not. The effects of changing salinity on synchrony (i.e., triploid induction) needs to be determined before commercial production can be pursued.

The goal of this study is to address the above two questions and to contribute to the development of a methodology for producing triploid oyster larvae in Louisiana using CB with emphasis on the effects of salinity. Execution of this research project requires a refinement of broodstock management and triploid induction techniques used in the Northeast for the Louisiana strain of *C. virginica*. This process will lead to the methodology for producing triploid oysters in Louisiana. The critical step of synchrony and how it is affected by changing salinity will be addressed, as well as CB dosage. Gonadal development and

cycling, which affects the availability of eggs, will also be addressed.

OBJECTIVES

My primary research objectives were:

1. To determine the variability of gonadal condition during seasonal gametogenesis, and between spawning and non-spawning broodstock using a Gonad/Body Ratio (GBR) and Developmental Staging (DS) (Null hypothesis: no difference in variability in the GBR during natural gonadal development of spawning and non-spawning broodstock; alternate hypothesis: variability is present). This objective is addressed in CHAPTER II; and,
2. To determine the effect of salinity and cytochalasin B dosage on triploid induction and survival (Null hypothesis: salinity and dosage do not affect triploid induction; alternate hypothesis: salinity and dosage do affect triploid induction). This objective is addressed in CHAPTERS III and IV.

CHAPTER II

ANALYSES OF GONADAL CYCLING BY OYSTER BROODSTOCK, *CRASSOSTREA VIRGINICA* (GMELIN), IN LOUISIANA

INTRODUCTION

Broodstock gonadal condition is fundamental to consistent production of oyster larvae (Lannan et al. 1980) and successful triploid induction (Downing and Allen 1987). Although temperature requirements for gonadal development and spawning have been reported for southern oysters (Hopkins 1931; Hopkins et al. 1953; Loosanoff 1969; Hayes and Menzel 1981; Gauthier and Soniat 1989), documentation of the relationship between gametogenesis and induced spawning of *C. virginica* in the Gulf region is limited. Also, southern oysters are multiple spawners and continue gametogenic development throughout their spawning season (Ingle 1951; personal hatchery observations). An understanding of gonadal cycling is critical because collection of viable eggs from mass spawning of adult *Crassostrea virginica* has been a problem at a Louisiana oyster hatchery, especially during mid-July through mid-August (Supan and Wilson 1993).

Oysters held nearshore at the hatchery on Caminada Bay, Louisiana had the propensity for gonadal redevelopment and respawning during the summers of 1990 through 1994.

Hypertrophic gonads with prominent genital canals beneath nearly translucent mantle tissue became typical about four weeks post-spawning, at an ambient salinity range of 15 to 30 ppt and seawater temperatures ranging from 30°C at dawn to 35°C by 1500 h on sunny days (Supan, unpublished data).

Such gonadal activity stimulated interest in histological examination of the hatchery's broodstock in an attempt to document such redevelopment and look at the relationship between induced spawning success and gonadal condition. The purpose of this study was to document broodstock gonadal development leading up to the spawning season and redevelopment or recycling between spawnings.

METHODS

Broodstock were collected from oyster reefs in Caminada Bay, Barataria Bay, Creole Bay, Chinaman Bayou, Bay Des Illettes, the lagoons of the Grand Terre Islands, and Long Lagoon, Louisiana and maintained in numbered containers nearshore at the hatchery. Ten oysters were randomly selected when brought to the hatchery, prior to each spawning attempt, and during the winter and spring of 1992-1993 for histological analyses. Temperature and salinity were noted during each sampling period.

A 4 to 5 mm cross-section of each oyster was removed just posterior to the labial palp-gill junction and

preserved in Davidson's fixative for histological sectioning (Howard and Smith 1983). A 4 μm section was obtained approximately 1,000 μm from the junction, mounted and stained with Gill's hematoxylin and eosin. This allowed the use of a standard field area of each oyster for comparison. The sections were characterized by two H-shaped structures (large appendix of the stomach caecum) ventrally located in the histological sections as described by Morales-Alamo and Mann (1989).

Qualitative Description

Individual sections were microscopically examined to determine the sex and gametogenic developmental stage of each oyster. Classifications included early development, late development, spawning, and advanced spawning and regression after Kennedy and Krantz (1982).

Quantitative Analyses

Gonad/Body Ratios (GBR) were generated from histological sections using ten equidistant measurements (transects) assigned laterally across each section to determine the gonadal width relative to body width (Kennedy and Battle 1964) (Figure 2.1). Oysters ($N = 200$) were analyzed over a two year period to determine GBR variability between transects and developmental stages.

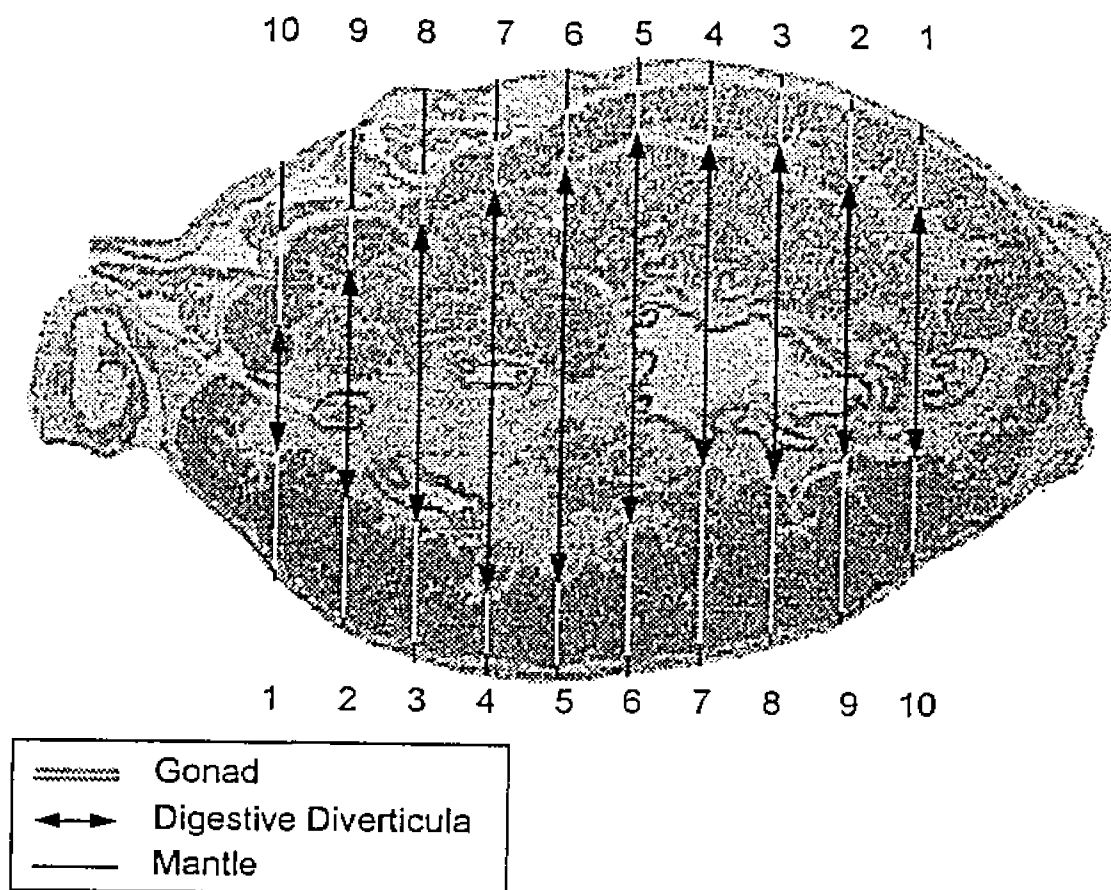


Figure 2.1. Drawing of histological transverse section through the mid-body region of an oyster. The 10 transects of measurements shown are used in determining the gonad/body ratio. Gonad tissue is represented in solid black. From Kennedy and Battle (1964).

GBR was also measured in groups of oysters used during normal hatchery operation to determine how GBR varied between spawning and non-spawning populations. During each spawning attempt, approximately 150 oysters were removed from nearshore containers, 10 were randomly selected for histology and the remainder were placed in a spawning table and exposed to ambient (30°-35°C) seawater for about 30 min. If necessary, the broodstock were then exposed to a gradual acclimation to 18°C for approximately 1 h, then re-exposed to ambient conditions for approximately 2 h to stimulate spawning. If temperature stimulation did not lead to spawning, the brood was exposed to a sperm suspension. Successful spawning was noted when a majority of the oysters had spawned, while unsuccessful spawning was characterized by little or no spawning activity.

The GBR's were analyzed using analysis of variance (ANOVA) with a two-way mixed factorial model (SAS Institute 1991). To test the difference between transects, the model included the GBR as the dependent variable, transects as a fixed effect, and gametogenic developmental staging and its interaction with transects as random effects. To test the difference between spawning attempts, the model included GBR as the dependent variable, spawning result as a fixed effect, and developmental stage and its interaction with the spawning result as random effects. The GBR's met the

assumptions of normality and variance homogeneity after angular transformation (Dowdy and Wearden 1991). Data was analyzed with the analyses of variance and Tukey's Honestly Significant Difference Procedure was used to test for differences in treatment means for the following main effects: transects, developmental stage and spawning result. Differences were declared to be significantly different at $\alpha = 0.05$.

RESULTS

Significant differences were found between the mean GBR's among transects ($P < 0.05$). Oysters that spawned generally had significantly greater GBR's than those that did not spawn.

Quantitative Analyses

Mean GBR's varied between transects. The ANOVA model only explained a small amount ($R^2 = 0.27$) of variability in the GBR's. The main effects of transect and developmental stage on GBR were highly significant ($P < 0.0001$, Table 2.1). Significantly different mean GBR's were found between transects 1 and 10, 2 and 9, and 3 through 8 ($P < 0.05$, Table 2.2). All developmental stages had significantly different mean GBR's ($P < 0.05$, Table 2.3).

TABLE 2.1.

Results of the analysis of variance:
effects of transects, developmental stage and
interaction (*) on gonad/body ratio
of *Crassostrea virginica*.

Sources of Variation	F-value	Prob>F
Transect	6.81	0.0001
Developmental stage	445.81	0.0001
Transect*D.Stage	0.34	0.9999

$R^2 = 0.27$

TABLE 2.2.

Results of the analysis of variance:
comparing mean gonad/body ratios of
Crassostrea virginica by transect.

Transect	Ratio*		Comparisons**
	Mean	SD	
1	0.5266	0.173	A
2	0.4824	0.152	B
3	0.4528	0.147	C
4	0.4392	0.139	C
5	0.4432	0.145	C
6	0.4389	0.138	C
7	0.4392	0.141	C
8	0.4610	0.147	C
9	0.4806	0.158	B
10	0.5204	0.179	A

* Ratio = $\text{Arcsin}(\sqrt{\text{Gonad Width}/\text{Body Width}})$.

** Tukey's Honestly Significant Difference ($\alpha = 0.05$).

SD = Standard Deviation.

N = 200 oysters.

TABLE 2.3.

Results of the analysis of variance:
 comparing mean gonad/body ratio of
Crassostrea virginica by developmental stage.

Developmental Stage	Ratio*		Comparisons**
	Mean	SD	
Early Development	0.3126	0.143	A
Later Development	0.4684	0.123	B
Spawning	0.5237	0.132	C
Advanced Spawning & Regression	0.3835	0.154	D

* Ratio = $\text{Arcsin}(\sqrt{\text{Gonad Width}/\text{Body Width}})$.

** Tukey's Honestly Significant Difference ($\alpha = 0.05$).

SD = Standard Deviation.

N = 200 oysters.

Because of the comparison between transects, only transects 3-8 were used in the model (GBR = spawning result, developmental stage, and their interaction) to determine differences in mean GBR's between spawning events of two groups of broodstock. Spawning condition had a significant effect ($P = 0.0419$) in the model indicating the GBR was greater in spawning groups than non-spawning groups (Table 2.4). Again, little of the variability in the GBR was explained by the model ($R^2 = 0.1755$) indicating the broad range of GBR's within the groups. The mean GBR during successful spawning attempts was significantly greater (mean = 0.5182, $P < 0.05$) than the mean GBR during unsuccessful attempts (mean = 0.4516) (Table 2.5).

Qualitative Analyses

After evaluating the histological sections for developmental stage (Kennedy and Krantz 1982), it became apparent that oyster gonads were recycling. Histological sections representing early development (Figure 2.2, A & B), later development (Figure 2.3, A & B), spawning (Figure 2.4, A & B) and advanced spawning-regression (Figure 2.5, A & B) were noticeably different than sections from recycling (Figures 2.6, A & B; 2.7, A & B; 2.8, A & B). The main difference was the presence and amount of atresia (i.e.,

TABLE 2.4.

Results of the analysis of variance:
effects of spawning result and developmental stage
and their interaction (*) on the gonad/body ratio.

Sources of Variation	F-ratio	Prob>F
Spawning result	18.11	0.0419
Developmental stage	12.99	0.0715
Sp.result*D.stage	2.70	0.0677
$R^2 = 0.1755$		

TABLE 2.5.

Results of the analysis of variance:
comparing mean gonad/body ratios of
Crassostrea virginica by spawning result.

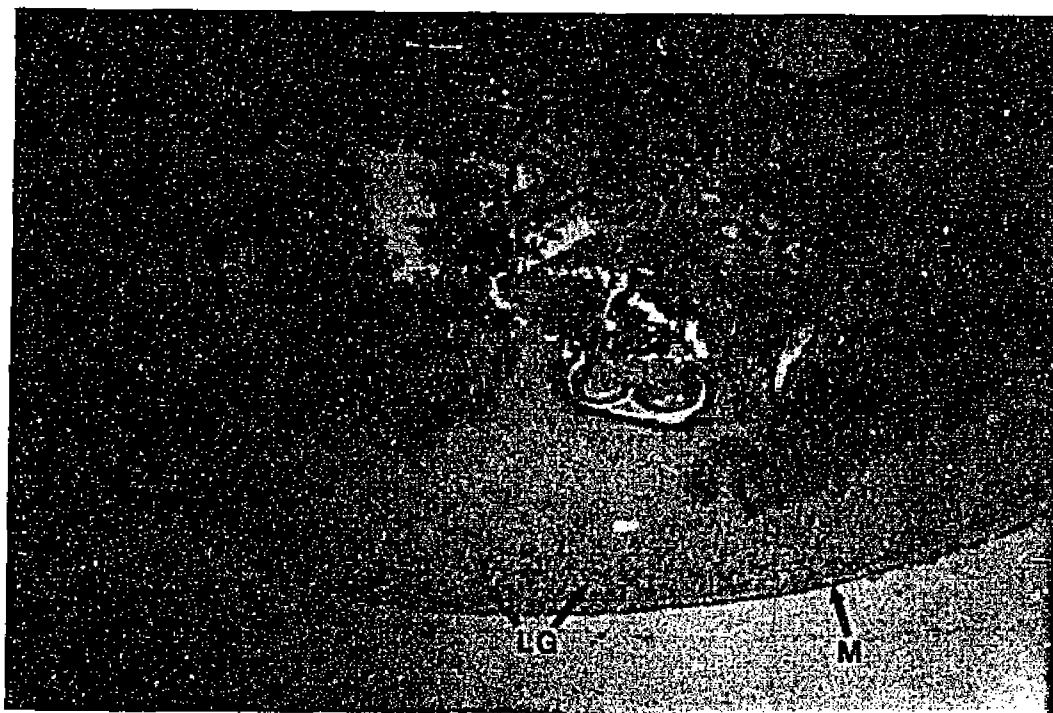
Spawning Result	Ratio*		Comparisons**
	Mean	SD	
Successful	0.5182	0.109	A
Unsuccessful	0.4516	0.122	B

* Ratio = $\text{Arcsin}(\sqrt{\text{Gonad Width}/\text{Body Width}})$.

