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Effect of estradiol-17B on the gonadal developemnt of diploid and triploid female eastern oysters

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EFFECT OF ESTRADIOL-17β ON THE GONADAL DEVELOPMENT OF DIPLOID AND TRIPLOID FEMALE EASTERN OYSTERS

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To my family

The Oyster

There once was an oyster
Whose story I tell,
Who found that some sand
Had got into his shell.
It was only a grain,
But it gave him great pain.
For oysters have feelings
Although they're so plain.
Now, did he berate
The harsh workings of fate
That had brought him
To such a deplorable state?
Did he curse at the government,
Cry for election,
And claim that the sea should
Have given him protection?
No---he said to himself
As he lay on a shell,
Since I cannot remove it,
I shall try to improve it.
Now the years have rolled around,
As the years always do,
And he came to his ultimate
Destiny---stew.
And the small grain of sand
That had bothered him so
Was a beautiful pearl
All richly aglow.
Now the tale has a moral;
For isn't it grand
What an oyster can do
With a morsel of sand?
What couldn't we do
If we'd only begin
With some of the things
That get under our skin.
Anonymous

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FOREWORD

The eastern oyster *Crassostrea virginica* (Gmelin 1791) provides an important commercial fishery along the Atlantic and Gulf coast of the United States, and for more than a century has been an important part of the local economy and cultural heritage of the state of Louisiana. Despite large annual landings, disease problems, environmental fluctuations, and reduced summer meat yields, have caused steady declines in production of oysters during the past decade. These declines have generated an interest for the production of oysters with enhanced growth and disease-resistant characteristics.

The production of polyploid oysters addresses some of these problems. Triploid oysters have an increased growth rate and higher meat yield as a result of decreased fecundity because energy reserves go to the production of meat rather than producing gonad. The lack of a reliable, consistent, and safe method for the induction of triploidy in eastern oysters has been a major obstacle for the production of triploid oysters on a commercial scale. In the Pacific oyster, *Crassostrea gigas*, the production of triploid oysters has been performed successfully by crossing diploid with tetraploid oysters. In the eastern oyster, this technique has not yet been successful because of the lack of tetraploid broodstocks. Tetraploidy in oysters has only been obtained by the chromosomal manipulation of mature oocytes from triploid oysters. The reduced fecundity of triploid eastern oysters has been a constraint for the production of tetraploid oysters.

The goal of this study was to evaluate the induction of ovarian maturation of triploid and diploid eastern oysters by using of the hormone, estradiol-17β, to produce viable oocytes to use in the production of tetraploid broodstocks. It has been demonstrated in several species of vertebrates and invertebrates that estradiol functions as an oocyte maturation hormone by stimulating the synthesis of vitellogenin.

This thesis is composed of five chapters that are organized in the following order. In the first chapter a description of general and reproductive biology of the eastern oyster *Crassostrea virginica* is provided, as well as a short history of the research done on invertebrate endocrinology, and the use of estrogens to enhance gonadal maturation in bivalves. It also states the general goal and objectives of the thesis. The second chapter describes a new technique to quantify gonadal development in histological slides based on image analysis of gonad-to-body ratio (GBR) and compares it to existing techniques. This new technique provides a fast and comparable estimate of gonadal quantity in oysters. The third chapter provides a qualitative description of the gonadal development in triploid oysters that allows categorization of the different gonadal stages. The fourth chapter, the core of the thesis, evaluates the effect of estradiol-17β on the gonadal development of triploid and diploid female eastern oysters. Finally, the fifth chapter provides a summary and general conclusion of the findings of this work, and suggests possible uses and applications in the aquaculture industry.

The results of his thesis have thus far yielded five published abstracts in conference proceedings, These conference presentations include: 1) Effect of the steroid Estradiol-17β on the gonadal maturation of diploid and triploid eastern oysters, 2003 Gulf Coast Reproductive Biology meeting, New Orleans, Louisiana; 2) Effect of Estradiol-17β on the gonadal maturation of the Eastern oyster *Crassostrea virginica*, 2004 Louisiana Chapter of the American Fisheries Society, Baton Rouge, Louisiana, which he was awarded first place in Best Abstract category and third place in Best Presentation category; 3) Steroid induced enhanced ovarian maturation in the Gulf coast oyster *Crassostrea virginica*, 2004 World Aquaculture Society, Honolulu, Hawaii; 4) Effect of Estradiol-17β on the female gonadal maturation of triploid Eastern oysters, 2005 Aquaculture America, New Orleans, Louisiana; and 5) Rapid estimation of gonad-to-body ratio

in oysters, 2005 Aquaculture America, New Orleans, Louisiana, which he was awarded second place in Best Poster category.

For consistency of presentation all chapters of this thesis have been prepared in the format of the *Journal of Shellfish Research*. It is anticipated that Chapters 2, 3, and 4 will be submitted for publication in peer-reviewed journals.

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ABSTRACT

Declines in annual oyster landings and problems associated with seasonal reduction of oyster meat yields have increased interest for development of techniques to produce oysters with enhanced growth characteristics. Research interest has been focused on developing improved lines by induction of polyploidy. The goal of the present study was to evaluate the enhancement of gonadal development of triploid oysters, by the use of the hormone estradiol 17-β (E_2) to produce viable eggs for the development of tetraploid broodstocks. Previous studies with the Pacific oyster, *Crassostrea gigas*, have shown: (1) the need to use the larger eggs of triploid females to accommodate a tetraploid nucleus, and (2) that E_2 appears to be responsible for ovarian maturation.

The objective of this research was to compare the effect of three dosages of E_2 (0.0, 37.5, 75.0 ng/g wet weight) on ovarian maturation of diploid and triploid eastern oysters, *Crassostrea virginica*, by measuring: 1) gonad-to-body ratio; 2) oocyte area; 3) levels of E_2 in the hemolymph; and, 4) by qualitatively staging gonadal development. Experiments were performed in August of 2003 and May and August of 2004 for 9, 12, and 15 days. Image analysis of histological sections (gonadal condition) and enzyme immunoassays (E_2 levels) were used to evaluate the effects of steroidal treatments.

There was no evidence of oysters in spawning condition in any of the control groups of the triploid oysters, yet oysters in spawning condition were found in the low and high dose treatments in percentages as high as 40%. For the diploid oysters, the effect of E_2 was less apparent due to their natural state of fecundity during the summer months. Concentrations of E_2 were measured in diploid and triploid oysters and their levels fluctuated from 13.8 to 29.1 for diploids and from 16 to 33.8 for diploids, with respect to the stage of gonadal maturation.

The overall response of triploid oysters to E_2 suggested that this hormone had a positive effect on ovarian maturation. These results can have direct applicability for the development of tetraploid broodstocks in the eastern oyster.

CHAPTER 1 - INTRODUCTION

The eastern oyster, *Crassostrea virginica*, is a member of the family Ostracea, class Bivalvia in the phylum Mollusca. This genus, together with the genera *Saccostrea* and *Ostrea*, comprise some of the most commercially important bivalve species in the world (NRC 2004).

The eastern oyster is an intertidal and subtidal bottom dweller that inhabits estuarine and coastal waters of the western Atlantic from the Gulf of St. Lawrence in Canada to the coasts of Argentina in South America (Carriker and Gaffney 1996). Its life cycle is divided in a larval planktonic stage ranging from 2 to 3 weeks and a sessile benthic stage. The planktonic stage can be subdivided in three major larval stages. After fertilization, a non-feeding "trochophore" larva develops. This larval stage last approximately 24h, during which the larvae reaches 50 to 60 μm in shell width. After the trochophore stage, the larvae develop into a "veliger" or "D-stage larva". These larvae possess a shell in the form of two valves with a straight hinge giving the larvae the appearance of the capital letter "D". In this stage, the larvae become planktotrophic and can reach 70 to 125 μm in shell width. After 12 to 20 days, the veliger larvae modify their morphology, forming an umbo that overhangs from the straight hinge line, and a foot with a retractor muscle that allows the larvae to crawl. During this "pediveliger" or "eyed-larva" stage the larvae s crawl on surfaces searching for suitable hard substrate where they settle, cement, and metamorphose into a small oyster called "spat". From the spat stage, the oyster acquires a sessile benthic form that is permanent through the rest of its life, typically forming assemblages called reefs, bars, or beds that range in size from a few to hundreds of acres (NCR 2003). Adult oysters are filter feeders, mainly feeding on phytoplankton and dissolved organic matter. They have a high tolerance to wide ranges of temperature, salinity, and turbidity (Shumway 1996), characteristics that make them an ideal specie for aquaculture.

Reproduction of the eastern oyster is seasonal, and is highly regulated by temperature (Loosanoff and Davis 1953). In the Gulf States, the reproductive season usually starts in late March to early April, with the beginning of gametogenesis. Spawning begins to occur in May and lasts until late August, but recycling of gonads with subsequent spawning is common as late as in October (Supan and Wilson 2001).

In this thesis, much of the work was based on the evaluation of gonadal development and understanding of oogenesis (female gametogenesis). The interplay of oogenesis and gonadal development, and how they proceed throughout the reproductive season is important to the understanding of this thesis.

Oogenesis refers exclusively to the process of egg maturation. It is a complex process in which primordial germ cells differentiate to become oogonia or oogonial cells, and later oocytes and eggs (Figure 1.1). These oogonial cells, besides forming a female gamete or haploid cell (cells with one set of chromosomes), also store cytoplasmic enzymes, messenger RNAs, organelles, and metabolic substrates that allow gametes to maintain metabolism and development. In oysters, oogonia are self-renewing stem cells that endure throughout the lifetime.

Gonadal development is a broader term that describes the changes that occur in the gonad throughout the inactive and active reproductive periods. The gonad is a tissue situated in the visceral mass between the digestive gland and the mantle, and in most eastern oysters, primordial gonadal tissues develop 8 to 12 weeks after settlement of the spat (Eble and Scro 1996).

Gonadal development can be divided in stages that include undifferentiated and sexually active stages (Figure 1.2). During the undifferentiated stage, the follicles, which are the primordial gonadal tissue, are small and separated, and oogonial cells cannot be distinguished

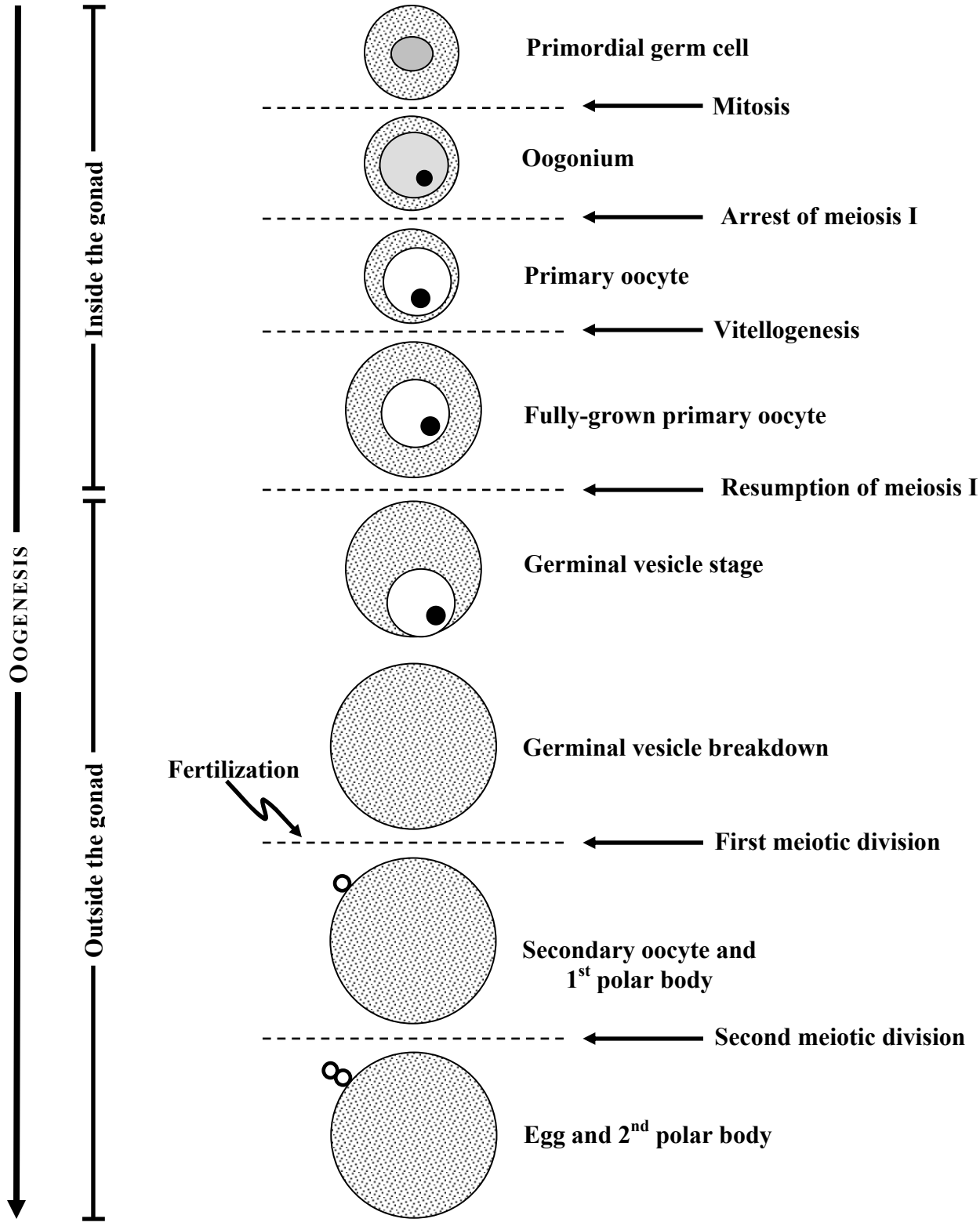

Figure 1.1 Diagram of the general progression that occurs during oogenesis

during this period. When sexual differentiation begins, the ovarian follicles enlarge and invade the surrounding vesicular connective tissue. At this point, the oogonia begin to divide rapidly and start to differentiate into primary oocytes, the nucleus enlarges and the volume of the primary oocytes increases as a result of nutrient accumulation (vitellogenesis). Before spawning occurs, the oocytes detach from the germinal epithelia and enter the lumen; these oocytes are approximately 40 μm in diameter. After the conclusion of spawning, the follicles shrink and surrounding connective tissues begins to grow, the gametes that remain in the follicles are eventually reabsorbed by hemocytes. After this process, the gonad becomes undifferentiated again and a new process of gonadal development begins when the appropriate biological and physical conditions occur again.

During the reproductive season, oysters expend most of their stored energy reserves, mainly glycogen, for the production, maturation, and spawning of gametes, a process that generates a reduction of as much as two-thirds of their body weight (Allen and Dowing 1986). This prolonged reproductive season causes reduction of meat yields during the summer months and has been a constant and costly constraint for oyster processors in the Gulf States (Supan 2000). The idea of developing triploid oysters came almost 25 years ago (Stanley and Allen 1981) as a response to address this problem and to provide profitable meat yields year round.

Triploidy is a condition where each cell contains three sets of chromosomes instead of the usual two sets of most animal somatic cells. This condition results in partial sterility as a result of an unbalanced chromosomal state causing irregularities during the meiotic divisions (Thorgard 1983). Triploid oysters are a valuable asset to aquaculture because they can retain most of their glycogen reserves during the reproductive months, and therefore, meat yields do not decrease during this period.

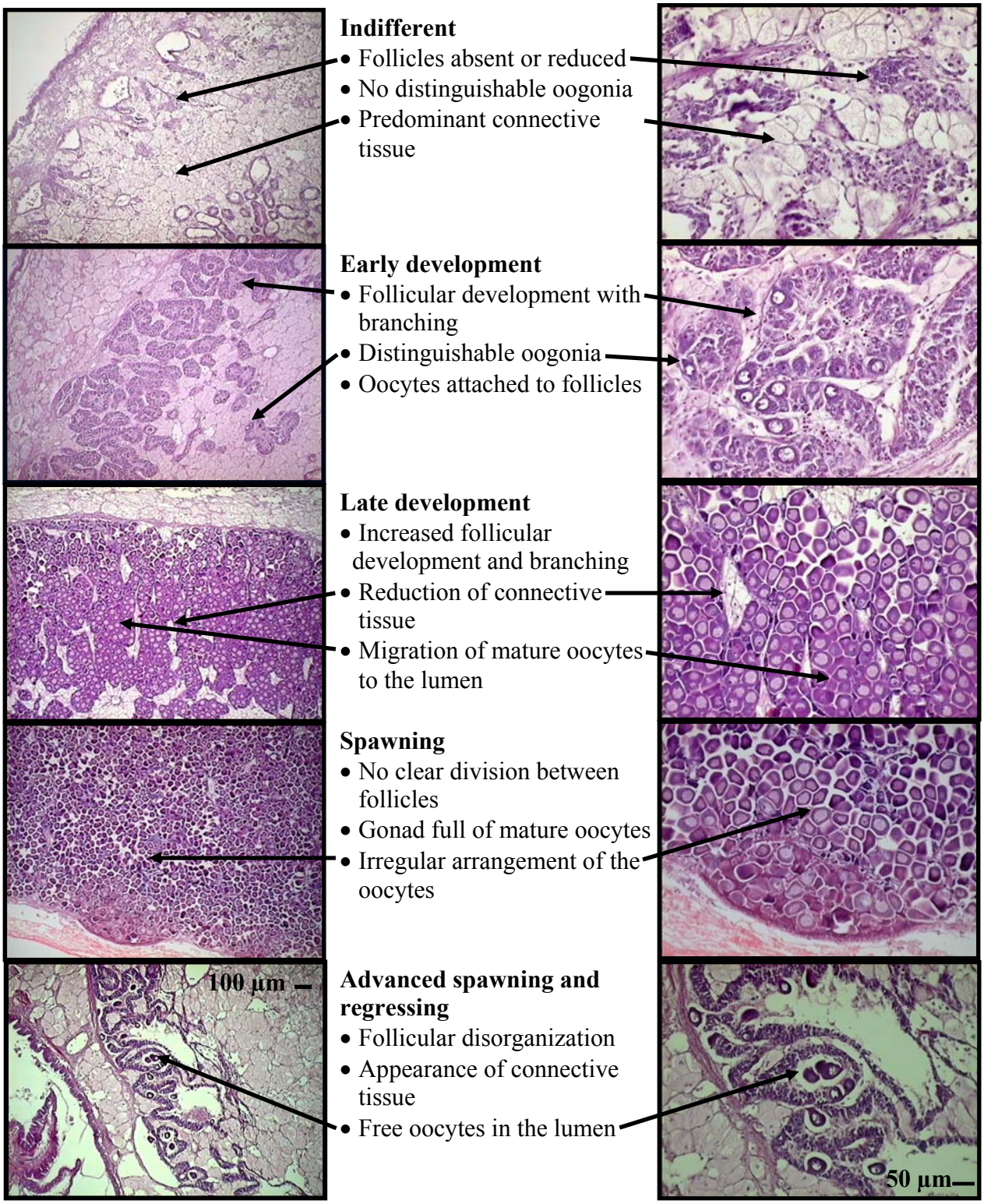

Figure 1.2 Photomicrographs of histological sections of the eastern oyster, *Crassostrea virginica*, showing different stages of gonadal development throughout the 2003 reproductive season at Grand Isle, Louisiana. This classification is based on the work of Kennedy and Krantz 1982.

Triploidy in oysters can be induced by several chemical and physical methods, but cytochalasin-B (CB) has been used most often because it has been proven to be more effective than other methods (Dowing and Allen 1987). Unfortunately, the use CB has several disadvantages including: 1) an induction success of less than 80%, 2) highly toxicity that poses risks for the untrained personal, and 3) larval mortality rates that are usually higher than normal diploid-diploid spawnings. Because of these negative factors, different methods to induce triploidy in oysters have been investigated.

In the Pacific oyster, *Crassostrea gigas*, triploids are now produced by crossing of tetraploid males with diploid females (Guo *et al*. 1996). This method is used to produce so-called "natural triploids" which yields 100% triploid oysters due to the chromosomal combination of diploid sperm with a haploid egg, and avoids the negative effects of CB. Another advantage is that triploid oysters produced by this method grow faster, 10% in size and 12% in meat weigth, than those produced with CB (Wang *et al.* 2002).