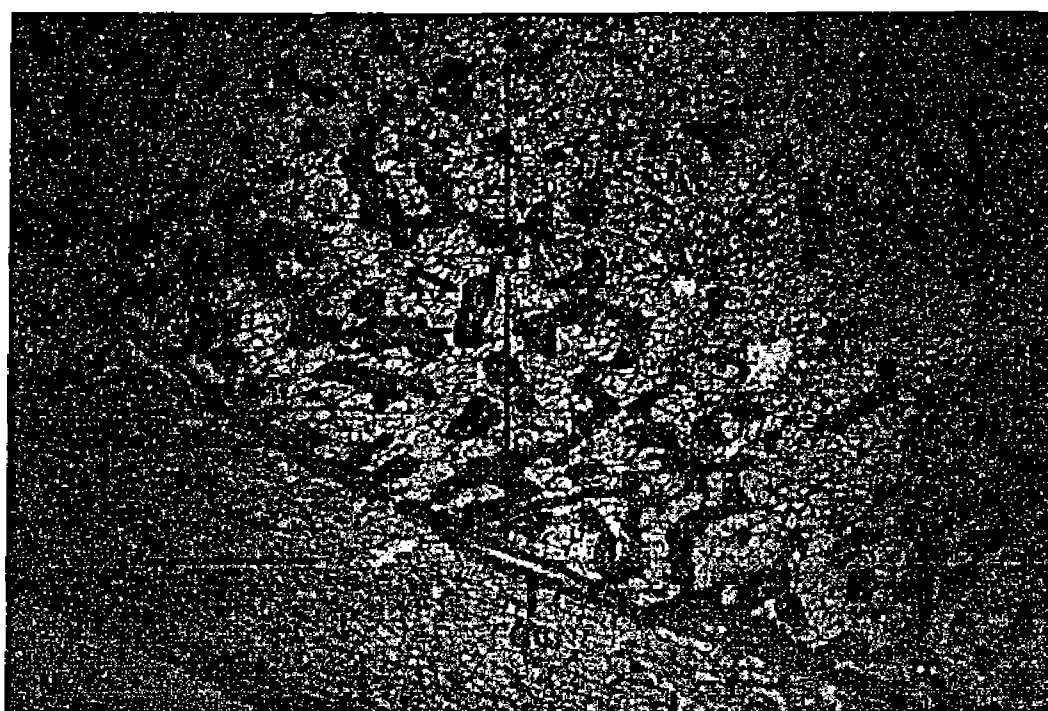
** Tukey's Honestly Significant Difference ($\alpha = 0.05$).

SD = Standard Deviation.

N = 54 oysters.

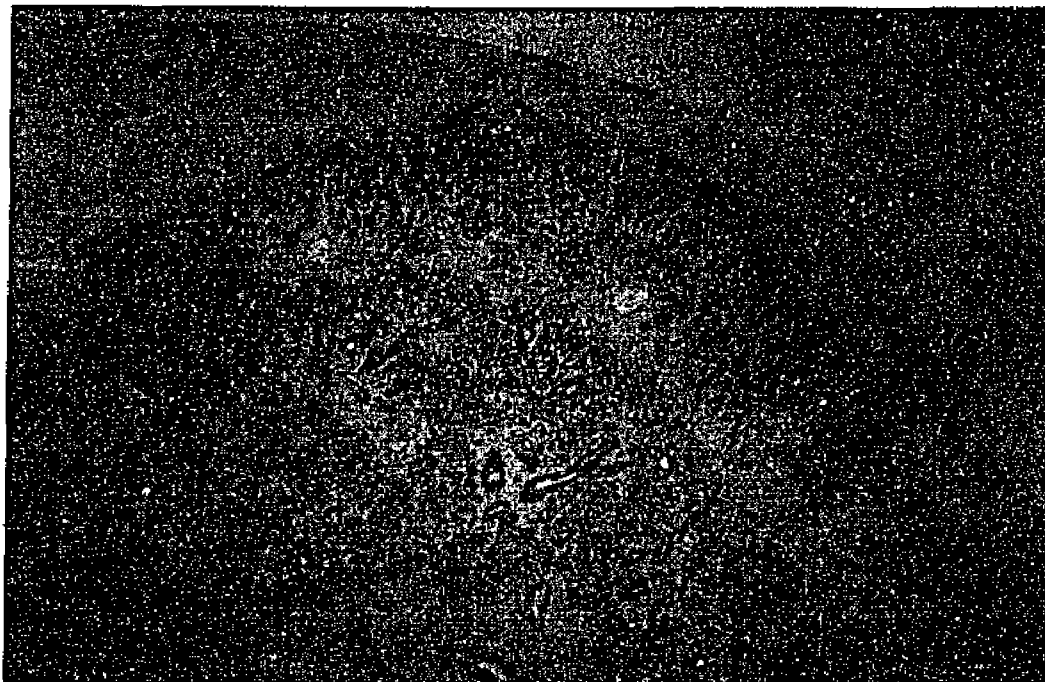


A

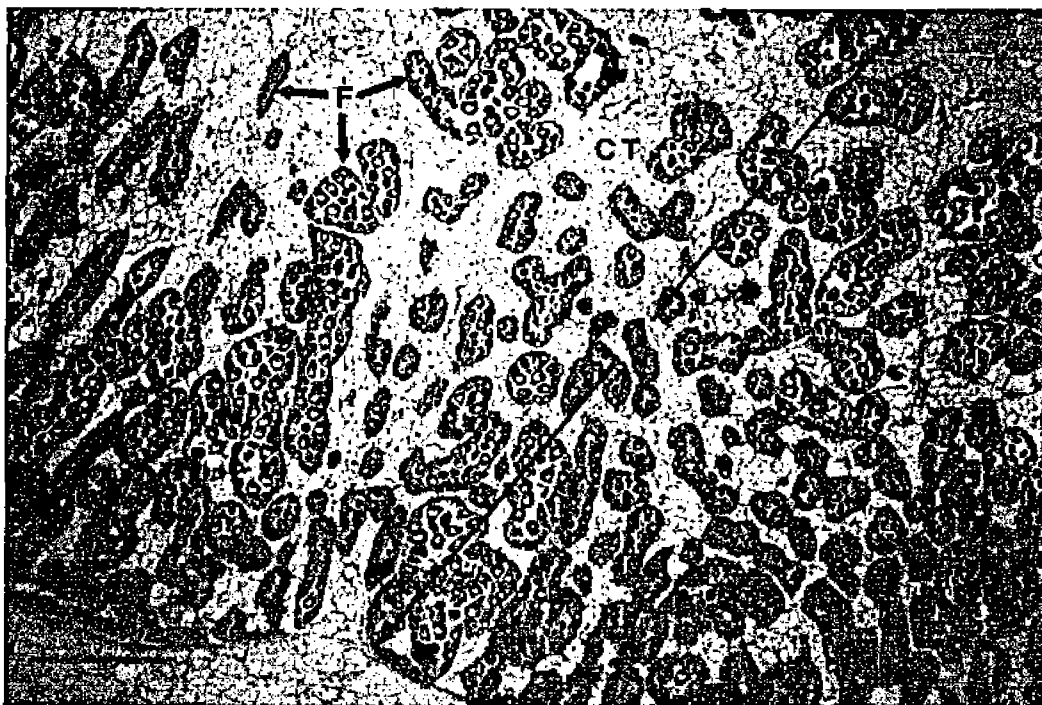


B

Figure 2.2 A & B. Photomicrographs of histological sections showing early gonadal development (March) of *C. virginica*, posterior-ventral region (A: bar = 200 μ m; B: bar = 30 μ m). LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, G = gonad, F = follicles, CT = connective tissue, GC = genital canal.



A

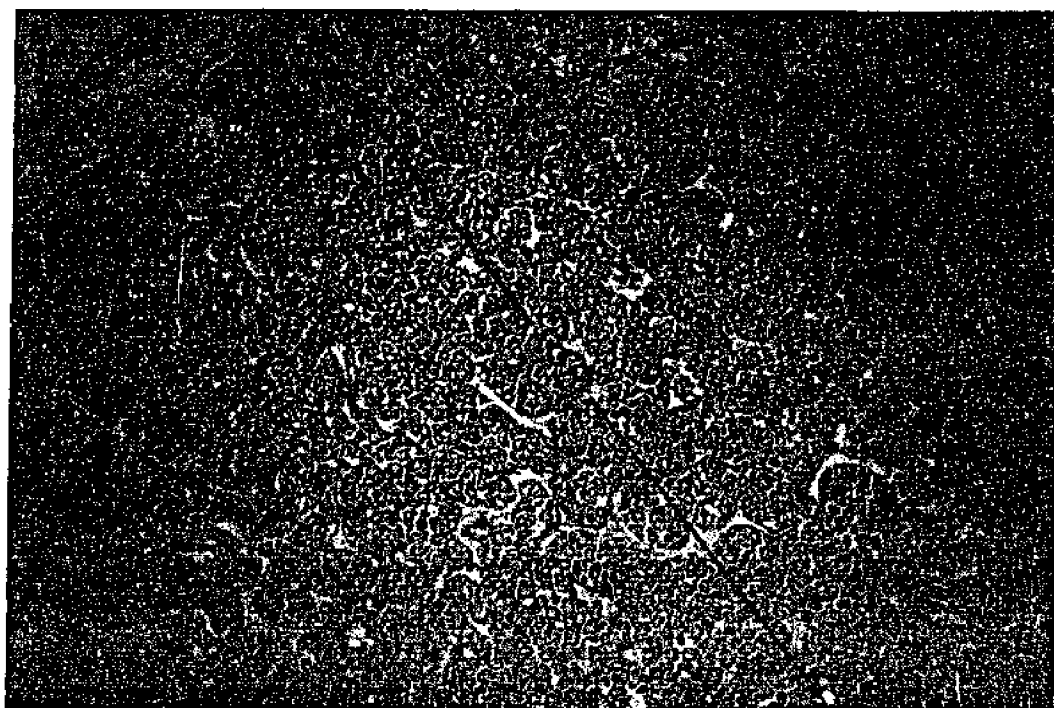


B

Figure 2.3 A & B. Photomicrographs of histological sections showing later gonadal development (April) of *C. virginica*, posterior-dorsal region (A: bar = 200 μ m; B: bar = 30 μ m). RG = right gonad, DD = digestive diverticula, M = mantle, G = gonad, F = follicles, CT = connective tissue.

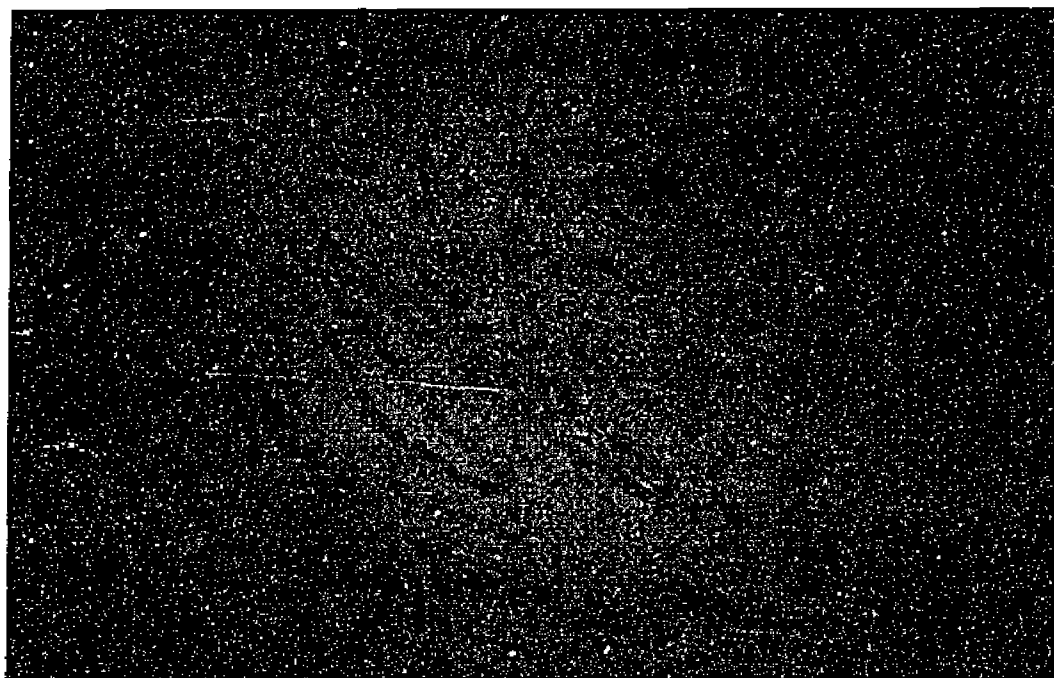


A

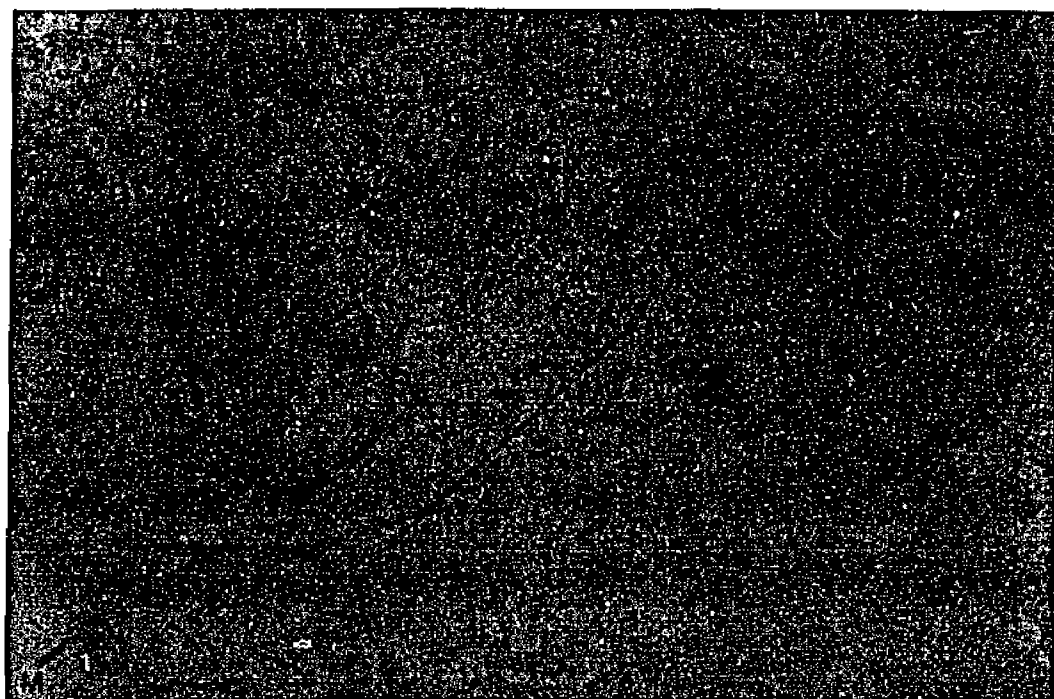


B

Figure 2.4 A & B. Photomicrographs of histological sections showing spawning gonadal development (June) of *C. virginica*, (A: bar = 200 μ m; B: bar = 30 μ m, posterior-dorsal region). RG = right gonad, LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, S = stomach, G = gonad, CT = connective tissue.