In oysters, tetraploidy is not a normal condition and several efforts have been explored to induce this condition (Guo *et al.* 1994). So far the only successful method to induce tetraploidy is by blocking the first polar body in eggs of triploid oysters fertilized with haploid sperm (Guo and Allen 1994). The disadvantage with this method is that it needs sexually mature triploid female oysters. Nevertheless, the method has been successful with several oyster species (Supan *et al*. 2000, Eudeline and Allen 2000, He *et al*. 2000). At the commercial level, triploidy production has only been successful in Pacific oysters, because triploid females of this species have an ample range of egg production, from 20,000 per individual to 20,000,000 (Guo and Allen 1994a). Although the commercial production of triploid oysters began in 1984 (Allen

1988), it was not until the development of "natural triploids" that the commercial interest for triploid oysters developed in hatcheries around the world (Nell 2002).

In the eastern oyster, the development of tetraploids has not been as successful as with the Pacific oyster. Part of the problem is that sexually mature female triploid oysters in this species occur rarely, about 10 in 1,600 (Supan *et al.* 2000). If successful production of tetraploid eastern oysters is to occur, methodologies are needed to induce sexual maturity in triploid females, rather than reliance on natural occurrence.

The reproduction of the eastern oyster, as of most other marine invertebrates, is regulated by a combination of exogenous factors (temperature, salinity, food availability) and endogenous factors (stored nutrients, endocrine and neuroendocrine compounds) (Giese and Pearse 1974). Although numerous experiments have reported the effect of exogenous factors on reproduction (Medcof and Needler 1941, Gauthier and Soniat 1989, Paulet and Boucher 1991), the study of endogenous factors, such as the function and occurrence of hormones, has been limited (Thompson *et al*. 1996).

Hormones are substances secreted and transported to other parts of that body to evoke physiological responses (Joosse and Geraerts 1983). In vertebrates the occurrence and action of hormones on sexual development has been extensively studied since the first experiments of Berthold in 1849, where he recorded the effects of castration on the development of secondary sexual characteristics in cockerels (Hadley 1992). In invertebrates, endocrinology has been biased toward the study of insects and crustaceans, due to the economic importance that they represent to agriculture and aquaculture (Tombes 1970). In mollusks, study of the function and occurrence of hormones, with the exception of gastropods and cephalopods, has been limited

(Joose and Geraerts 1983), but the economic importance of several species of bivalves has caused an increased interest of the study of endocrinology.

Hormones can be categorized into different groups depending on their chemical structure, the place in the body where they are produced, and on the physiological effect they cause. Among these groups, steroids are a group of hormones that play an important role in reproduction. Steroids are organic compounds of adrenal or gonadal origin which share in common a basic structure of four carbon rings (Norris 1980). The most commonly occurring steroids in vertebrates are the corticoids, progestogens, androgens, and estrogens (Hadley 1992). Their common names and functions are giving in Table 1.1. In this thesis, interest will be focused on estrogens due to their role in female reproduction development.

Table 1.1 Common vertebrate hormones and functions

Category	**Commonly occurring steroids**	**Function**
Corticoids	Cortisol Corticosterone Cortison	Carbohydrate metabolism
Progestogens	Pregnenolone progesterone	Maintenance of pregnancy Induction of sexual receptivity
Androgens	Testosterone Androstenedione Dehydroepiandrosterone	Stimulate development of male characteristics (Masculinizing agents)
Estrogens	Estradiol-17β Estrone Estriol	Stimulate development of female characteristics (Feminizing agents)

Estrogens are a group of hormones synthesized by the reproductive organs and adrenal glands in females and in lesser quantities in males. In most vertebrates, estrogens are responsible for reproductive processes, such as gonadal differentiation, maturation, and oogenesis; and in mammals they are also responsible for secondary sexual characteristics. The principal estrogens found in females are estradiol, estrone, and estriol. From these, estradiol-17β (E_2) is the major estrogen produced by the ovary in vertebrates and is responsible for oocyte maturation and development of secondary sexual characteristics (Sadlier 1974). In invertebrates, it has been suggested that its main function is the regulation of vitellogenesis (Couch *et al*. 1987).

Studies on the endocrine control of reproduction in crustaceans have found that some groups have the ability to synthesize vertebrate-type steroids such as progesterone and E_2, and that levels fluctuate closely in accordance with the condition of the ovaries (Pavlof and Goy 1990, Subramoniam 1999). In bivalves, E_2 is believed to be directly involved in the regulation of yolk protein formation (Matsumoto *et al*. 1997). In the Pacific oyster and the Japanese scallop, *Patinopecten yessoensis*, presence of estrogens such as estrone, E_2, and estriol has been demonstrated (Matsumoto *et al.* 1997). Although the mechanism of action is not well understood, there is evidence that shows that these hormones are linked to the reproductive cycle and play an important role in gametogenesis (De Longcamp *et al*. 1974). For example, levels of E_2 increase significantly in the hepatopancreas of the red mud crab, *Scylla serrata*, at the onset of vitellogenesis (Warrier *et al*. 2001). In cultured ovarian pieces of the starfish *Asterina pectinifera*, the oocyte diameter increased significantly when the tissues were incubated with E_2 (Takahashi and Kanatani 1981). In the giant tiger shrimp, *Penaeus monodon*, the highest levels of E_2 were found in maturing ovaries (Quinitio *et al.* 1994). This estrogen was found to be

capable of accelerating glycogenolysis and of accelerating sexual maturation in female Pacific oysters (Mori 1969, 1972), and was also demonstrated to be one of the factors controlling vitellogenesis in the ovary of this species (Li *et al*. 1998).

Information on the effect and occurrence of E_2 in the eastern oyster *C. virginica* is not available, and could be of great value in the sexual maturation of triploid female eastern oysters. Experiments in this thesis were designed to evaluate at the effect of exogenous estradiol-17β on the ovarian maturation of diploid and triploid eastern oysters, with the purpose of producing viable oocytes to use for the production of tetraploid broodstocks. The first objective of this thesis was to develop a rapid method to quantitatively estimate gonadal development in oysters that was comparable to the existing slower methods. The second objective was to create a qualitative scale to stage the gonadal maturation of triploid oysters, and the third objective, was to evaluate the effect of E_2 on the gonadal development of diploid and triploid eastern oysters.

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CHAPTER 2 - COMPARISON OF METHODS TO ESTIMATE GONAD-TO-BODY RATIO IN OYSTERS

According to the Food and Agricultural Organization, more than 90% of global oyster production comes from aquaculture (FAO 2002). Methods to assess gonadal condition of oyster broodstocks are important to maintain a dependable supply of larvae for culture. Although histological sectioning is expensive and time consuming, and therefore not a practical tool for hatchery evaluation of broodstock (Supan and Wilson 2001), its use is essential in detailed studies that evaluate gonadal development.

Although several methods have been used to quantify gonadal condition in oysters, it is commonly performed by use of histological transverse sections. Some of these measure only gonadal and body widths in predetermined transects (Kennedy and Battle 1964), others determine the gonadal and body areas by planimetry (Morales-Alamo and Mann 1989), or by techniques based on computer image analysis of specific areas of the gonad (Heffernan and Walker 1989). The abundance of different methods and a lack of standardization makes it difficult to compare published results, and raises concerns that some of the apparent differences in gonadal estimations are due to methodological differences.

Image analysis has been used to quantify gonadal development in oysters and other bivalves in previous studies and its reliability has been demonstrated (Heffernan and Walker 1989, Buchanan 2001, Delgado and Perez-Camacho 2003). The novelty of the methods tested in this chapter is in the use of digital morphometric image analysis based on simple computer software. The goal of this work was to identify a fast and reliable method to quantify gonadal condition of eastern oysters, *Crassostrea virginica*, based on a gonad-to-body ratio (GBR) that could be comparable to the methods currently employed. The objectives of this study were to: 1) compare the GBR obtained by a standard transect method (Supan and Wilson 2001) to three

methods that determine gonad and body areas by computer image analysis; 2) compare GBR values obtained from the four methods for different gonadal stages; and 3) compare the time required for used of each method.

MATERIALS AND METHODS

The eastern oysters used in this study were produced at the Sea Grant Grand Isle Bivalve Hatchery (29º15'12"N, 90º03'26"W) on Caminada Bay, Louisiana, in June of 2002, and were collected in August of 2003, during the spawning season for this species. The 50 oysters used for this experiment were selected randomly from a population held in natural waters, and represented the normal stages of gonadal development present during this portion of the spawning season (Figure 2.1).

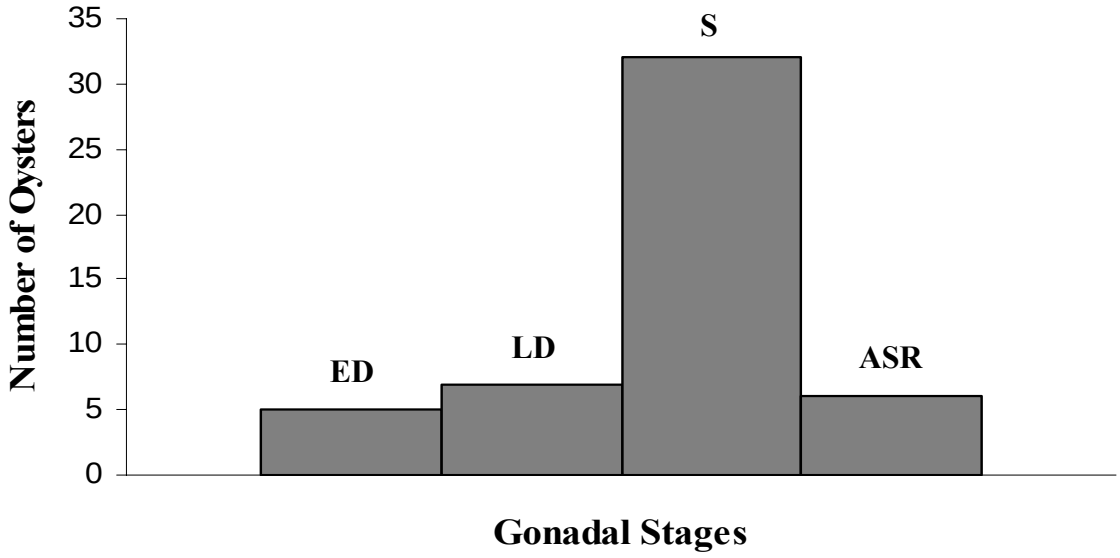

Figure 2.1 Distribution of gonadal stages for the oysters studied. ED = early development, LD = late development, S = spawning, ASR = advanced spawning and regressing (N = 50).

Histology

Sectioning of the oysters for histological comparison was done according to Morales-Alamo and Mann (1989), (SOP-1). The oyster meats were removed from the shells and a 4-mm thick cross-section posterior to the gill-palp junction was dissected, placed in a tissue cassette (Omnisette, Fisher-Scientific, Pittsburg, Pennsylvania) and preserved in Davidson's fixative (SOP-2) (Humason 1967). To prepare the samples for histological processing, an automated tissue processor (Leica TP1050, Mayer Instruments, Houston, Texas) was used. Tissue samples were dehydrated in a stepped alcohol series (70%, 80%, 95%, and 100%), cleared in xylene and embedded in paraffin (Paraplast, Tyco/Healthcare Group LP, Mansfield, Massachusetts). By use of a microtome (Shandon, Thermo Electron Corp. Pittsburg, Pennsylvania), a 4-µm section approximately 1 mm from the gill-palp junction was cut and mounted on a negatively charged microscope slide. Each of the slides were placed on a tray inside an autostainer (Leica Autostainer XL, Mayer Instruments), where the paraffin was removed and the tissues were stained for 30 min with hematoxylin (Anatech Ltd., Battle Creek, Michigan) and counterstained for 1 hr with eosin Y (Anatech, Ltd.). Cover slips were mounted above the histological samples with permount (Fisher-Scientific, Fair Lawn, New Jersey).

Transect Method

The GBR was calculated following a routine methodology (Supan and Wilson 2001) that used ten equidistant transects across the section, excluding the mid-dorsal and mid-ventral regions of the gonad, and determining the gonadal width relative to the body width on each transect (Figure 2.2A). The average GBR value from transects 3 to 8 was used to determine the total GBR of each oyster (SOP-3).

Image Analysis

For the three image analysis methods, the same histological section of each oyster used for the transect method was digitized on a scanner (Epson Perfection 1640SU, Epson America, Inc. Long Beach, California) at a resolution of 300 dpi and imported into Photoshop 7.0 (Adobe Inc, San Jose, California). The scanned images were magnified 12 times and analyzed using commercially available image analysis software (Metaview 6.1, Universal Imaging Corporation, Downingtown, Pennsylvania). The outline of the body following the outer margin of the mantle was traced by use of a digital pen and a drawing tablet (Hyper Pen 12000U, Aiptek Inc. Irvine California) connected to a personal computer (Dell precision workstation 360, Dell Inc. Austin, Texas). The gonad was also traced following the inner and outer margins and avoiding the interfollicular space to the extent allowable by the magnification of the digital image (SOP – 4). The image analysis software automatically calculated the areas of the body and the gonad by counting the number of pixels contained within the traced areas. The first image analysis method (no curvature: NC) calculated the GBR by comparing the area of the gonad to the area of the body only in a section of the slide comparable to the section used in the transect method, excluding the mid-dorsal and mid-ventral regions of the gonad (Figure 2.2B). The second method (no gills: NG) calculated the GBR comparing the total area of the gonad to the area of the body excluding the gills (Figure 2.2C). The third method (total area: TA) calculated the GBR comparing the total area of the gonad to the total area of the body including gills (Figure 2.2D).

Based on the gametogenic classification described by Kennedy and Krantz (1982) (Chapter 1), the oysters were classified into four different groups: early development (ED), late development (LD), spawning (S), and advanced spawning and regression (ASR), and the GBR values estimated within these groups were compared by the four methods. The time spent

performing each method was recorded and compared to determine the effort required for each method.

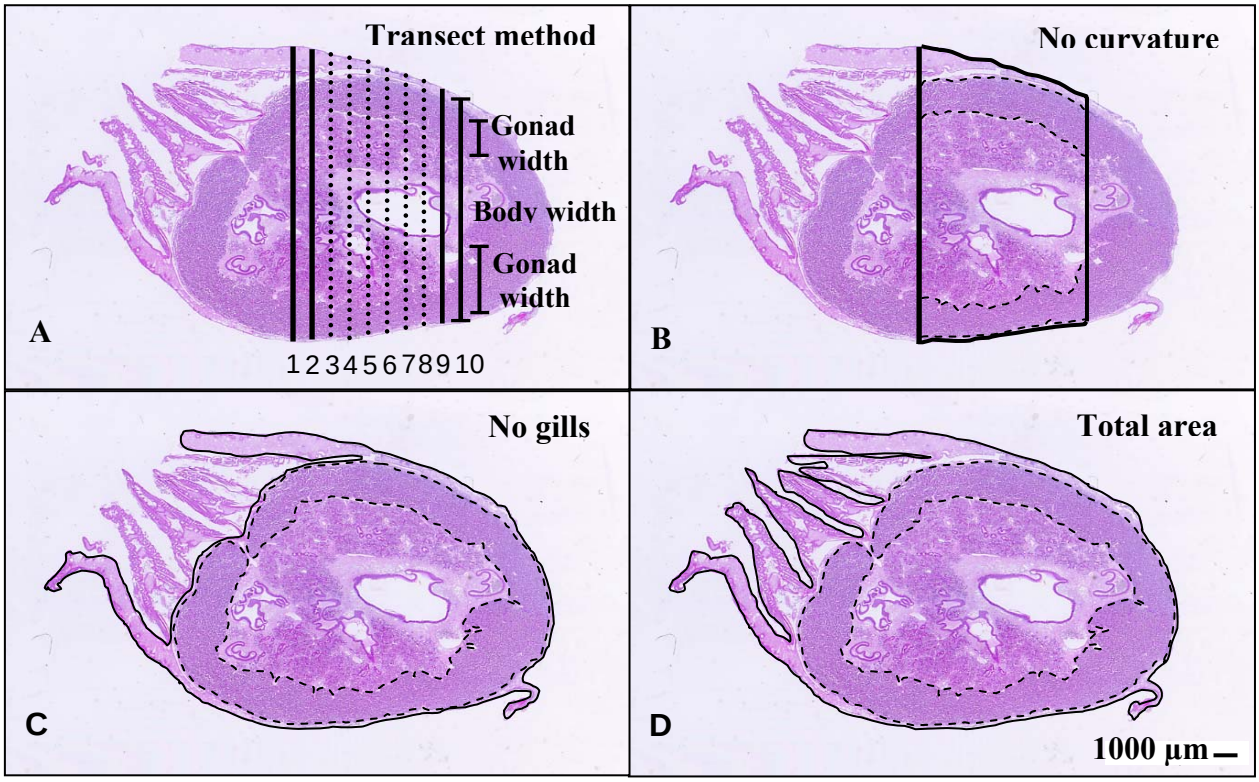

Figure 2.2 Diagrammatic representation of the four methods used to determine the gonad-to-body ratio on transects and areas associated with histological sections. Solid lines outline the body area; dashed lines outline the gonadal area.

Statistical Analysis

The GBR values obtained with each method were analyzed and compared using the Friedman test (Zar 1974), and Dunn's post-hoc procedure was used to test for differences among the four methods. An ANOVA followed by a Tukey's post-hoc test was performed to test for differences among the time required to performed each method. A significance level of $\alpha = 0.05$ was used in all statistical analysis.

RESULTS

Determination of the GBR of the oyster cross sections required that the various tissue regions be easily distinguishable. The gonadal tissue was located in a ring shape at the periphery of the body mass, but separated from the exterior by the intervening mantle tissue (Figure 2.3). The prominent characteristic of the gonad for these studies was the presence of gametes and darker basophilic staining of the gonadal tissue. The central region of the sections contained the stomach, digestive diverticula, and intestinal branches. The gill tissue was confined to the ventral region of the animal and appeared on only one side of the tissue sections. The "body" area included all tissues in the cross sections (as defined by the particular method used) including the gonadal tissues.

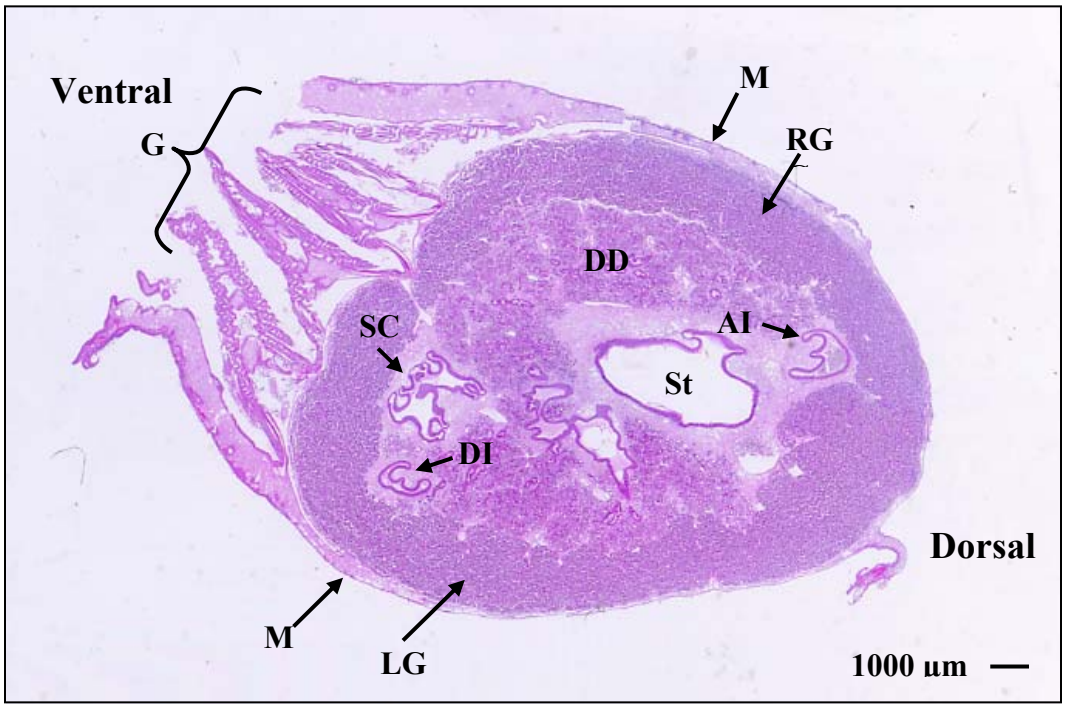

Figure 2.3 Anatomical features in a transverse histological section of the eastern oyster. AI = ascending intestine, DI = descending intestine, DD = digestive diverticula, SC = stomach caecum, St = stomach, LG = left gonad, RG = right gonad, M = mantle, and G = gills.

There were no significant differences ($P > 0.05$) among the GBR values derived from two of the image analysis methods tested in this work (TA and NC) and the transect method (Table 2.1). The differences among the GBR values obtained for any oyster by the TA, NC, and TM methods were never greater than 10%. The NG method was significantly different ($P < 0.05$) from the other three methods and gave higher estimations of the GBR values 80% of the time. These values were always at least 10% higher than the values derived from the other methods.