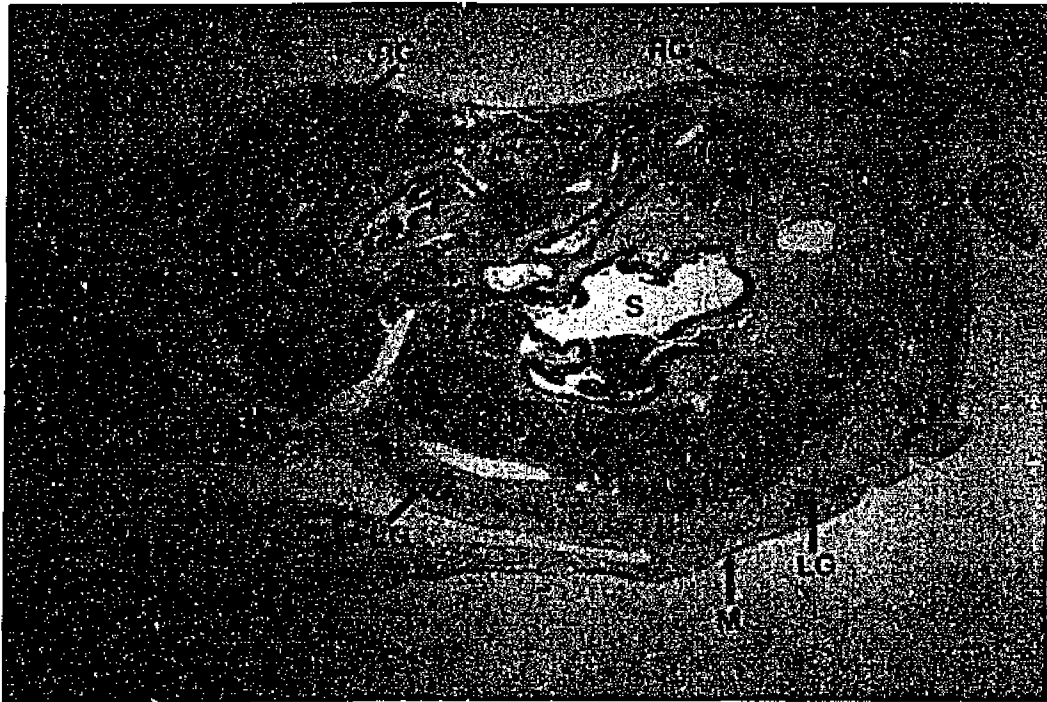


A

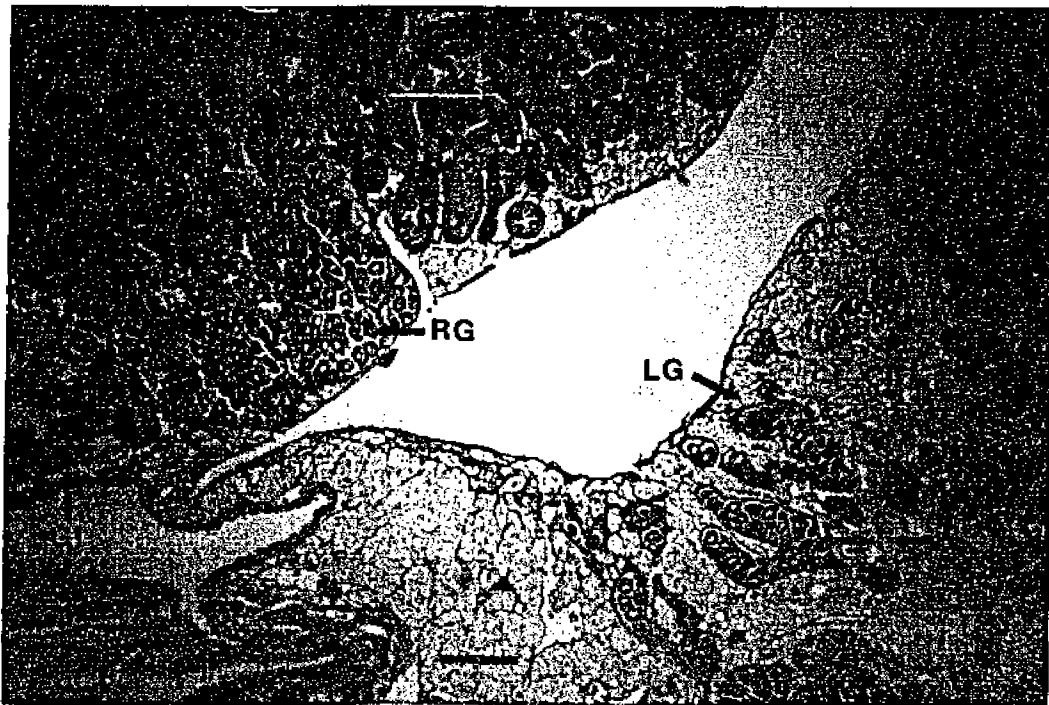


B

Figure 2.5 A & B. Photomicrographs of histological sections showing advanced spawning and regression gonadal development (August) of *C. virginica*, posterior-ventral region (A: bar = 200 μ m; B: bar = 30 μ m). LG = left gonad, DD = digestive diverticula, GC = genital canal, M = mantle, GI = gill, G = gonad, CT = connective tissue, F = follicle.

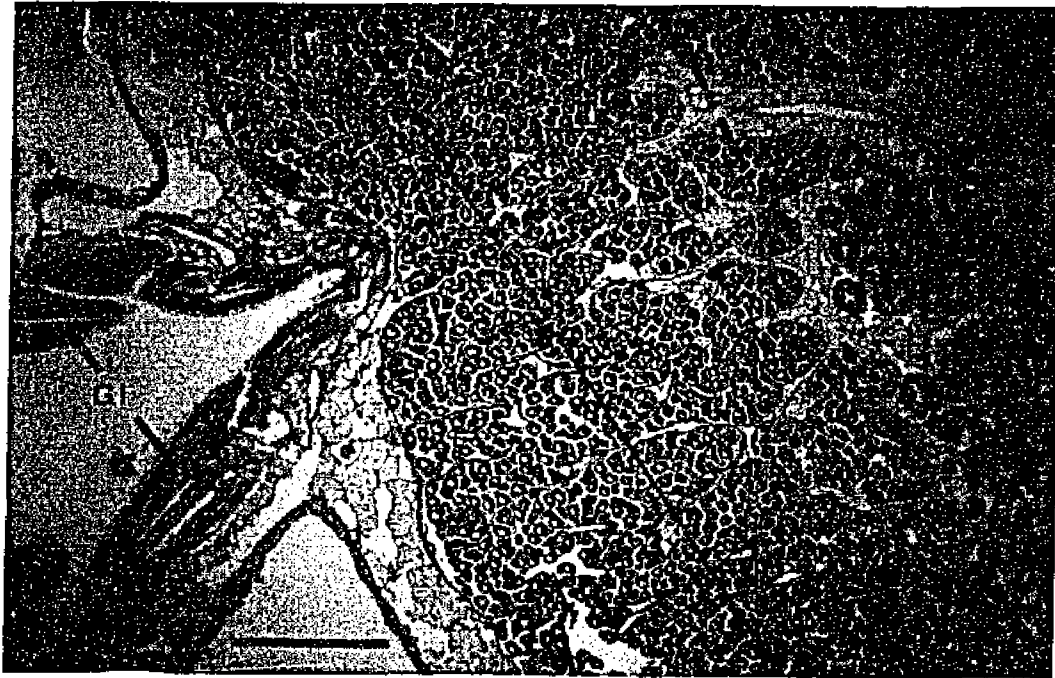


A

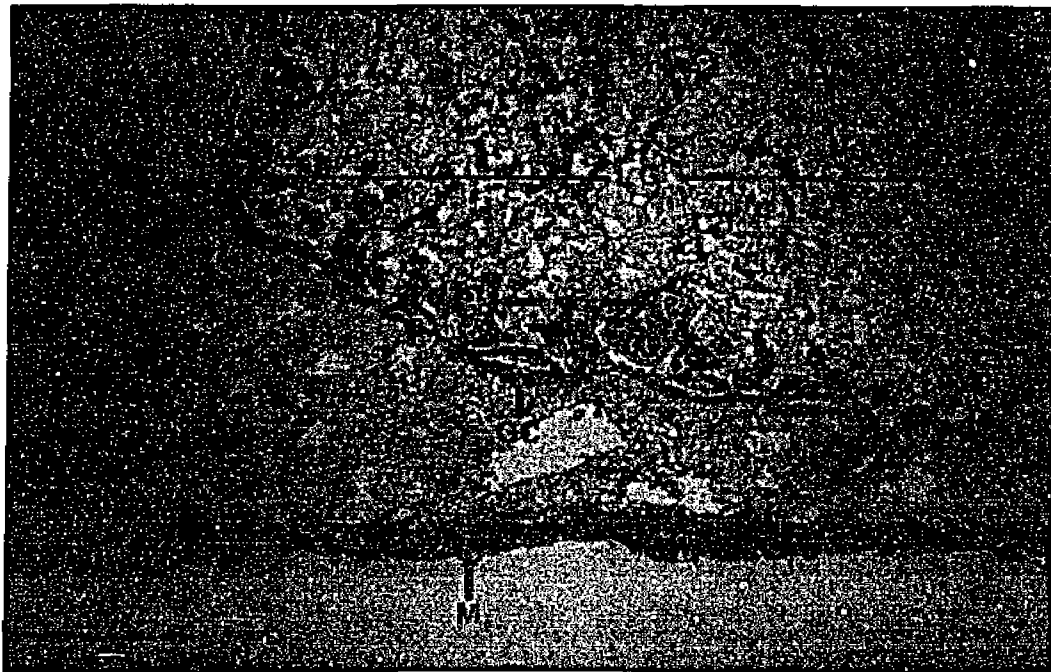


B

Figure 2.6 A & B. Photomicrographs of histological sections showing gonadal recycling (October) of *C. virginica* (A: bar = 200 μ m; B: bar = 30 μ m, ventral region). RG = right gonad, LG = left gonad, DD = digestive diverticula, S = stomach, M = mantle, GI = gill, CT = connective tissue, F = follicle.



A

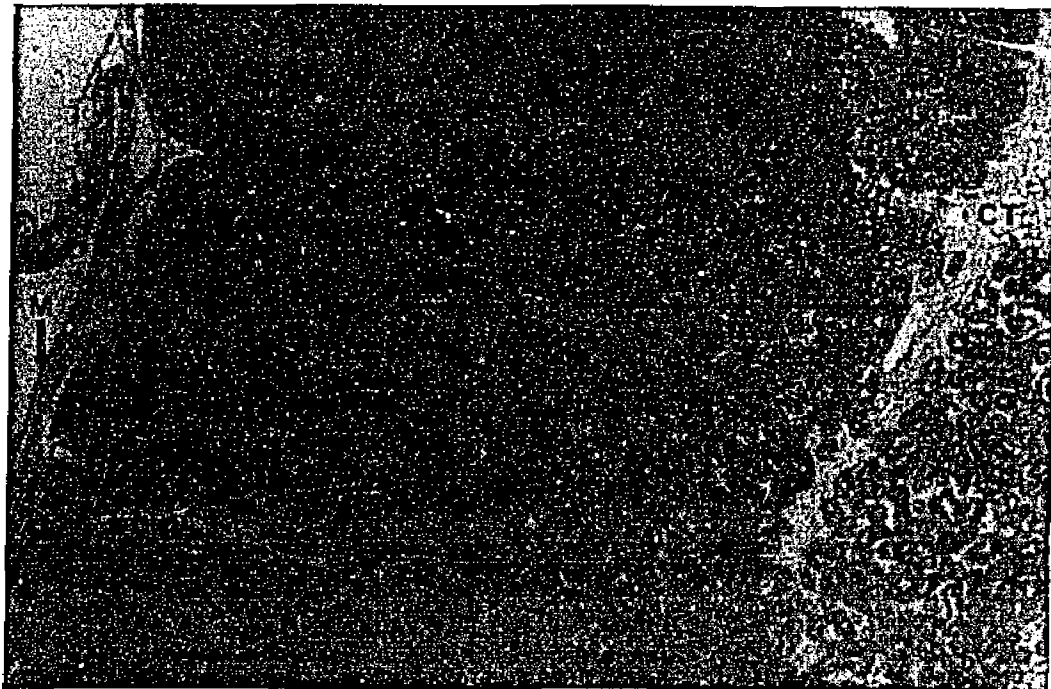


B

Figure 2.7 A & B. Photomicrographs of histological sections showing gonadal recycling (October) of *C. virginica* (A: bar = 200 μ m, ventral region; B: bar = 200 μ m, ventral region). RG = right gonad, LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, CT = connective tissue, F = follicle, GC = genital canal.



A



B

Figure 2.8 A & B. Photomicrographs of histological sections showing gonadal recycling (August) of *C. virginica* (A: bar = 200 μ m; B: bar = 200 μ m, dorsal region). RG = right gonad, LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, CT = connective tissue, S = stomach, GC = genital canal.