Table 2.1 Statistical comparison among the different methods to obtain the gonad-to-body ratio (GBR)

	GBR		
Method	**Mean**	**± SE***	**Comparisons****
Transect method	0.213	0.011	A
Total area	0.237	0.013	A
No gills	0.258	0.013	B
No curvature	0.214	0.012	A

*SE: standard error. **Different letters indicate significant differences among treatments, as indicated by Dunn's test ($\alpha = 0.05$). N=50

When the oysters were separated based on their gonadal stages, there were no significant differences ($P > 0.05$) among the four methods when the GBR values were small (≤ 0.10). When the GBR values were high (≥ 0.14), there were significant differences among the methods (Table 2.2) similar to those observed when the values were pooled for all of the oysters.

Comparison of the time required to obtain the GBR with each method was significantly different ($P < 0.05$) among the transect method and the three image analysis methods. On average, the TA method required 20%, and the NC method 10%, of the time required for the transect method (Table 2.3).

Table 2.2 Statistical comparison among the different methods to obtain the gonad-to-body ratio (GBR) at different gonadal stages.

Method	GBR		Comparisons**
	Mean	± SE*	
Early development			
Transect method	0.082	0.015	A
Total area	0.072	0.010	A
No gills	0.089	0.007	A
No curvature	0.069	0.005	A
Late development			
Transect method	0.174	0.022	Abc
Total area	0.178	0.016	Ac
No gills	0.203	0.014	B
No curvature	0.168	0.022	C
Spawning			
Transect method	0.251	0.011	A
Total area	0.289	0.012	A
No gills	0.309	0.013	B
No curvature	0.258	0.011	A
Advanced spawning and regression			
Transect method	0.148	0.015	A
Total area	0.162	0.013	Ab
No gills	0.186	0.016	B
No curvature	0.158	0.012	Ab

*SE: standard error. **Different letters indicate significant differences among treatments, as indicated by Dunn's test ($\alpha = 0.05$).

Table 2.3 Time comparison among the different methods used to obtain the gonad-to-body ratios.

Method	Time (min)		Comparisons**
	Mean	± SE*	
Transect method	14.7	0.3	A
Total area	3.1	0.2	B
No gills	2.7	0.1	B
No curvature	1.6	0.1	C

*SE: standard error. **Different letters indicate significant differences among treatments, as indicated by Tukey's test ($\alpha = 0.05$).

DISCUSSION

The goal of this study was to develop a fast and reliable method to quantitatively estimate gonadal condition in oysters based on a gonad-to-body ratio. To achieve this, three methods were developed to calculate the area of the gonad and body on histological sections with computer image analysis. These methods were compared among themselves and with a reference transect method that has been used in several studies to report gonadal condition.

Of the image analysis methods tested, the TA and NC methods were the fastest and most reliable compared to the transect method. Although the TA and NC methods gave results similar to the transect method, the NC method was susceptible to visual error at the time of selecting the area to be measured, potentially resulting in greater variation when performed by different technicians.

There were no differences among the methods to estimate GBR values when the ratios were small, (early development, and advanced spawning and regression). This could be because the differences were too small to resolve (< 0.02), but it is important to note that because the number of oysters in the ED and ARS stages were small, the statistical power may have been

insufficient to detect differences among the methods. In this study, this population of oysters was chosen because the frequency of stages sampled represented the frequencies observed normally during the spawning season. Future studies should address in more detail the discriminating power of GBR values across gonadal stages.

There were several advantages of the computer image analysis methodologies tested in this work. They significantly reduced the amount of time required to determine the GBR because they were less labor intensive, required only a minimum knowledge of computer software, and the GBR values obtained were not different from those of the transect method, allowing direct comparison of results among studies.

Another important difference between the image analysis methods tested in this experiment compared to the image analysis methods used in other studies is that these methods utilize the complete area of the histological section to estimate the GBR instead of only a few fields of the section, which reduces the possibility of erroneous estimation due to sampling error or asymmetrical development of the gonad. The use of the total area (TA) method was the most successful and therefore is the method recommended because it gives comparable results to other existing methods in the literature, is fast (requiring an average of 3 min to complete the GBR of one oyster), and once the histological characteristics of the body and gonad have been established on the slide, the method is not subject to visual errors by the operator.

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CHAPTER 3 – GONADAL DESCRIPTION OF FEMALE EASTERN OYSTERS

The eastern oyster, *Crassostrea virginica* (Gmelin 1791), provides an important commercial fishery along the Atlantic and Gulf coast of the United States, with average annual landings of 12.5 metric tons representing $70 millions in dockside value (NMFS 2003). Despite large annual landings, there has been a steady decline in production during the past decade due to overharvesting, disease, habitat loss, environmental fluctuations, and low summer meat yields (Supan 2000). Production declines have generated interest in the production of oysters with enhanced growth and disease resistance characteristics (Supan 2000). The production of triploid oysters addresses some of these problems. Triploidy refers to the condition of a cell or organism having three sets of chromosomes (Guo and Allen 1994). Triploid oysters have an increased growth rate during the reproductive season as a result of decreased fecundity because energy reserves go to the production of meat rather than in producing gametes.

Gametogenesis in diploid eastern oysters has been well studied and various classifications have been proposed to describe gonadal development (Loosanoff 1942, Kennedy and Battle 1964, Kennedy and Krantz 1982). In contrast, only a few studies have been performed addressing reproductive development of triploid oysters, and these have been concentrated on the Pacific oyster, *Crassostrea gigas* (Allen et-al 1986, Allen and Downing 1986, 1990). The studies specific to the eastern oyster have been conducted in northern latitudes where water temperatures are low and spawning seasons are shorter than in the south, factors that could influence gonadal condition due to the correlation between water temperature and gonadal maturation (Gauthier and Soniat 1989).

Most of the studies that describe gametogenic development in triploid oysters have been focused on evaluating reproductive sterility due to interference of meiosis by the possession of

three chromosome sets. It is known that sterility is a common feature of induced triploidy aquatic vertebrates (Thorgaard, 1986), but in aquatic invertebrates, especially bivalves, sterility is not absolute and the production of mature gametes, although in most cases reduced, is not uncommon. In a study done to evaluate gametogenesis in three species of triploid bivalves (*M. arenaria, C. gigas, C. virginica*), retarded gonadal development was reported as the general pattern of development for all three species. Nevertheless, the production of oocytes occurred, and in some animals in substantial numbers (Allen 1987). Experiments on the reproductive potential of triploid Pacific oysters showed that the gametes were capable of fertilization (Guo and Allen 1994). This suggests that triploid bivalves have the capability to produce viable gametes.

Estimating gonadal development is important in studies that address reproduction, and estimates based solely on quantitative parameters, although important and necessary because they provide a quantifiable parameter, do not give enough information on the actual condition or the stage of development. A combination of quantitative and qualitative parameters is necessary to provide an accurate estimate of the reproductive condition of an organism.

The goal of this study was to create a descriptive scale of gonadal development for triploid female eastern oysters and to estimate the reproductive condition during the spawning season based on the quantity and quality of the gonad. The objectives were to: 1) describe and classify by stages the gonadal development of diploid and triploid eastern oysters collected during August of 2003, and May and August of 2004, 2) estimate gonad quantity based on a gonad-to-body ratio, and 3) combine qualitative and quantitative descriptions of gonadal development to graphically categorize the potential reproductive condition.

MATERIALS AND METHODS

Production of Triploids

The triploid oysters used in this study were produced at the Sea Grant Grand Isle Bivalve Hatchery (29º15'12"N, 90º03'26"W) on Caminada Bay, Louisiana in June of 2002, following the methodology described by Supan *et al.* (2000). Eggs from ripe diploid oysters were stripped from the gonads and hydrated in 0.45-µm filtered ambient seawater (FSW) for 1 h. The eggs were fertilized at a concentration of approximately 10 sperm per egg and monitored until appearance of the first polar body in at least 50% of the eggs. A concentration of 0.5 mg of cytochalasin B (CB) dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) was added to each liter of eggs to induce triploidy. After 10 min, the eggs were rinsed with FSW over a 15-µm screen to remove excess sperm, and were soaked in a solution of seawater containing 0.05% of DMSO for 15 min to wash the CB from the eggs. The eggs were rinsed again with FSW to remove the DMSO and were placed in 36,000-L tanks for larval culture. The larvae were set on oyster shell cultch (1-8 mm) that was deployed near the shore of Caminada Bay on August 2002 on floats for off-bottom culture in ambient water.

Ploidy Determination

Flow cytometry was used with all oysters in this study to verify ploidy. From each oyster a 0.2-ml hemolymph sample was collected from the adductor muscle sinus by inserting a 23-ga needle (Becton Dickinson Co. Franklin Lakes, New Jersey) through a notch in the shell. The hemolymph was immediately placed into microcentrifuge tubes (Dot scientific Inc. Lippincott Burton, Michigan) and placed on ice until analysis, to prevent hemocyte clumping (SOP-5).

For ploidy analysis, the cells were prepared according to the method of Krishan (1975). One hundred microliters of oyster hemolymph were mixed with 35 µl of blood diluted in phosphate buffered solution (1 X 10^6 cell/ml) of male Nile tilapia (Oreochromis *nilotica*) (SOP-6) and stained with 1 ml of a solution containing 0.05mg/ml of propidium iodide (Sigma-Aldrich, St. Louis, Missouri), 38mM sodium citrate (EM Science Inc, Gibbstown, New Jersey), 0.1% of Triton X-100 (Mallinckrodt Specialty Chemicals Co, Paris, Kentucky) (SOP-7), and 100 µl of an RNase solution (Sigma Chemicals Co. St. Louis, Missouri) (SOP–8). The samples were mixed and filtered through a 35-µm screen and analyzed by flow cytometry after 15 min of incubation in the dark. The tilapia blood was used as an internal reference to correct for factors that can cause variations in DNA readings.

Analyses were performed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, California) with an air-cooled argon laser at a wavelength of 488 nm. Individual nuclei of lysed hemocytes and erythrocytes were analyzed at a rate of 100-200 particles per second (SOP-9). The fluorescence of each particle analyzed was transmitted as an analog signal to a computer, and the signal was digitized to generate pulse-height histograms that represented a minimum of 30,000 cells. Cellquest Software (Becton Dickinson) was used to calculate the fractional mode channel (peak channel) of each histogram fluorescence peak. Measurement of oyster DNA content was expressed as picograms of DNA per cell in relation to an assigned value of 2.00 pg/cell for fresh red blood cells from channel catfish *Ictalurus punctatus* that was used as an external reference (Tiersch *et al*. 1990). Oyster DNA content was calculated by the formula: DNA content = (O/T)(T/C) x 2.0; where O, T, and C, are the fractional mode channels of the oyster, tilapia and catfish, and 2.0 is the DNA content value in picograms of the channel catfish.

Histology

Sectioning of the oysters for histological comparisons was done according to Morales-Alamo and Mann (1989) (SOP–1). The oyster meats were removed from the shells and a 4-mm thick cross-section just posterior to the gill-palp junction was dissected, placed in a tissue cassette (Omnisette, Fisher-Scientific, Pittsburg, Pennsylvania) and preserved in Davidson's fixative (Humason 1967) (SOP–2). An automated tissue processor (Leica TP1050, Mayer Instruments, Houston, Texas) was used to prepare the samples for histology. Tissue samples were dehydrated in a stepped alcohol series (70%, 80%, 95%, and 100%), cleared in xylene and embedded in paraffin (Paraplast, Tyco/Healthcare Group LP, Mansfield, Massachusetts). With a microtome (Shandon, Thermo Electron Corp. Pittsburg, Pennsylvania) a 4-µm section approximately 1 mm from the gill-palp junction was cut and mounted on a negatively charged microscope slide. Each of the slides was placed on a tray inside an autostainer (Leica Autostainer XL, Mayer Instruments), where the paraffin was removed and the tissues were stained for 30 min with hematoxylin (Anatech Ltd., Battle Creek, Michigan) and counterstained for 1 hr with eosin Y (Anatech Ltd.). Cover slips were mounted above the histological sections with permount (Fisher Scientific, Fair Lawn, New Jersey).

Quantitative Analysis

Gonad-to-body ratios were estimated by comparison of gonad and body areas calculated by image analysis (Chapter 2) (SOP-4). To do this, the histological sections were digitized on a scanner (Epson Perfection 1640SU, Epson America, Inc. Long Beach, California) at a resolution of 800 dpi and imported into Photoshop 7.0 (Adobe Inc, San Jose, California). The scanned images were magnified 12 times and analyzed using image analysis software (Metaview 6.1, Universal Imaging Corporation, Downingtown, Pennsylvania). To calculate the body area, the

outline of the body following the outer margin of the mantle and gills was traced by use of a digital pen and a drawing tablet (Hyper Pen 12000U, Aiptek Inc, Irvine, California) connected to a personal computer (Dell precision workstation 360, Dell Inc, Austin, Texas). The gonad was also traced following the inner and outer margins avoiding the interfollicular space to the extent allowable by the magnification of the digital image. The image analysis software automatically calculated the areas of the body and the gonad by counting the number of pixels contained within the traced area.

Gonadal Description

Complete gonad sections from each histological slide were microscopically examined to describe the gonadal development of each oyster. Classifications used to stage the gonadal development included early development, late development, spawning, and spawned (adapted from Kennedy and Krantz 1982). For the triploid oysters, the same classification was followed with the inclusion of two more stages, defined as indifferent and reduced development.

Statistical Analysis

A comparison of GBR values of each stage of gonadal development in each ploidy was conducted to test for differences among the stages, using a one-way analysis of variance (Statistical Analysis Software system Version 9 for Windows®, SAS Institute Inc., Cary, North Carolina). The model included GBR as the dependent variable and stage as the fixed effect. Also, comparison of the GBR values of diploid and triploid oysters at the same stage of development was performed, to find quantitative differences between the two ploidies. A Tukey-Kramer test was used to compare stage differences, and these were considered to be significant at $P < 0.05$. The GBR values met the assumption of normality and homogeneity of variance after square root transformation (Kutner *et al*. 2005).

RESULTS

Gonadal Description

Diploid Female Oysters

The gonadal condition of the diploid female samples evaluated during the two summers was representative for that time of the year in coastal Louisiana. The gonads of the majority of oysters (68%) were found to be in spawning condition, defined as gonads full of non-pendant (not connected to the follicle wall) mature oocytes, with a disrupted follicular arrangement or no clear division between follicles (Figure 3.1).

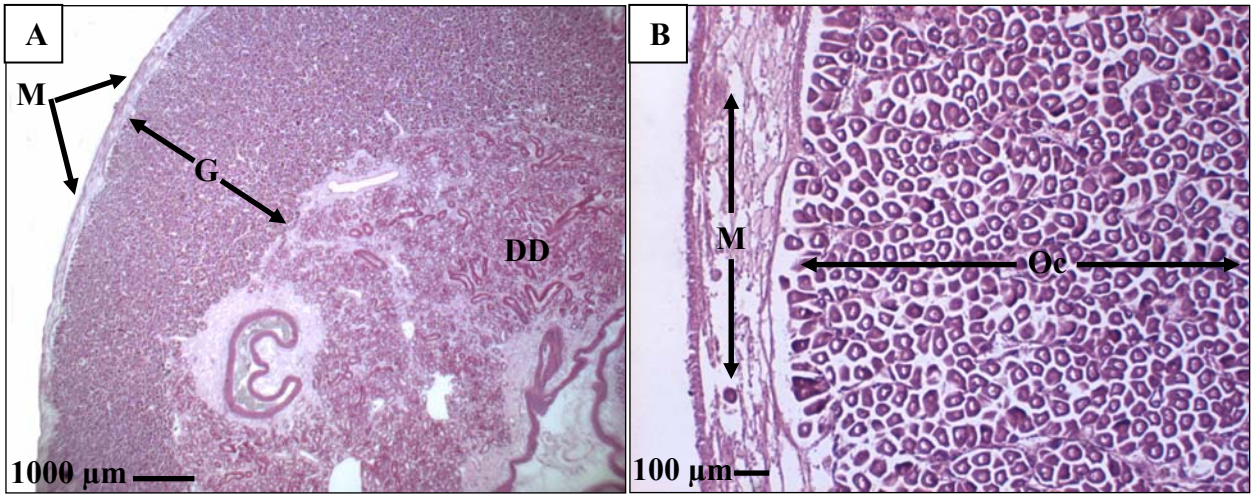

Figure 3.1 A & B Photomicrographs of a histological section showing a female diploid eastern oyster in spawning condition (Stage V). M: mantle; G: gonad; DD: digestive diverticula; Oc: oocytes.

Triploid Female Oysters

Gonadal development in the triploid eastern oysters during both summers was retarded compared to diploids, but the gonadal condition was able to be categorized into six descriptive stages based on the degree of development as defined below:

Stage I.- Indifferent: In this stage, follicular development was absent or reduced with no follicular branching, dominant interfollicular space (connective tissue), and no evidence of oogonia or distinguishable sex cells were observed in the follicles (Figure 3.2).

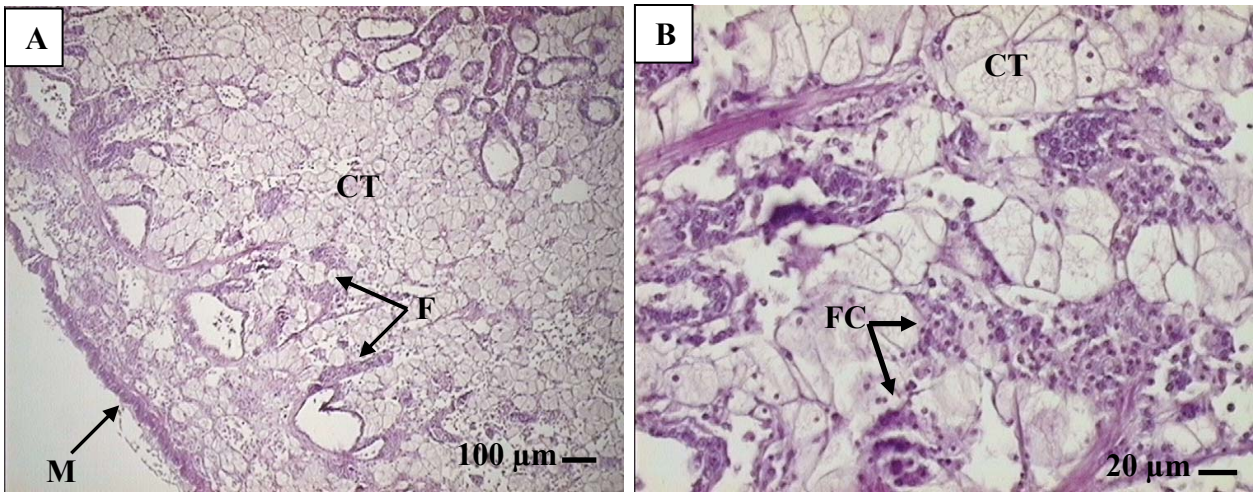

Figure 3.2 A & B Photomicrographs of a histological section showing an indifferent gonadal condition (Stage I) of a female triploid eastern oyster. M: mantle; CT: connective tissue; F: follicles; FC: follicular cells.

Stage II.- Reduced Development: Follicular development was arrested with little or no follicular branching, and dominant interfollicular space. Few oocytes were present (1 to 20 in the whole histological section), but they were mature with a large distinct germinal vesicle. All oocytes were inside the follicles and in general did not appear to be pendant to the follicular wall. The follicles that had oocytes usually had only one and this was surrounded by undeveloped germinal cells. This was the typical gonadal condition for the majority of the triploid oysters (Figure 3.3).

Stage III.- Early Development: In this stage, the gonad presented follicular development with some branching and a prominent reduction in the interfollicular space.

Oogonia were observable and the oocytes present inside the follicles were still pendant (Figure 3.4).