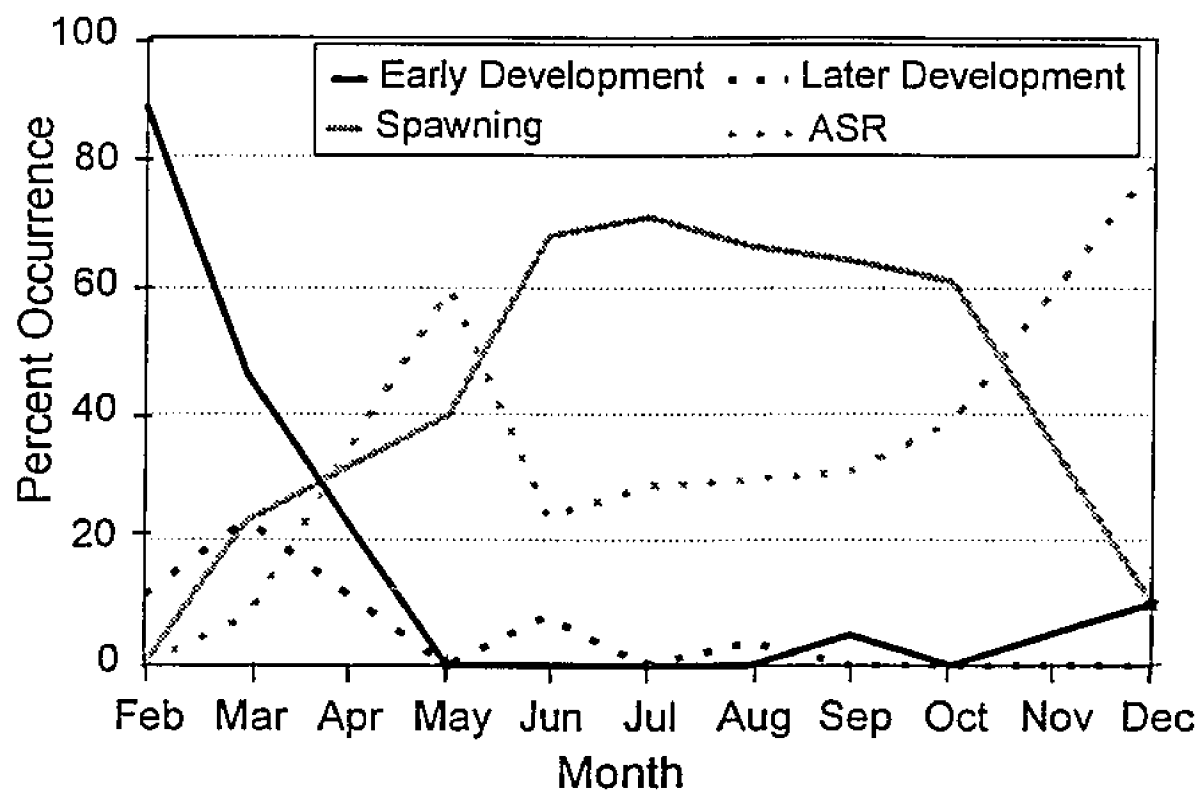


Figure 2.9. Percent occurrence of gonad developmental stage of *Crassostrea virginica* by month. * denotes advanced spawning and regression.

cellular debris and amoebocytes in nearly empty follicles and surrounding connective tissue) typically found in advanced spawning-regression sections (Figure 2.5). Also evident was advanced proliferation of developing follicles typically found in early and later development sections (i.e., enlarged germinal cells and young pendant primary oocytes in females and stratified germinal epithelia consisting of spermatogenic stages in males [Kennedy and Battle 1964]).

A histological section of an oyster during early development (Figures 2.2, A & B) was representative of the gonadal condition of 88.9% of the oysters sampled from containers held in nearshore waters of Caminada Bay during February, 46.2% during March, 4.9% during September and 10% during December (Figure 2.9).

A histological section of an oyster during later development (Figures 2.3, A & B) was representative of the gonadal condition of 11.1% of the oysters similarly sampled during February, 23.1% during March, 7.9% during June, and 3.7% during August (Figure 2.9).

A histological section of an oyster during advanced spawning and regression (Figures 2.5, A & B) was representative of the gonadal condition of 7.7% of the oysters similarly sampled during March, 60% during May, 23.7% during June, 28.6% during July, 29.6% during August,

30.9% during September, 38.9% during October, and 80% during December (Figure 2.9).

Figures 2.6 through 2.8 represent gonadal recycling. The follicles of the left gonad depicted in Figure 2.6(B) and those depicted in Figure 2.7(B) are identical to those found during early and later development (Figures 2.2 and 2.3). Atresia is evident in the dorsal region of the right and left gonads in Figure 2.6(A) and in the nearby connective tissue and follicles in Figures 2.6(B) and 2.7(B). This atresia is similar to that found in advanced spawning and regression (Figure 2.5). The right gonad in Figure 2.6, Figure 2.7(A), and Figure 2.8 are similar to the gonads in spawning stage oysters in Figure 2.4, with no noticeable atresia nor reduction in oocyte organization within the follicles (Kennedy and Krantz 1982).

DISCUSSION

Quantitative Results

Comparison of GBR's between transects indicated the transects from the dorsal and ventral regions of the gonads were more variable and less representative of maturation state of the oyster. The difference is likely a result of the shape of the right and left gonads (Figure 2.1). Bias is inflicted with use of straight lines to determine GBR's of the curved left and right gonads in the dorsal and

ventral region. When using the techniques of Kennedy and Krantz (1982), variability in the mean GBR/oyster can be reduced by using only transects 3 through 8 (Table 2.2). Use of imaging enhancement techniques could provide a more unbiased quantification of GBR's in those regions (Heffernan and Walker 1988).

The ranking and significant differences of mean GBR's by developmental stage was as expected (Table 2.3). Oysters in spawning condition should have higher GBR's than non-spawning oysters. Follicles anastomose through the surrounding connective tissue during gonadal development (Figures 2.2-2.4) increasing the mean GBR (Table 2.3). The creamy-white appearance of a sexually mature oyster is due to the hypertrophy of the gonad (Kennedy and Battle 1964).

Fecund oysters are prolific spawners. Galtsoff (1964) reported an average of $57.6 \text{ million eggs} \pm 44.6 \text{ (SD)}/\text{spawn}$ of 9.2 to 13 cm (in height) oysters, with females capable of spawning 2 to 3 times during a 6-week period. Such spawning activity is reflected in the mean GBR's in Table 2.3, which depict gonadal attrition between spawning (0.5237) and advanced spawning and regression (0.3835).

GBR's of oysters that successfully spawned were also significantly higher than those that did not spawn (Table 2.5). Such results are expected, yet the model explained little of the variation, while developmental stage was not

significant (Table 2.4). Morse et al. (1978) found that reproduction and spawning in abalones, oysters, clams and other commercially important molluscs are regulated by prostaglandins. During spawning, the hormone is released in the water, stimulating gregarious spawning by others. They found that traces of hydrogen peroxide in the spawning tank stimulate spawning by providing free oxygen radicals necessary in an enzymatic step of prostaglandin synthesis. Hormonal concentration, genetic variation (Lannan et al. 1980) and food availability to broodstock are examples of the many sources of variation in oyster spawning activity.

Qualitative Results

A high percentage of the oysters examined were in early development at the beginning of the year; this stage decreased from May through November, rose during September, and increased again in December.

A short occurrence of later development in the spring during the sharp rise in the occurrence of spawning stage shows how oysters held in the nearshore waters of Grand Isle rapidly developed towards spawning condition. The percent occurrence of later development fluctuated during the summer.

The percent occurrence of spawning stage gonadal development depicts an expected curve; over 60% occur during

May through October, with reductions in the winter and spring. A similar autumn gonad state has also been documented in Louisiana (Gauthier and Soniat 1989).

One would think that a line depicting an advanced spawning-regression (ASR) gonadal stage would sharply increase from June through December, but this is did not occur here.

The dramatic drop in ASR occurrence and the rather steady occurrence of the spawning stage, as well as fluctuations in the percent occurrence of early and later developmental stages during the summer months, may illustrate recycling during June through October. This may or may not be evidence of spawning in the wild since the samples were taken from suspended broodstock coerced to spawn at the hatchery during the study. Nevertheless, the results show how gonadal recycling may be occurring in the broodstock held at the Grand Isle hatchery.

The description of ASR by Kennedy and Krantz (1982) included the occurrence of early gametogenic stages remaining on the follicle walls and the reappearance of connective tissue in the interfollicular areas with invading phagocytic cells. The use of an additional developmental stage, recycling, including the presence of early and later development characteristics plus the presence of atresia, is

recommended to improve the characterization of gonadal development in Louisiana.

CHAPTER III

THE EFFECT OF SALINITY ON THE SYNCHRONY OF POLAR BODY DEVELOPMENT IN FERTILIZED OYSTER EGGS (*CRASSOSTREA VIRGINICA* [GMELIN])

INTRODUCTION

Despite exhaustive efforts at site selection, oyster hatcheries are commonly located in estuaries which have changing water conditions that vary and affect production. The external environment during broodstock conditioning and ploidy manipulation may play a critical role in the production of triploid oyster larvae. Although temperature is generally regarded as the dominant factor controlling the metabolism of aquatic poikilotherms, salinity is also important. In marine invertebrates, the degree of tolerance to changing salinity often varies during ontogeny (Kinne 1971). Parental haline exposure during gametogenesis and/or just prior to spawning greatly affects larval oyster survival, especially during early development to D-stage (Davis 1958; Muranaka and Lannan 1984). Salinity was found to have a strong influence on meiotic division in *C. gigas* (Lu 1986), which is the critical period for ploidy manipulation (Downing and Allen 1987).

Production and grow-out of triploid *C. virginica* began in 1993 at Grand Isle, Louisiana during major flooding by the Mississippi River. Since salinity fluctuated at the

hatchery site during high Mississippi River discharges, the effect of changing salinity on triploid induction was questioned. The following experiment was conducted to test the null hypothesis that changing salinity during broodstock conditioning and embryonic development has no effect on the synchrony of polar body development of *C. virginica* eggs.

METHODS

Experiments were conducted by exposing fertilized eggs to 10 ppt, 20 ppt and 30 ppt artificial seawater after a one week broodstock acclimation period at 13 ppt, 20 ppt and 30 ppt.

Broodstock were collected from Caminada Bay, held in a recirculating seawater system and fed algal paste (*Chaetoceros muelleri*) three times per day, based on a feeding regime of ≤ 10 ug dry wt/mL (Bolton 1982).

At the beginning of each experiment, ten oysters were randomly selected from the holding system and prepared for histological examination to evaluate overall oyster gonadal condition of the population for comparison. Histological sections of oysters were prepared using the techniques of Howard and Smith (1983). Gonad/Body Ratios (GBR) were determined from ten equidistant measurements of gonadal width relative to body width (Kennedy and Battle 1964) (Figure 2.1). Gonadal developmental stage estimates used

descriptive stage characterization by Kennedy and Krantz (1982). A standard field area of each oyster was used for comparison (Morales-Alamo and Mann 1989).

Gametes were obtained for each experiment similar to Allen and Bushek (1992). Oysters were collected randomly from the holding system, opened, gender determined microscopically from gonad smears, and sexes were separated. Gonad ripeness was visually determined by the presence of prominent genital canals. Oysters were individually dry-stripped (i.e., without water) of gametes to ensure simultaneous hydration and fertilization. The resulting eggs were washed of gonadal debris with seawater from the holding system by passage through a 75 μm screen and retention on a 15 μm screen. The resulting sperm were retained in a beaker after being similarly washed of gonadal debris by passage through a 15 μm screen.

The eggs and sperm were pooled separately at the beginning of hydration and later mixed for fertilization, noting hydration and fertilization times.

For each experiment, equal aliquots of fertilized eggs were distributed into three replicate 100 mL beakers containing 10 ppt, 20 ppt, and 30 ppt artificial seawater held in a water bath at 28°C. Subsamples of embryos from each beaker were microscopically counted and examined for polar body formation at approximately 5 min intervals for

one hour or until about 50% of the embryos exhibited polar body I (PBI). Examination times (development time) were recorded, as well as total embryo counts, those at PBI stage and at further development (\geq PBII).

Data Analyses

Data were analyzed with an analysis of covariance using Statistical Analysis Software (SAS) (SAS 1991). Development stage proportions (synchrony) met the assumptions of normality and variance homogeneity after angular transformation (Dowdy and Wearden 1991). The dependent variable, synchrony, was defined as (Equation 3.1):

$$(PBI-PBII)/\text{embryos} \quad (3.1)$$

where,

PBI = no of embryos observed at meiosis I

PBII = no of embryos observed past meiosis I

embryos = total no of embryos counted.

The model included treatment and broodstock salinities as class variables, development time as a covariate, and their interactions.

The Bonferroni pairwise comparison procedure was used to test for significant differences ($\alpha = 0.00138$) between mean synchrony levels of treatment*broodstock interactions at mean development time (Neter et al. 1990).

The rate of change (slope) of synchrony for each interaction was determined by plotting (Equation 3.2):

$$ES = \beta_{\text{OTS|BS}} + \beta_{\text{TS|BS}}(DT) \quad (3.2)$$

where;

ES = the expected synchrony

$\beta_{\text{OTS|BS}}$ = the intercept for each treatment salinity
* broodstock salinity interaction

$\beta_{\text{TS|BS}}$ = the slope for each treatment salinity *
broodstock salinity interaction

DT = development time.

Gonad/Body ratios were compared by an analyses of variance. The ratios also met the assumptions of normality and variance homogeneity after angular transformation.

RESULTS

Generally, high levels and rates of synchrony were achieved when the treatment salinity \geq broodstock salinity, except when the broodstock salinity was 13 ppt.

Covariance Model

The covariance model proved to be appropriate in defining the relationship between synchrony and changing salinity. The model explained a high degree of variation ($R^2 = 0.797$). The significance of the independent variables, the covariate and their interactions are noted in Table 3.1. The singular effect of broodstock salinity

on synchrony was statistically significant ($P = 0.0172$), yet the treatment salinity effect ($P = 0.0513$) and their interaction ($P = 0.8351$) was not significant. The covariate (development time) and its individual and collective interactions with broodstock and treatment salinities were highly significant ($P < 0.0001$) (Table 3.1).

Synchrony Level and Rate of Change

The level of synchrony at mean development time for each treatment salinity*broodstock salinity interaction is listed in Table 3.2. Bonferroni pairwise comparisons ($\alpha = 0.00138$) grouped the level of synchrony attained at broodstock*treatment salinity interactions of 20|30 ppt, which produced the highest level of synchrony, 20|20 ppt and 30|30 ppt as statistically the same. All other salinity interactions were not significantly different and all produced poor synchrony (Table 3.2).

The slopes of the regression lines derived from development time*treatment salinity*broodstock salinity interactions on synchrony showed similar results (Table 3.3). Broodstock*treatment salinity interactions of 20|20 ppt, 30|30 ppt and 20|30 ppt produced significantly greater slopes ($P < 0.0001$). The steepest slope (0.0110

TABLE 3.1

Results of the analysis of covariance:
effects of broodstock and treatment salinities, development
time (covariate) and their interactions (*) on synchrony
of *Crassostrea virginica* development.

Sources of Variation	DF	F-ratio	Prob>F
Treatment Salinity	2	3.11	0.0513
Broodstock Salinity	2	4.33	0.0172
Treatment*Broodstock	4	0.36	0.8351
Development Time	1	85.23	0.0001
D.Time*Treatment Sal.	2	14.75	0.0001
D.Time*Broodstock Sal.	2	23.91	0.0001
D.T.*T.Sal.*Brdstck.Sal.	4	6.20	0.0003

$R^2 = 0.7973.$

TABLE 3.2

Results of the analysis of covariance:
 comparing LSMean synchrony of *Crassostrea virginica*
 development by broodstock*treatment salinity interactions.

Broodstock Salinity	Treatment Salinity	Synchrony**		Comparisons***
		LSMean	SE	
13	10	0.084	0.026	A
13	20	0.044	0.022	A
13	30	0.015	0.020	A
20	10	0.014	0.017	A
20	20	0.164	0.016	B
20	30	0.224	0.018	B
30	10	0.038	0.021	A
30	20	0.083	0.023	A
30	30	0.146	0.021	B

** Synchrony = $\text{Arcsin}(\sqrt{(\text{PBI}-\text{PBII}/\text{Embryos})})$.

*** Bonferoni Pairwise Comparison Procedure ($\alpha = 0.00138$).
 SE = Standard Error.

TABLE 3.3

Results of the analysis of covariance:
 slope estimates of synchrony of *Crassostrea virginica*
 development by broodstock*treatment salinity interactions.

Broodstock Salinity	Treatment Salinity	Synchrony**		Prob> T
		Slope	SE	
13	10	0.0002	0.0008	0.8047
20	10	0.0005	0.0012	0.6577
30	10	0.0020	0.0015	0.1708
13	20	0.0006	0.0009	0.4952
20	20	0.0065	0.0010	0.0001
30	20	0.0035	0.0013	0.0099
13	30	0.0004	0.0010	0.6738
20	30	0.0110	0.0011	0.0001
30	30	0.0067	0.0012	0.0001

** Synchrony = $\text{Arcsin}(\sqrt{(\text{PBI}-\text{PBII}/\text{Embryos})})$.

SE = Standard Error.

change in synchrony/min.) was demonstrated at a broodstock salinity of 20 ppt and a treatment salinity of 30 ppt (Table 3.3; Figure 3.1). Development time interactions with broodstock salinity*treatment salinities of 20|20 ppt and 30|30 ppt produced nearly identical slopes (0.0063 and 0.0065) and were next highest (Table 3.3; Figures 3.2 and 3.3). All other interactions were grouped together by comparison ($\alpha = 0.00138$, Table 3.3) and produced slopes at nearly zero, with negative slopes produced at lower confidence limits (Figures 3.5-3.9), except for the broodstock salinity*treatment salinity interaction of 30|20 ppt (Figure 3.4).

Gonad/Body Ratios

There were no significant differences in the gonad/body ratios within the broodstock population during the study ($P < 0.05$).

DISCUSSION

Egg Appearance

The results were somewhat predictable by the physical appearance of the treated eggs. Eggs from a lower broodstock salinity placed into a higher treatment salinity had shrunken membranes, hence, poor synchrony was expected (Table 3.2). When the opposite occurred, the eggs looked more normal, yet synchrony was affected (Table 3.2).

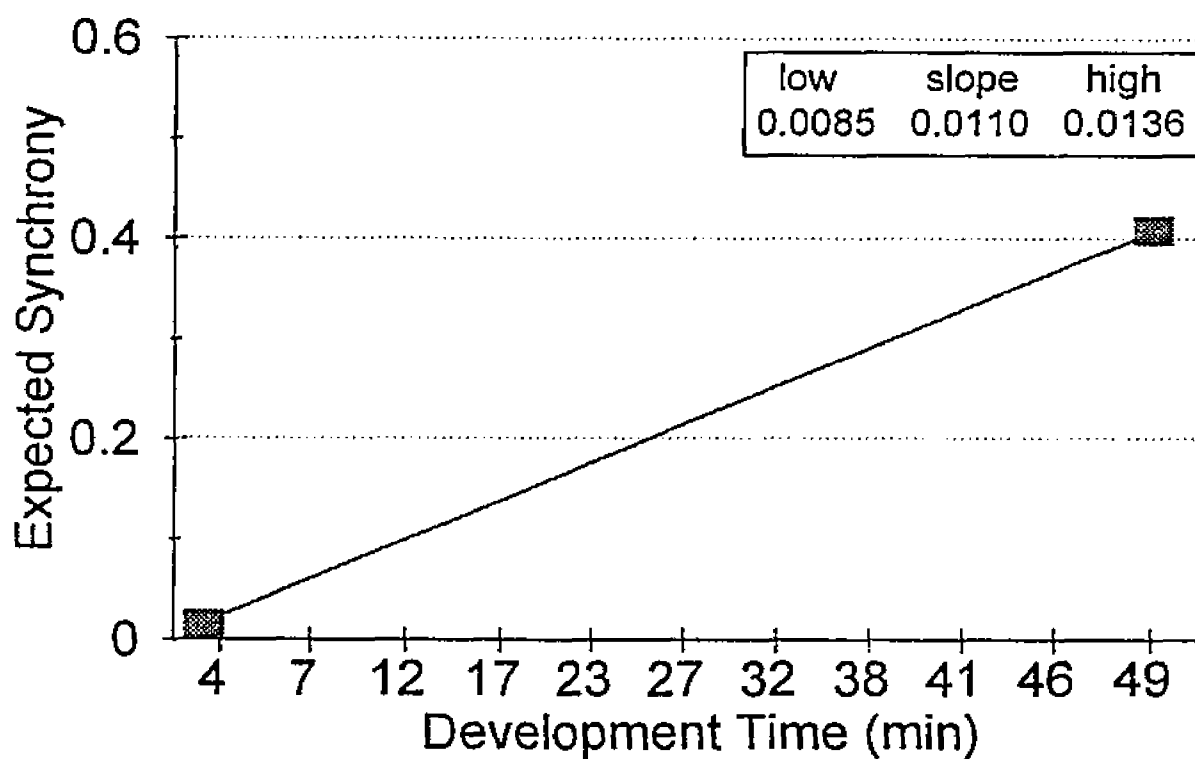


Figure 3.1. Regression of development time*treatment salinity (30 ppt)*broodstock salinity (20 ppt) interaction on synchrony, with slope and confidence limits.

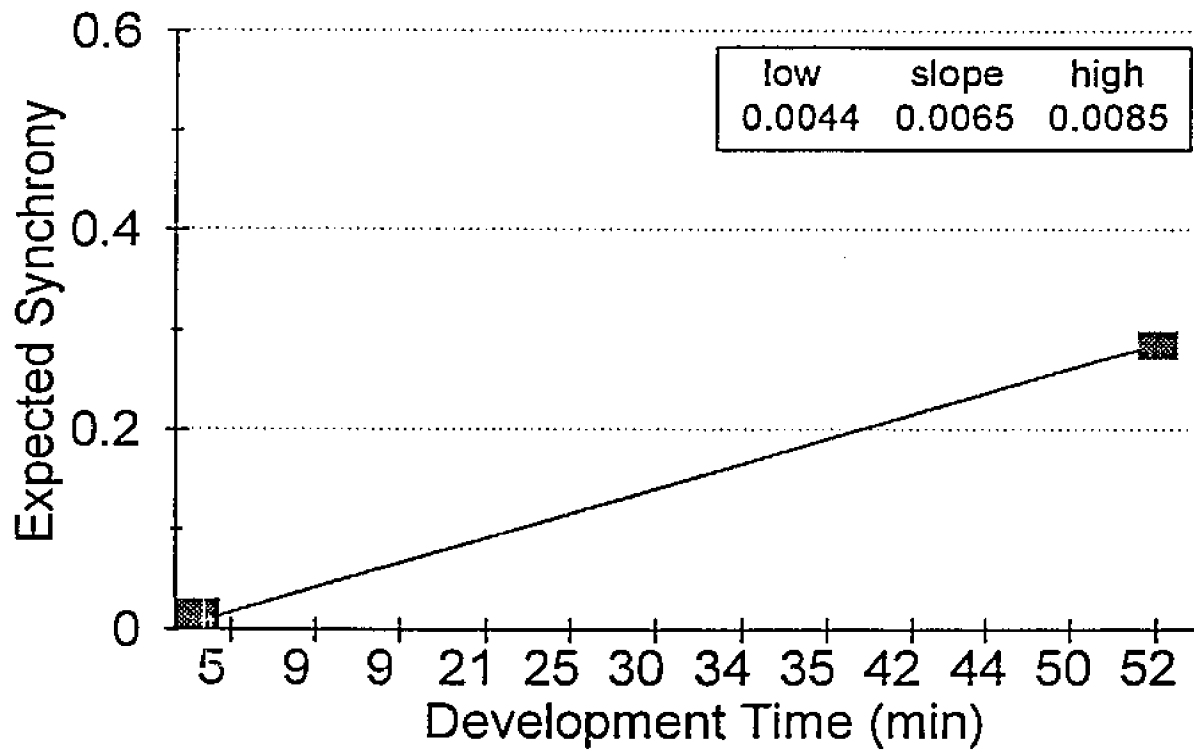


Figure 3.2. Regression of development time*treatment salinity (20 ppt)*broodstock salinity (20 ppt) interaction on synchrony, with slope and confidence limits.

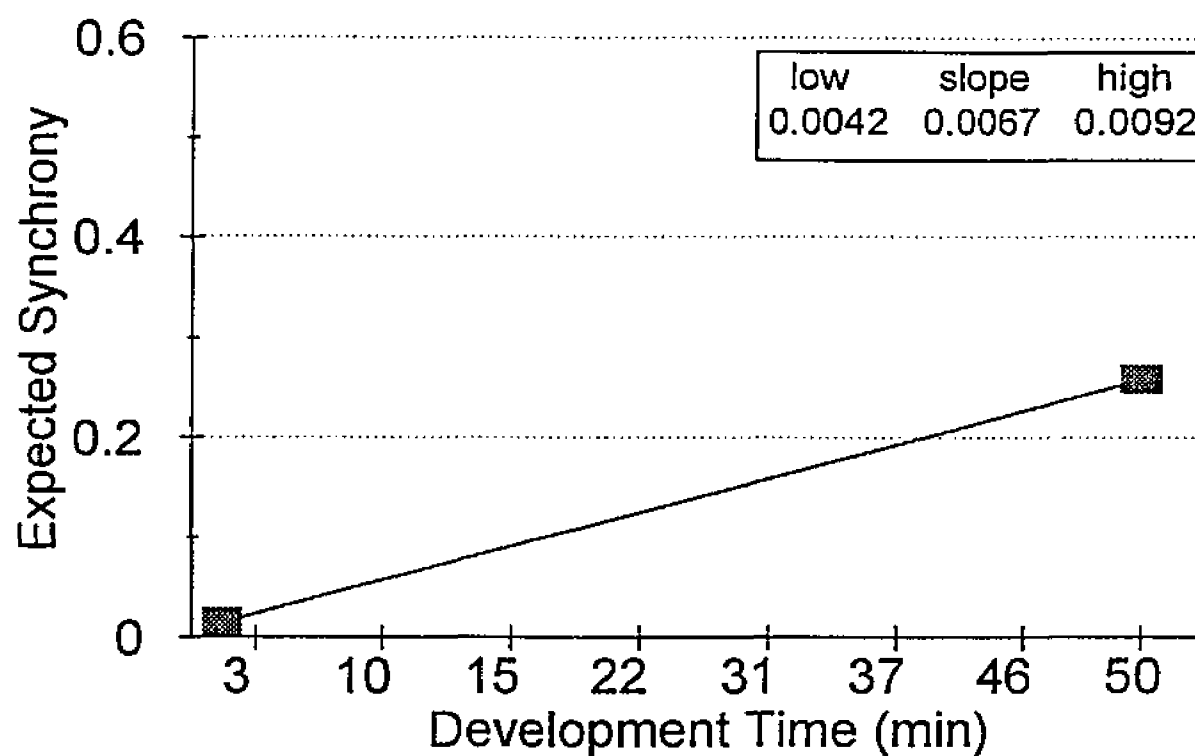


Figure 3.3. Regression of development time*treatment salinity (30 ppt)*broodstock salinity (30 ppt) interaction on synchrony, with slope and confidence limits.

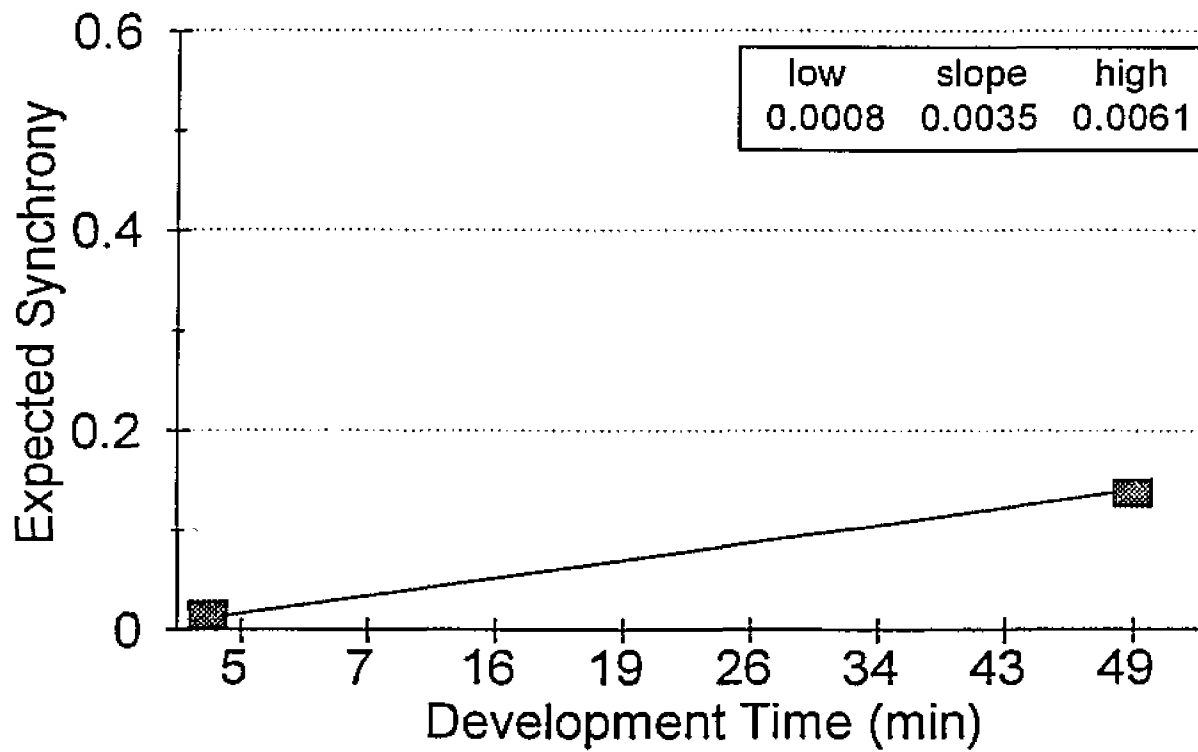


Figure 3.4. Regression of development time*treatment salinity (20 ppt)*broodstock salinity (30 ppt) interaction on synchrony, with slope and confidence limits.