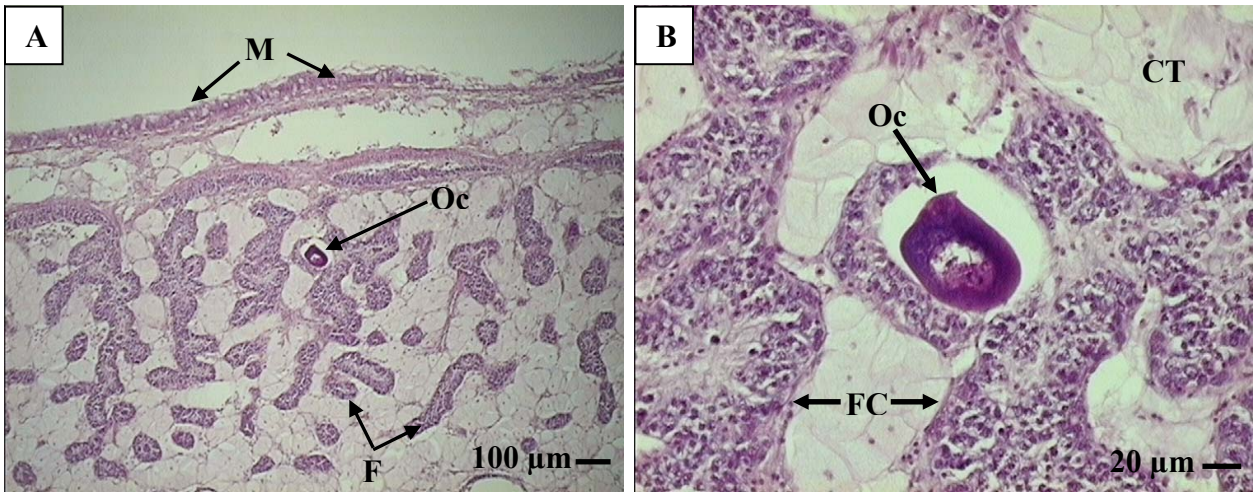

Figure 3.3 A & B Photomicrographs of a histological section showing a reduced gonadal condition (Stage II) of a female triploid eastern oyster. M: mantle; Oc: oocyte; F: follicles; CT: connective tissue; FC: follicular cells.

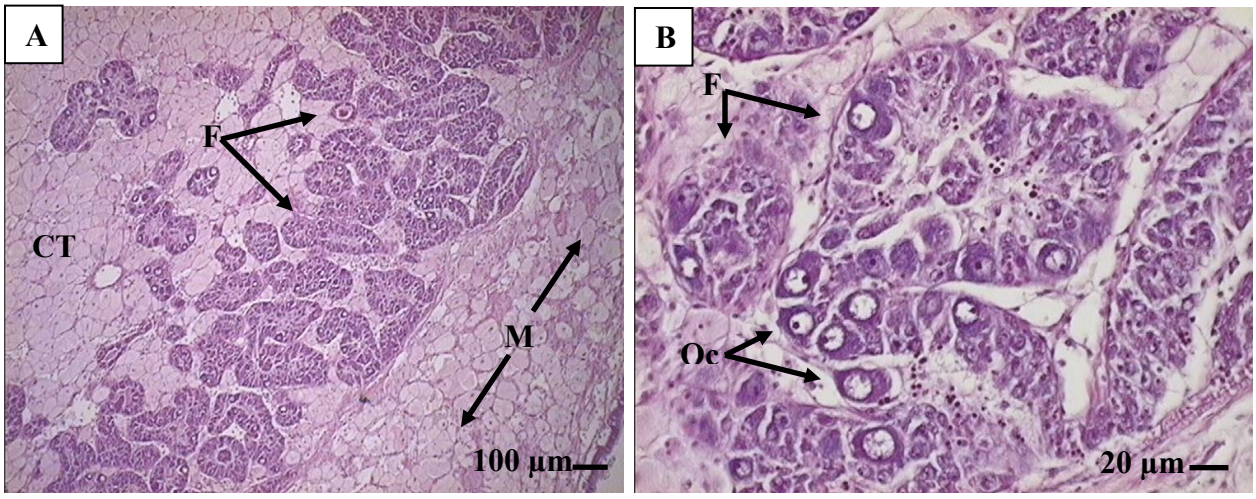

Figure 3.4 A & B Photomicrographs of a histological section showing an early development gonadal condition (Stage III) of a female triploid eastern oyster. M: mantle; CT: connective tissue; F: follicles; Oc: oocytes.

Stage IV.- Late Development: In this stage, there was an increase in the size and number of follicles, significant branching and a reduction of interfollicular space compared to the early development stage. Several mature oocytes were observed inside the follicles usually projected into the lumen. There were some developing pendant oocytes (Figure 3.5).

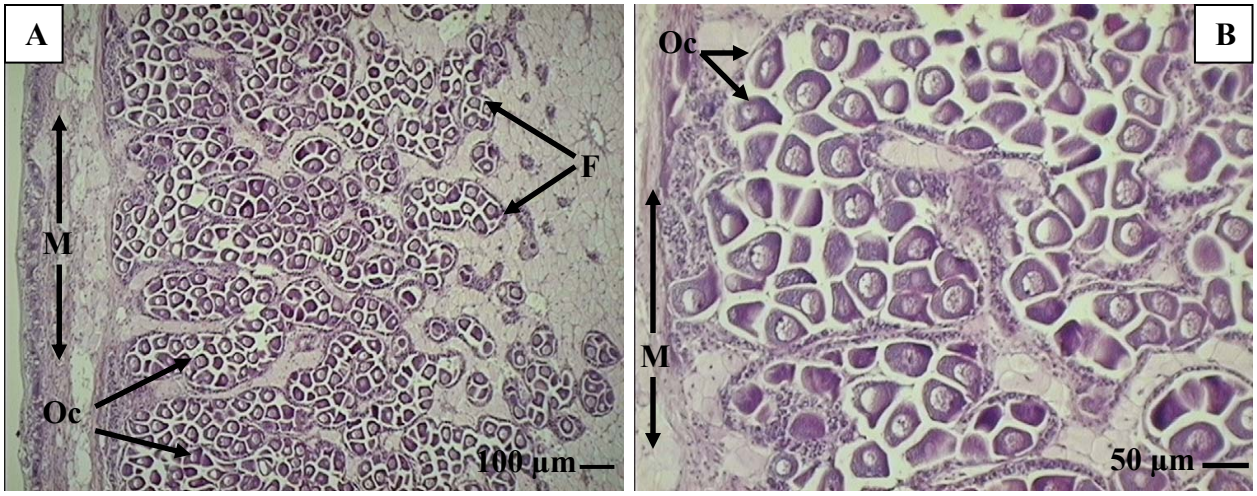

Figure 3.5 A & B Photomicrographs of a histological section showing a late development gonadal condition (Stage IV) of a female triploid eastern oyster. M: mantle; Oc: oocytes; F: follicles; CT: connective tissue.

Stage V.- Spawning Condition: In this stage, the gonad had follicles full of oocytes with prominent clear germinal vesicles in most of them, and almost no interfollicular space, no clear division between follicles. The oocytes presented an irregular arrangement and only a few developing oocytes were pendant (Figure 3.6)

Stage VI.- Spawned: Gonads in this stage presented follicular disorganization, extensive interfollicular space, and the appearance of connective tissue in the interfollicular areas. Numerous mature oocytes were observed free in the lumina and in the genital canals (Figure 3.7).

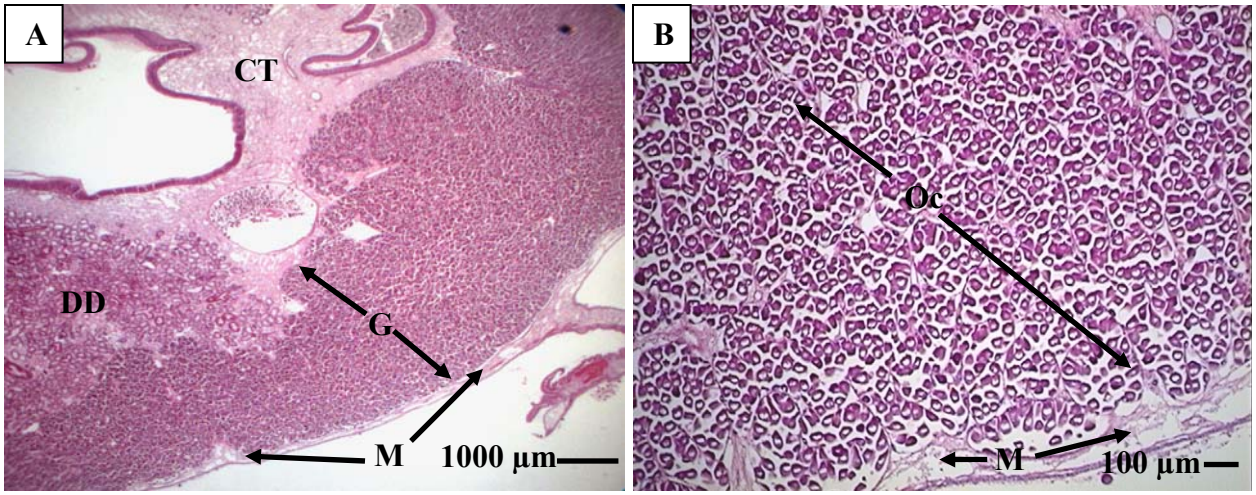

Figure 3.6 A & B Photomicrographs of a histological section showing a spawning gonadal condition (Stage V) of a female triploid eastern oyster. M: mantle; G: gonad; DD: digestive diverticula; CT: connective tissue; Oc: oocytes.

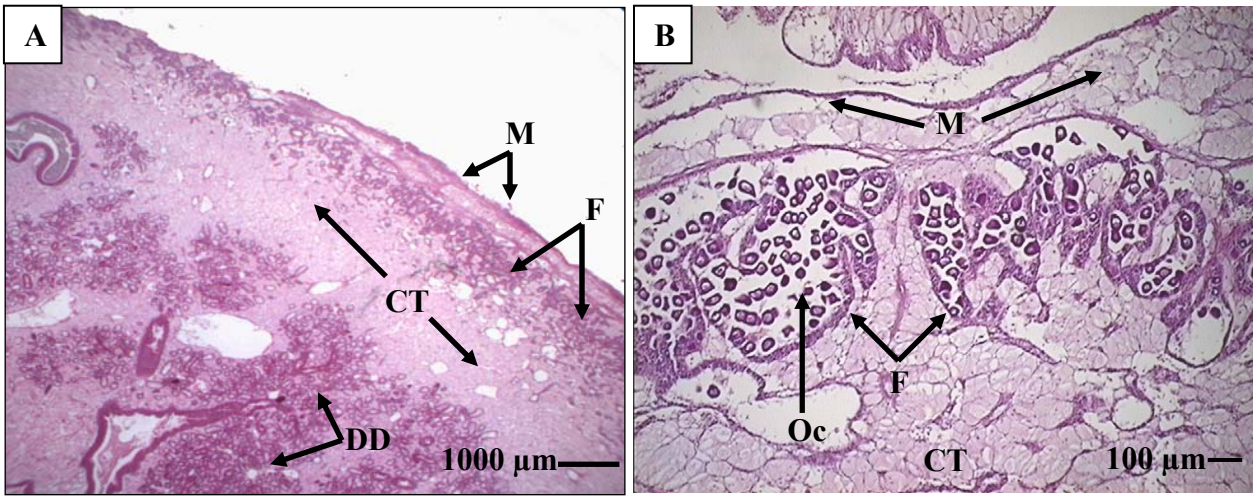

Figure 3.7 A & B Photomicrographs of a histological section showing a spawned gonadal condition (Stage VI) of a female triploid eastern oyster. M: mantle; F: follicles; CT: connective tissue; DD: digestive diverticula; Oc: oocytes.