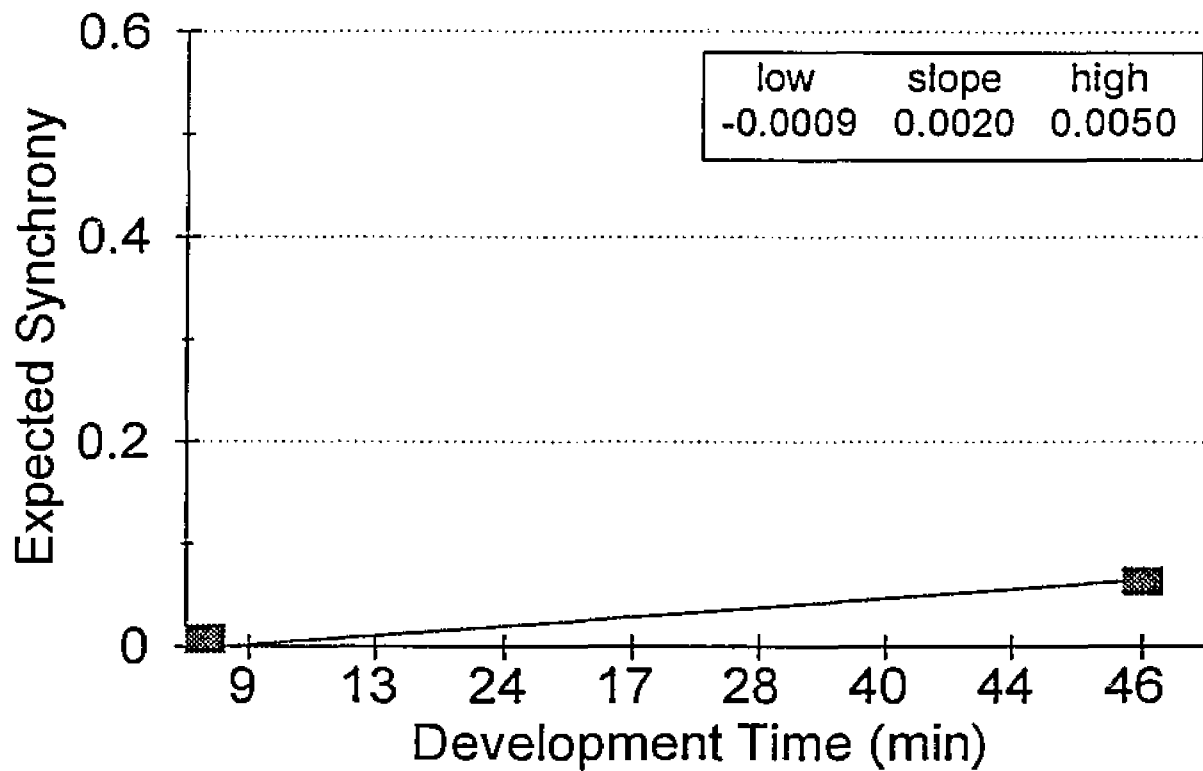


Figure 3.5. Regression of development time*treatment salinity (10 ppt)*broodstock salinity (30 ppt) interaction on synchrony, with slope and confidence limits.

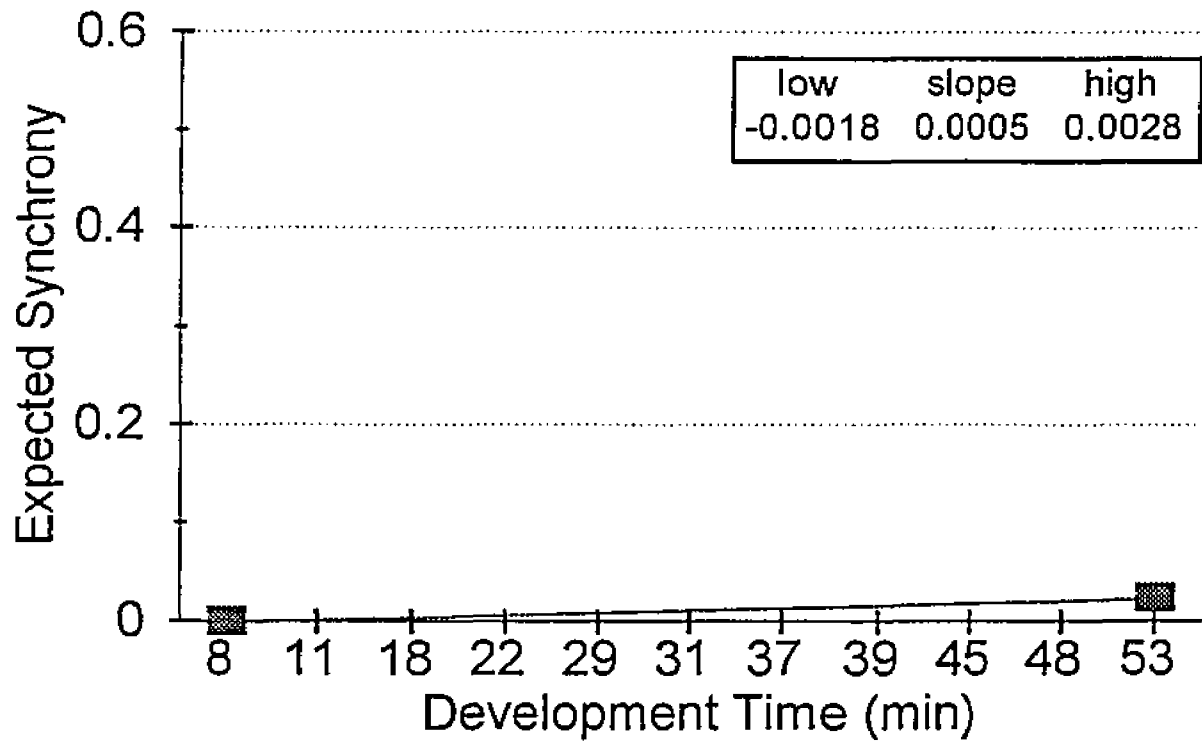


Figure 3.6. Regression of development time*treatment salinity (10 ppt)*broodstock salinity (20 ppt) interaction on synchrony, with slope and confidence limits.

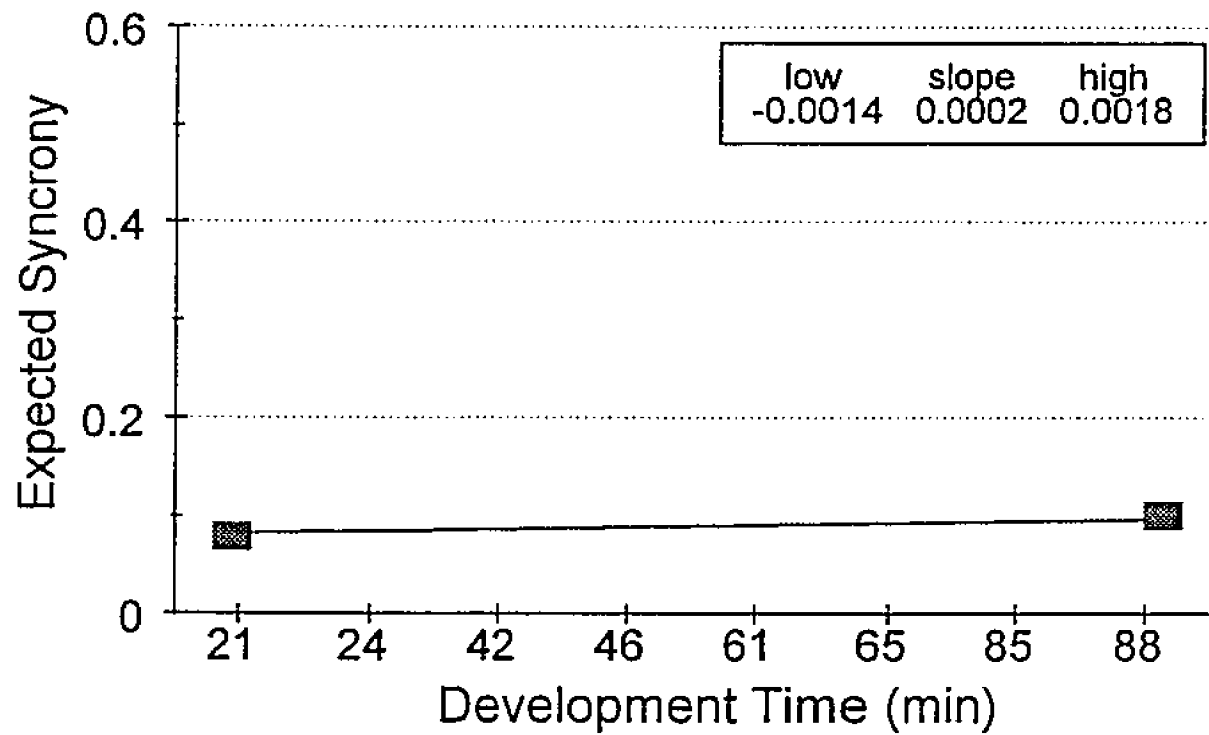


Figure 3.7. Regression of development time*treatment salinity (10 ppt)*broodstock salinity (13 ppt) interaction on synchrony, with slope and confidence limits.

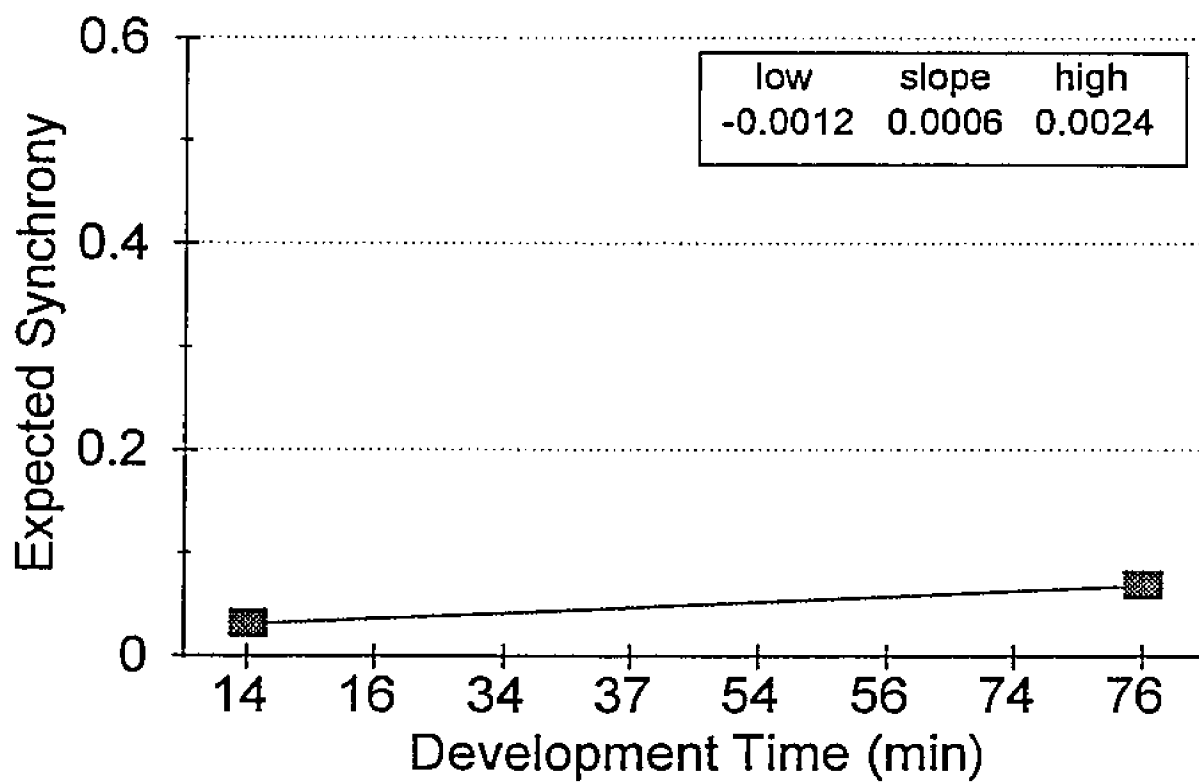


Figure 3.8. Regression of development time*treatment salinity (20 ppt)*broodstock salinity (13 ppt) interaction on synchrony, with slope and confidence limits.

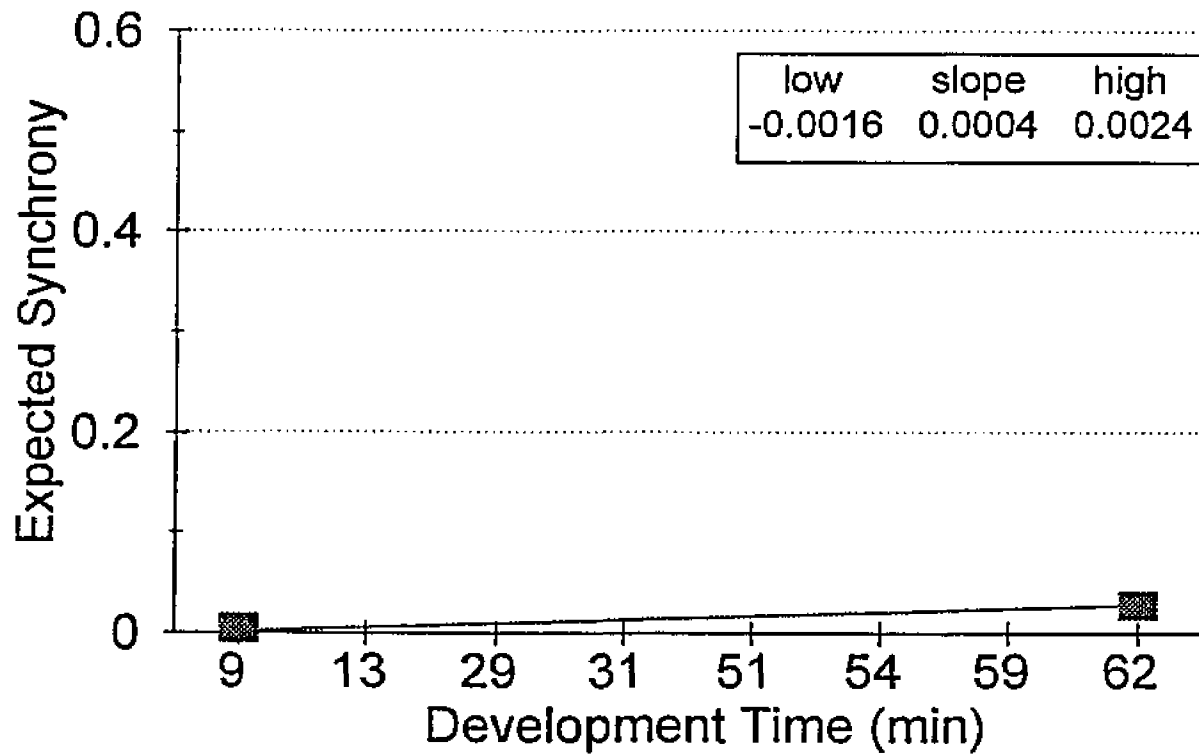


Figure 3.9. Regression of development time*treatment salinity (30 ppt)*broodstock salinity (13 ppt) interaction on synchrony, with slope and confidence limits.

Where broodstock and treatment salinities were the same, eggs looked unaffected and increased levels and rates of change of synchrony occurred (Table 3.2, Figures 3.2 and 3.3).

Salinity Effects

In general, when eggs were treated at the same salinity as the broodstock, synchrony rate and level were greater than at higher or lower treatment salinities (Table 3.2, Figures 3.1-3.9). The exception was at a broodstock | treatment salinity regime of 20|30 ppt. Lu (1986) found meiotic stage duration times decreased equally with increasing salinity at constant temperatures with fertilized *C. gigas* eggs; the rate of meiosis increased with increasing salinity.

A broodstock salinity of 13 ppt did not produce a high level synchrony (Table 3.2). Since the eggs appeared normal at a treatment salinity of 10 ppt, compared to those at 20 ppt and 30 ppt, a higher level of synchrony was expected and occurred, yet all three were grouped together with other low levels of synchrony ($\alpha = 0.00139$, Table 3.2).

There were no significant differences between the mean Gonad/Body Ratios (GBR) of the ten oysters sampled from the holding system prior to each experiment ($P > 0.05$). Since

the selection of oysters for each experiment was biased for gonad ripeness, the GBRs' are an inappropriate variable for use in the model, however, they did show that the mean gonadal condition of the oyster population was the same when the oyster samples were taken and should not have contributed a great deal to random error between the samplings. Also, it eliminates the supposition that the 13 ppt broodstock salinity was inadequate for gonad maturation. Therefore, the poor synchrony exhibited at that salinity was a true result of the salinity interactions.

A reduction in the level and rate of change of synchrony may be the result of a number of factors, such as osmotic condition, the occurrence of particular ions (Shumway 1977) or genetic influences. Maturity of the gametes, environmental factors and genetic constitution of the eggs have been reported to cause variation in the time required for *C. virginica* eggs to be fertilized and to reach different stages of meiosis (Galtsoff 1964). Lower temperature or concentrations of divalent ions Ca^{++2} and Mg^{++2} were found to decrease the rate of chromosomal movement by affecting the polymerization of microtubulin, which is involved in spindle formation (Lu 1986). Lower concentrations of such ions could be achieved by the osmotic effect of placing oyster eggs into a hypotonic condition,

decreasing the rate of chromosomal movement and affecting synchrony. Variance in larval survival is comprised of genetic and non-genetic influences, which includes the rearing environment and broodstock management (Lannan et al. 1980).

A direct relationship between haline exposure and embryonic survival has been reported for other species as well as *C. virginica*. Cain (1973) found salinity had more effect than temperature on early development of *Rangia cuneata*; eggs have less tolerance the further away they are from spawning conditions. This was also the case for *Mulinia lateralis* embryos, *C. virginica* and *Mercenaria mercenaria*, *Nereis divesicolor* and other invertebrates (Cain 1973).

Implications on Wild Oyster Production

The authors of these studies support the paradigm that although many adult estuarine organisms are truly euryhaline and exist in a broad salinity range, a narrower range is more conducive for gametogenesis and more so for embryonic development. For substantial recruitment to occur, a suitable salinity range for embryonic development must occur during spawning. The resulting planktonic cohort can then be transported to a broader salinity range. Ecologically, this would help perpetuate the population, since estuarine

isohalines change during given years due to conditions in the watershed (i.e., rainfall, river discharges, drought). This is substantiated by trends in wild oyster seed production in Louisiana, which is most correlated to suitable salinity regimes (Chatry et al. 1983). Since meiosis (I and II) occurs in oyster eggs after fertilization, it is the earliest stage in embryonic development and its haline inhibition could have lasting effects on development and survival.

Hatchery Management

Spawning techniques and haline conditions at a hatchery can have an effect on both diploid and triploid larval production. Strip-spawning has long had a reputation for poor results, yet Allen and Bushek (1992) report success if ripe gonads are available. It is especially useful with *C. virginica*, since natural spawning on demand can be quite frustrating; strip-spawning provides immediate evaluation. What must be further considered is the salinity from which the broodstock is obtained and the ambient conditions at the hatchery. This study recommends that broodstock should be haline acclimated prior to spawning to achieve greater embryonic survival and ploidy manipulation. Oysters that are chosen for ripe gonadal condition from other locations, however, may spawn when placed back into ambient waters. A

broodstock holding system with reduced water temperature at ambient salinity may be use to inhibit spawning. Otherwise, strip-spawning into an appropriate salinity may be more useful. If commercial-size (e.g., 30,000 L) larval rearing tanks are used, however, one would have the burden of adjusting large volumes of water to eliminate detrimental haline conditions for later embryonic development. Of course, a large capacity broodstock conditioning system that is managed for optimum gonadal development is most desirable (Lannan et al. 1980).

CHAPTER IV

THE EFFECT OF CYTOCHALASIN B DOSAGE ON THE SURVIVAL AND PLOIDY OF *CRASSOSTREA VIRGINICA* (GMELIN) LARVAE

INTRODUCTION

Cytochalasin B (CB), a cytokinetic inhibitor, was first used to produce triploid *Crassostrea virginica* and *Crassostrea gigas* (Thunberg) over a decade ago (Stanley et al. 1981; Allen 1986). Optimal treatments for high triploidy induction have been reported for *C. gigas*, based on temperature, dosage, application timing and duration, at 1 mgCB/1mL dimethyl sulfoxide (DMSO)/L of seawater for 20 min at 25°C, when 50% of the eggs are at meiosis I (Downing and Allen, 1987; Allen et al., 1989). Since *C. virginica* is less fecund than *C. gigas* (Allen 1988), lower dosages and treatment times of 0.5 mgCB/L for 15 min at 25°C (Shatkin and Allen 1990) and 0.25 mgCB/L for 10 to 15 min, at 27° to 29°C (Barber et al. 1992) have been suggested to increase the survival of embryos while maintaining high triploidy induction.

During 1993, work began to test the feasibility of triploid *C. virginica* production in Louisiana, based on the premise that higher summertime meat yields resulting from triploidy could be profitable for the oyster industry. Triploid induction, using 0.5 mg/L CB, has been variable

with commercial size broods (≥ 4 million eyed larvae) achieved with $\leq 85\%$ triploidy. Interaction between the induction salinity, broodstock source salinity, and development time on meiotic synchrony have been identified as major causes of variation (Chapter III). During the first summer of commercial production attempts, survival of CB-treated embryos was $\leq 5\%$ compared to $\leq 21\%$ diploid controls using stripped gametes.

The objective of this study was to investigate the effect of CB dosage on survival and triploidy induction of *C. virginica* to provide further information on the subject and to determine variability by experimental replication.

METHODS

Survival and ploidy of oyster larvae were estimated after exposing embryos to CB dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L for 10 to 15 minutes, with 0.05% DMSO and ambient seawater as controls. Since timing did not permit true replication, the experiment was conducted three times on the same day with the same procedures and partially stripping the same male oysters; only different females were used.

Preparation of Gametes

Gametes were obtained for each experiment similar to Allen and Bushek (1992). Oysters were collected from

nearshore containers, opened, and gender and possible hermaphroditism were determined microscopically using gonad smears. Gonadal ripeness was visually determined by the presence of prominent genital canals. Ten female and three male oysters were grouped separately to avoid sperm contamination.

Eggs were obtained from three of the ripe female oysters, randomly chosen for each experiment. Oysters were individually dry-stripped (i.e., without water) of eggs to ensure simultaneous hydration and fertilization. The resulting eggs were pooled and washed of gonadal debris with filtered (1 μ m) ambient (24 ppt) seawater (FAS) by passing through a 75 μ m Nytex screen, retained on a 15 μ m screen, placed in a beaker and brought to a 1 L volume.

The eggs were kept at 28°C in a water bath throughout hydration, enumeration, fertilization and treatment. Hydration time was 60 min for each experiment, during which time the pooled eggs were enumerated to obtain a random experimental aliquot of about 8 million eggs, which was then brought to a 1 L volume with FAS. After fertilization of the experimental aliquot, 200 mL of eggs were used for each of the five treatment beakers containing 800 mL of FAS, bringing the treatment concentration to about 1.5 M eggs/L. After treatment, each beaker of eggs was eventually put into separate, labeled culture vessels and brought to a 15 L

volume of FAS, bringing the culture concentration to 15 embryos/L.

Three male oysters were partially stripped for each experiment. The resulting sperm were retained in a beaker after being similarly pooled and washed of gonadal debris by passage through a 15 μ m screen.

Fertilization and Treatment

Eggs were fertilized at about 10 sperm/egg and distributed into the five treatment beakers. Eggs were regularly stirred, counted and examined microscopically for polar body formation from individual beakers at appropriate intervals. Treatments began when approximately 50% of the eggs reached PBI (24 to 31 min).

Treatments consisted of stirring the individual concentrations of CB and DMSO into the labeled treatment beakers of developing embryos. The dosages were (in order of application) 0.5 mg/L, 0.25 mg/L and 0.125 mg/L (0.5 mL, 0.25 mL, 0.125 mL of a 1 mgCB/1 mL DMSO stock solution), with 0.05% DMSO (v/v) dissolved in FAS and FAS as controls. Ten minutes after the beginning of the 0.5 mg treatment, those treated embryos were drained onto a 15 μ m screen, rinsed with FAS and washed in 0.05% DMSO (v/v) for 15 min. It took 5 min to complete the process with the other treated embryos. In the same order, the embryos were then rinsed

again with FAS and placed into individual culture vessels containing aerated FAS and equal volumes (from the same culture) of *Isochrysis* aff. *galbana* clone CISO and brought to a 15 L volume. Thus, all the eggs-embryos received the same soak, treatment and wash times and were handled in a similar manner.

Embryos were incubated for 48 h at ambient temperature to D-stage larvae. Initial embryo counts were obtained using a 1 mL subsample from each culture vessel in triplicate prior to beginning the next experiment. Afterward, the larvae were thoroughly mixed and counted as before. The vessels were individually drained onto a 40 μ m screen and subsamples were placed into 1.5 mL centrifuge tubes and shipped overnight to Dr. Standish K. Allen at Rutgers University's Haskin Shellfish Research Laboratory for ploidy determination using flow cytometry (Chaiton and Allen 1985).

Data Analyses

Differences between treatment means for both survival and percent triploidy were determined by analysis of variance using SAS (SAS 1991). The percent triploidy was calculated from the relative proportion of triploid cells to the total number analyzed by the curve-fitting program ModFit (Verity Software House, Topsham, Maine, USA) (Allen

and Bushek 1992). Survival and percent triploidy met the assumptions of normality and variance homogeneity after angular transformation (Dowdy and Wearden 1991). The models included survival and percent triploidy as separate dependent variables, and treatments and experimental replicates as independent variables. Tukey's Honestly Significant Difference Procedure was used to test the significance between the treatments and experiments ($\alpha = 0.05$).

RESULTS

Generally, equal triploidy and survival results were obtain with 0.5 mg/L and 0.25 mg/L CB dosages.

Percent Triploidy

The model (% triploidy = treatments, experiments) proved to be very appropriate in defining the relationship between the treatment effects and triploidy. The model explained a high degree of variability ($R^2 = 0.9616$). The treatment effect was highly significant ($P < 0.0001$), as expected, and the experimental replication effect was not significant ($P = 0.5291$) (Table 4.1). Comparison procedures on mean percent triploidy (angular transformation) found no significant differences between the 0.125 mgCB/L dosage and the two controls, as well as between the 0.25 mgCB/L and 0.5 mgCB/L dosages (Table 4.2).

TABLE 4.1.

Results of the analysis of variance:
effect of cytochalasin treatment and experimental
replication on the percent triploidy of oyster larvae.

Sources of Variation	DF	F-ratio	Prob>F
Treatment	4	49.70	0.0001
Experiment	2	0.69	0.5291

$R^2 = 0.9616.$

TABLE 4.2.

Results of the analysis of variance:
comparing mean triploidy of *Crassostrea virginica*
larvae by treatment.

Treatment	Triploidy*		Comparisons**
	Mean	SD	
0.125mg cb	0.3594	0.110	A
0.25 mg cb	0.9052	0.063	B
0.5 mg cb	0.9783	0.156	B
control w/o dmso	0.1862	0.009	A
control w/ dmso	0.0949	0.084	A

* Triploidy = $\text{Arcsin}(\sqrt{(\% \text{Triploid}) (0.01)})$.

** Tukey's Honestly Significant Difference ($\alpha = 0.05$).
SD = Standard Deviation.

Untransformed mean percentages were $13\% \pm 6.7\%$ triploidy for 0.125 mgCB/L, $61.8\% \pm 6.2\%$ for 0.25 mgCB/L, and $68.2\% \pm 14.1\%$ for 0.5 mgCB/L. The 0.05% DMSO control had a mean triploidy of $1.4\% \pm 1.3\%$ and the FAS control was $3.4\% \pm 0.3\%$, representing error caused by "noise" during the ploidy analyses (Figure 4.1).

Figure 4.1 depicts untransformed percent triploidy by treatment and experiment. The graph visually represents the variability that occurs in triploid induction, particularly when different females are used, yet transformed data revealed no significant difference ($P < 0.05$) (Table 4.2).

Survival

The model (survival = treatments, experiments) also proved to be appropriate in defining the relationship between the treatment and experimental replication effects on survival. The model explained a reasonable amount of variation in survival ($R^2 = 0.7172$). Both effects were highly significant ($P < 0.0001$, Table 4.3). There were significant differences in mean survival between all three experiments (Table 4.4). No significant difference was found between the two controls. Survival was also not significantly different between the three CB treatments and averaged 19.5% less than the mean survival of the controls (Table 4.5).

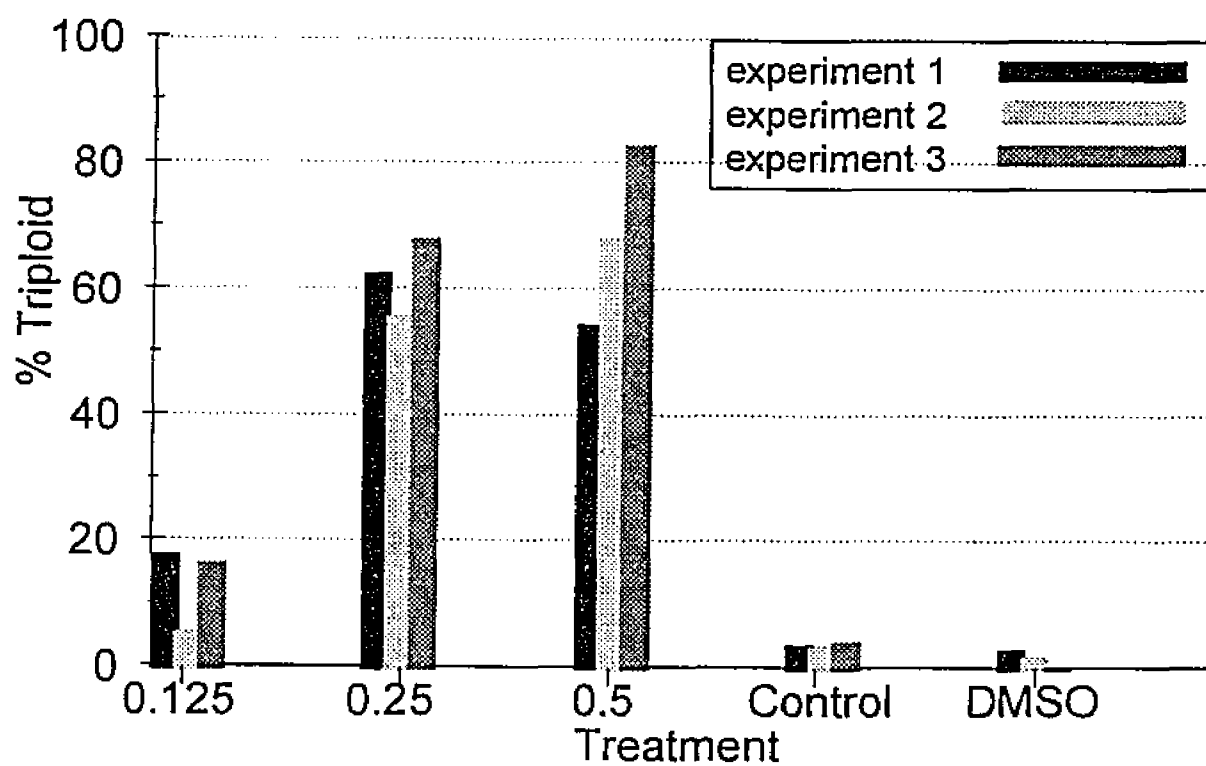


Figure 4.1. Percent triploidy of D-stage oyster larvae by treatment and experiment.

TABLE 4.3.

Results of the analysis of variance:
effect of cytochalasin treatment and
experimental replication on survival of oyster larvae.

Sources of Variation	DF	F-ratio	Prob>F
Treatment	4	11.54	0.0001
Experiment	2	25.11	0.0001
$R^2 = 0.7172.$			

TABLE 4.4.

Results of the analysis of variance:
comparing mean survival of *Crassostrea virginica*
larvae by experiment.

Experiment	Survival*		Comparisons**
	Mean	SD	
1	0.6211	0.095	A
2	0.4998	0.089	B
3	0.7658	0.216	C

* Survival = $\text{Arcsin}(\sqrt{\text{Normal Larvae/Embryos}})$.

** Tukey's Honestly Significant Difference ($\alpha = 0.05$).

SD = Standard Deviation.

TABLE 4.5.

Results of the analysis of variance:
 comparing mean survival of *Crassostrea virginica*
 larvae by treatment.

Treatment	Survival*		Comparisons**
	Mean	SD	
0.125mg cb	0.5961	0.1204	A
0.25 mg cb	0.5795	0.0860	A
0.5 mg cb	0.4748	0.0913	A
control w/o dms0	0.7334	0.2035	B
control w/ dms0	0.7585	0.2126	B

* Survival = $\text{Arcsin}(\sqrt{\text{Normal Larvae/Embryos}})$.

** Tukey's Honestly Significant Difference ($\alpha = 0.05$).

SD = Standard Deviation.

Figure 4.2 depicts untransformed survival by treatment and experiment. The results of experiment III represent the expected inverse relationship between survival and CB dosage. All three experiments illustrate how DMSO exposure has virtually no effect on survival. The difference in survival between replicates again portrays the variability that occurs, particularly when different females are used, with mean survival being significantly different ($P \leq 0.05$, Table 4.4).

DISCUSSION

These results concur with previously reported findings: 0.25 mgCB/L (Barber et al. 1992) and 0.5 mgCB/L (Shatkin and Allen 1989) are appropriate dosages for inducing triploidy in *C. virginica*, while variation may depend on egg quality, or is random, or both (Allen and Bushek 1992).

Treatment Recommendations

In this study, there was no statistical difference in triploidy or survival between the two higher CB dosages. With the cost of CB at about \$10/mg, economics dictate that the lower effective dosage is more suitable, at 28°C for 10 min. With a range of 54% to 82% triploidy (0.5 mgCB/L) versus 55% to 67% (0.25 mgCB/L), one is inclined to use the higher dosage. Greater triploidy probably would have occurred at a longer (15 min) treatment time, as in previous

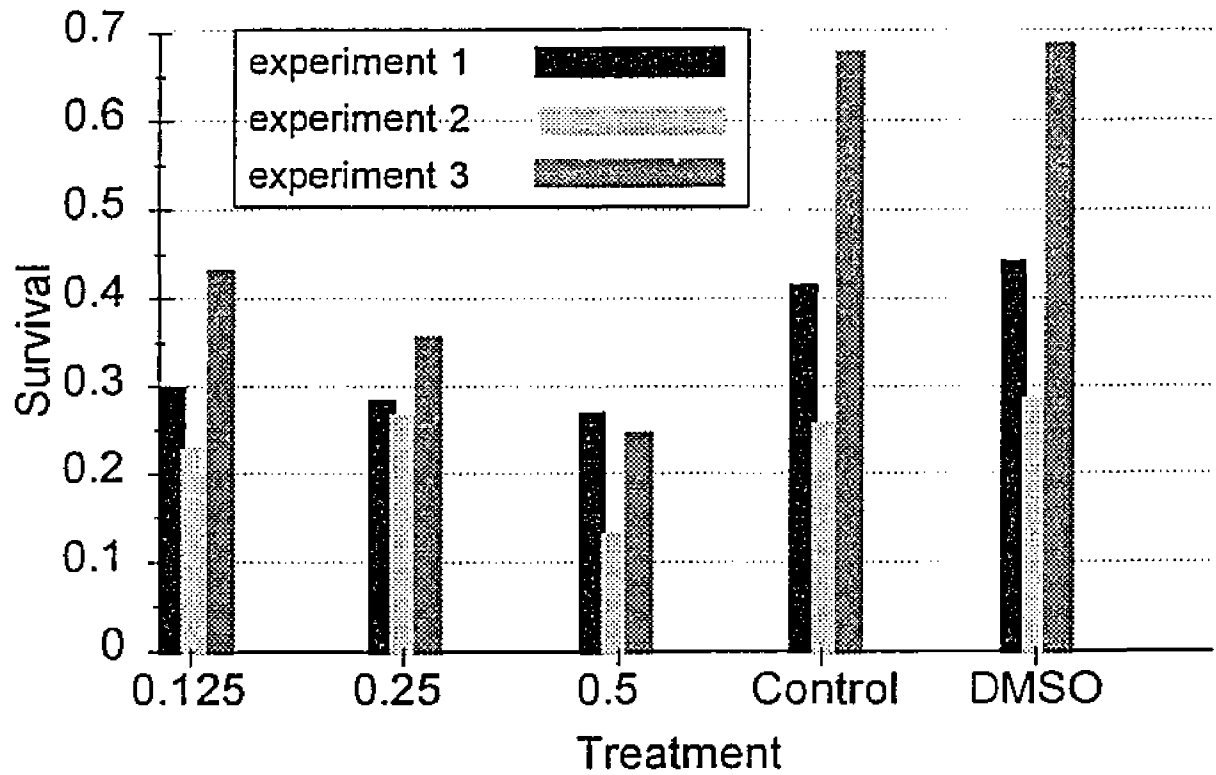


Figure 4.2. Survival of embryos to D-stage oyster larvae by treatment and experiment.

research, and possibly would have resulted in lower survival.

The question of dosage duration may be answered by using a developmental benchmark to cease treatment. For maximization of triploid production, embryos should be exposed to CB for a period of time long enough to have the highest proportion possible undergo meiosis but short enough to minimize mortality (Barber et al. 1992). Allen and Bushek (1992) attributed their low variance in triploidy to using meiosis I as a benchmark to begin treatment, claiming to have effectively removed meiotic rate as a factor. This study points out, however, that the meiotic rate is still affecting variability in triploidy, even with using the same treatment time for each group of eggs from different females. A developmental benchmark is also necessary to terminate treatment when using CB. Observation of a 100 mL subsample of eggs just prior to and during treatment, until the eggs reach a benchmark (i.e., 50% meiosis II or some percent beginning cleavage) would be more effective than a set treatment time. This benchmark could be used for *C. virginica* or *C. gigas*, and needs to be determined to improve the efficiency of utilizing CB for triploid induction.

The real answer to improving induction efficiency is the development and use of tetraploid broodstock.

Tetraploid male oysters produce diploid sperm and when used to fertilize eggs from diploid females, 100% triploid offspring are possible (Guo and Allen 1994).

CHAPTER V

CONCLUSIONS

Three critical factors affecting the production of triploid oyster larvae using cytochalasin B were addressed in this study: (1) the gonadal condition of the broodstock; (2) the salinity conditions before and during meiotic development, and; (3) the cytochalasin B dosage. All three factors must be understood before consistent, commercial-scale production of triploid larvae can be achieved in Louisiana.

Gonad condition is very important to the production of any animal, and oysters are no different. Additional documentation of oyster gametogenesis in Louisiana has now been provided in order to better understand gonad recycling. The use of an additional developmental stage, recycling, including the presence of early and/or later development characteristics plus the presence of atresia, is recommended to improve the characterization of gonadal development in Louisiana during histological analyses. Understanding gonadal recycling will allow greater success at egg acquisition for hatchery efforts, particularly for triploid production since low survival of early embryos to D-stage larvae occurs after ploidy manipulation. Although histological analysis is not a practical tool for everyday

hatchery management, oysters with prominent genital canals and hypertrophic gonads are suitable and obvious traits of egg abundance. Broodstock represented by visible digestive diverticula, or gonads with a "spotty" appearance are either spent or recycling, and should be returned to the water for further development before use. A large capacity broodstock conditioning system that is managed for optimum gonadal development is most desirable.

The salinity of the seawater the broodstock is obtained from, and the salinity during induction have significant effects on successful production of triploid larvae. Broodstock should be acclimated to induction salinity conditions prior to use. Otherwise, poor synchrony of polar body formation will occur and few embryos will be in the correct stage of development for induction (i.e., 50% PBI).

Either 0.25 mg/L or 0.5 mg/L are appropriate cytochalasin B dosages for triploid induction of *C. virginica*. Both will give suitable triploidy and larval survival results.

COMMERCIAL-SCALE PRODUCTION OF TRIPLOID OYSTER LARVAE IN LOUISIANA

The production and sale of triploid oyster larvae for use in remote setting systems may be the answer to a commercial hatchery's economic viability in Louisiana. Hatchery and remote setting technologies are not be

economically competitive when located in areas where periodic wild oyster seed production. These technologies must produce an oyster that is more valuable than nature can provide to be economically feasible. Triploid oysters have the potential to be a valuable summer crop in the Gulf region if grow-out produces the expected (high yielding meats from July through September). Only until then will there be a demand for triploid *C. virginica* larvae. If that occurs, then the following recommendations should be considered.

Having enough eggs to produce commercial-size broods of pediveligers is the name of the game in triploid production. An appropriately engineered broodstock holding system with ≥ 8 sack (1.5 bu/sack) capacity is recommended. Obtain broodstock during mid-April ideally from mid-estuary locations where there is less incidence of *Perkinsus marinus*. Maintain broodstock at 28°C and at ambient salinity, assuming that ambient salinity is suitable for gonadal maturation (e.g., >15 ppt). If ambient salinity is ≥ 20 ppt, then hold broodstock at 5 ppt less than ambient. Feed broodstock using regular changes of raw seawater and $\geq 10\mu\text{g}$ dry wt/mL of algal paste 2 to 3 times per day (*Chaetoceros* sp., *Thalassiosira pseudonana* (3-H), *Skeletonema* sp. or other appropriate algal food [see Bolton

1982; Donaldson 1991])). Periodically check broodstock for feeding (i.e., the presence of crystalline styles [see Galtsoff 1964]) and dark digestive diverticula. If broodstock mortality occurs, check for *Perkinsus* and lower temperature and/or salinity appropriately. Use broodstock with prominent genital canals. Stripped female oysters averaged 71 million eggs \pm 33 million per female.

During 1994, triploid induction using a recommended dosage and treatment time of 0.5 mg/L CB for 10-15 min resulted in commercial broods of \geq 4 million eyed larvae with \leq 85% triploidy. Survival of CB-treated embryos using stripped gametes was \leq 5% compared to \leq 21% diploid controls. Triploid larvae generally took 10-15 days to become pediveligers, compared to 10-12 days for diploid siblings. Setting success was equally good (e.g., 20%). Plan accordingly.

FUTURE RESEARCH NEEDS

The use of a developmental benchmark for terminating CB treatment is needed to deal with the variability in meiotic rate between batches of eggs. Research is needed to identify such a benchmark in order to obtain consistent, successful ploidy manipulation (e.g., high percentage triploidy or tetraploidy) with minimum mortality.

The development and use of frozen gametes from tetraploid broodstock would revolutionize triploid production. Straws of frozen diploid sperm could be shipped anywhere, thawed, and used with eggs from diploid females to produce 100% triploid offspring. The development of tetraploid *C. virginica* broodstock and cryogenically preserved gametes are currently proposed (1995) for funding to Sea Grant. Although the commercial feasibility of triploid *C. virginica* production in Louisiana is still being evaluated, triploid *C. gigas* have proven to be beneficial to the oyster industry in the Pacific Northwest and warrants the investigation and development of tetraploid broodstock.

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VITA

John Eric Supan was born May 19, 1953 in Glen Dale, West Virginia. He graduated from Bishop Donahue Memorial High School in 1971. Afterward, he graduated from Wheeling Jesuit College, Wheeling, West Virginia, with a B.S. in Biology (Pre-Med) in 1975. Two years later, he married Karen Franklin of Cameron, West Virginia and began graduate studies at the Gulf Coast Research Laboratory, Ocean Springs, Mississippi, where he began developing expertise in oyster culture, depuration, management and industrial development. He received an M.S. in Biology (Marine) from the University of Southern Mississippi in 1981. After working for three years in fisheries assessment and monitoring of Mississippi Sound, ten years as a part-time consultant-interviewer of marine recreational finfish surveys along the Gulf and six years as a marine advisory agent for the Louisiana Cooperative Extension Service, he transferred within Louisiana State University to pursue a doctorate as a research associate in the Office of Sea Grant Development, where he is currently employed. John received his Ph.D. in Wildlife and Fisheries Science in May, 1995 from the School of Forestry, Wildlife and Fisheries at LSU. John, Karen and their two children currently reside near Covington, Louisiana.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: John Eric Supan

Major Field: Wildlife and Fisheries Science

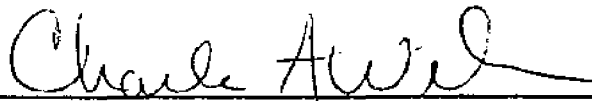
Title of Dissertation: The Production of Triploid Oyster Larvae
(Crassostrea virginica [Gmelin]) in Louisiana

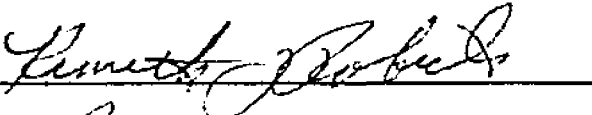
Approved:


Major Professor and Chairman

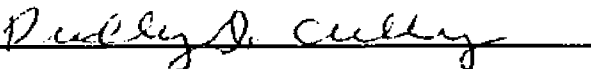

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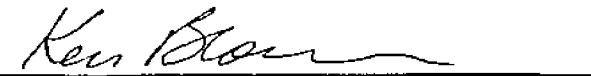
EXAMINING COMMITTEE:











Date of Examination:

April 3, 1995