Quantitative Analysis

In the diploid oysters, only the GBR value of the oysters at Stage III was not significantly different ($P > 0.05$) to the oysters at Stages I and VI, the rest of the stages were significantly

different (Table 3.1). In the triploid oysters, only those with a gonadal condition of Stage V had a significantly different ($P \leq 0.05$) GBR value from the oysters at other gonadal stages (Table 3.2).

The comparison diploid and triploid GBR values of the same stages showed that only the oysters with gonads at Stage IV had a significantly different GBR value ($P < 0.05$).

Table 3.1 Statistical comparison of the gonad-to-body ratio (GBR) by developmental stage in diploid eastern oysters (N = 212).

	GBR		
Gonadal stage	**LS mean***	**SD****	**Comparison*****
Undifferentiated (I)	0.068	0.043	A
Early development (III)	0.119	0.054	Ad
Late development (IV)	0.208	0.067	B
Spawning (V)	0.320	0.073	C
Spawned (VI)	0.141	0.062	D

* LS mean: Least significant mean; **SD: Standard deviation; ***Different letters indicate significant differences among developmental stages, as indicated byTukey-Kramer test ($\alpha = 0.05$).

Potential Reproductive State

The descriptive classification of the diploid and triploid gonad condition with their respective GBR values were combined to graphically illustrate the reproductive potential of eastern oysters based on the quality and quantity of the gonad (Table 3.3). In general, the GBR values of diploid oysters were higher than those of triploid oysters at later stages of development (Stages IV, V, and VI), but only the gonads at Stage IV had significantly different ($P < 0.05$)

GBR values. At earlier stages (I and III), the GBR values of triploid oysters were within the same range as the diploid oysters.

Table 3.2 Statistical comparison of the gonad-to-body ratio (GBR) by developmental stage in triploid eastern oysters (N =215).

	GBR		
Gonadal stage	**LS Mean***	**SD****	**Comparison*****
Indifferent (I)	0.063	0.033	A
Reduced development (II)	0.070	0.029	A
Early development (III)	0.079	0.034	A
Late development (IV)	0.094	0.046	A
Spawning (V)	0.257	0.105	B
Spawned (VI)	0.078	0.042	A

*LS mean: Least significant mean; **SD: Standard deviation; ***Different letters indicate significant differences among developmental stages, as indicated by Tukey-Kramer test (α = 0.05).

DISCUSSION

Normal summer gonadal condition (Stage V) was mostly found in the diploid eastern oysters examined during May and August of 2003 and 2004 for Louisiana waters. In triploid eastern oysters, although different degrees of gonadal development were found, the typical characteristic was oysters with reduced gametic and gonadal development (Stage II). A similar pattern of gonadal development was reported for cultured triploid Pacific oysters (Allen and Downing 1990).

Table 3.3 Distribution of gonadal condition by gonad-to-body ratio (GBR) in diploid (blue) and triploid (yellow) eastern oysters, and their intersection (green).

Stage / GBR	I	II	III	IV	V	VI
0.0-0.05						
0.06-0.10						
0.11-0.15						
0.16-0.20						
0.21-0.25						
0.26-0.30						
0.31-0.35						
0.36-0.40						
0.41-0.45						
0.46-0.50						
0.51-0.55						

Although a reduced gonadal development (Stage II) was expected for the triploid oysters, it is not clear why only a few primary oocytes matured even when the gonad had visible oogonial cells and large reserves of glycogen. The theory of chromosomal disruption during meiotic divisions does not fully explain the reason for the arrested development of oocytes because meiotic divisions begin normally after the oocyte has been fertilized, after vitellogenesis has occurred. This suggests that factors that affect the mobilization of energy reserves and nutrients

(vitellins) into gamete must have a larger impact than genetic aberrances on the maturation of oocytes.

Although the gonadal development found in the majority of triploid oysters was reduced and in those oysters that developed beyond Stage II, the gonadal material was less abundant when compared to diploid oysters, a gradual gonadal development in triploid oysters similar to diploids was observed, suggesting that both follow the same path of gonadal development albeit at different rates, allowing similar categorization.

Although the inclusion of a Stage II helped to describe part of the gonadal development in triploid oysters and to differentiate between a reduced development and an early developing gonad (Stage III), the comparison of these two stages based on GBR values showed no significant differences. In subsequent chapters of this thesis, Stages II and III of the triploid oysters were merged into a single stage, (Stage III) to allow comparison with diploid oysters and facilitate statistical analysis.

The fact that some triploid eastern oysters were found in Stage VI with clear signs of having spawned, even in cases were the gametes were not mature, was evidence that triploid oysters were capable of spawning regardless of the quantity or quality of gonadal material. This suggests that the factors that regulate spawning might not be strongly dependent on the state of gonadal maturity.

None of the eggs found in the mature triploid females during these experiments were fertilized, but based on the quality of the gonad and the characteristics of the gametes it could have been possible that some of these gametes would have been capable of fertilization. In triploid Pacific oysters mature eggs have been able to be fertilized and to produce viable larvae (Guo and Allen 1994). Thus, based on the gonadal and gametic characteristics of the ovaries of

triploid eastern oysters, it can be predicted that oysters with GBR values of 0.2 or higher are potentially capable of reproduction.

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CHAPTER 4 – EFFECT OF ESTRADIOL-17β ON OVARIAN DEVELOPMENT OF DIPLOID AND TRIPLOID EASTERN OYSTERS

In vertebrates, estradiol is the major steroid hormone secreted by the ovary. Estradiol 17-β (E_2) is a steroid hormone from the family of estrogens that also includes estrone and estriol, and together with estradiol are the most important estrogens found in vertebrates (Sadleir 1974). In mammals, estradiol is secreted during the follicular or preovulatory phase of the ovary and plays an important role in oocyte and follicle maturation (Hadley 1992). In oviparous vertebrates like the chicken, transcriptional activation of the vitellogenin gene in liver cells is estrogen dependent (Edinger *et al.* 1997). In fish, estradiol functions as an oocyte maturation hormone by stimulating the synthesis of vitellogenin, a precursor of yolk (Wallace and Selman 1981). In a study done with tilapia, *Oreochromis mossambicus,* to measure the effect of E_2 on the synthesis of vitellogenins, it was found that the levels of two vitellogenin proteins in the plasma increased after the fish were injected with estradiol, indicating the importance of estrogens in the process of oocyte maturation (Tekemura and Kim 2001).

In marine invertebrates, the presence of estrogens in several tissues has also been demonstrated and the effect on gonadal development seems to be similar to that in vertebrates (Takahashi and Kanatani 1981, Subramoniam 1999, Warrier *et al*. 2001). Several species of decapod crustaceans have shown the ability to synthesize steroid hormones similar to vertebrate E_2 to stimulate vitellogenesis (Pavlof and Goy 1990). Studies on the tiger shrimp (*Penaeus monodon*), have shown that the levels of estradiol and progesterone in the hemolymph and in tissues such as ovary and hepatopancreas correlated positively with progressive stages of ovarian maturity (Quinitio *et al*. 1994). The same pattern was observed for the red mud crab, *Scylla serrata*, where the levels of E_2 and progesterone increased sharply in the ovary and hepatopancreas at the onset of vitellogenesis (Warrier *et al.* 2001).

In bivalves, the presence of estrogen-like steroids has also been reported. Studies on gonadal estrogen profiles done with the Pacific oyster, *Crassostrea gigas*, and the Japanese scallop, *Patinopecten yessoensis*, identified the presence of estriol, estrone, and E_2, with E_2 being found at higher concentrations in the ovary and increased with sexual maturation (Matsumoto *et al*. 1997). Other studies have also demonstrated that E_2 is capable of accelerating sexual maturation in female Pacific oysters (Mori 1969). In this same species, E_2 treatments *in vivo* causes significant increases in oocyte diameter and vitellin content and appeared to be one of the major factors that controlled vitellogenesis (Li *et al.* 1998).

In the United States, the oyster industry has traditionally depended on fisheries based on public reefs or private lease systems to supply the domestic market. Although private leasing has been considered as a form of aquaculture because it involves the manipulation and transportation of juvenile oysters to desirable growing areas, it is an extensive form of culture that offers little control over the outcome of transported animals. As aquaculture evolves, species with enhanced characteristics such as faster growth and disease resistance will be more desirable, especially as culture practices are transformed from extensive artisan methodologies to intensive and more industrialized practices.

Production characteristics of traditional concern are growth rates and meat yields. In oysters, a drastic reduction of body tissue occurs in summer months in Louisiana coastal waters due to the natural spawning cycle. During spring maturation and spawning, glycogen stores are depleted as the gametes mature. The glycogen stores are not fully replenished during the summer and meat yields decline accordingly.

One solution that addresses this natural phenomenon came almost 25 years ago when triploid oysters were produced with the purpose of enhancing growth rates and increasing meat

yields (Stanley *et al.* 1981). Triploidy is a genetic condition where the organism has three sets of chromosomes (3N) instead of two (2N), which is the normal condition for most animals (Guo and Allen 1994). The result of triploidy in oysters is an almost complete reproductive sterility due to an abnormal chromosomal paring at the meiotic divisions (Thorgaard 1986). The absence of maturing gametes allows energy reserves of the oysters to go to somatic (meat) development rather than gonadal growth (reproduction). The option of producing triploid oysters to overcome problems of reduced meat yields in the summer has proven to be effective, to the point that on the west coast of the United States, 30% of the Pacific oysters grown are triploids (Nell 2002) and this percentage is likely to increase with increased demand for more and better quality products, especially during the summer months.

Three methods have been used to induce triploidy in animals: 1) manipulation of the environment (physical); 2) addition of chemicals to interfere with meiosis (chemical), and 3) mating between diploid and tetraploid animals (natural triploids). In oysters, the most commonly used method is the addition of chemicals to newly fertilized eggs to prevent the release of a polar body, causing retention of an extra set of chromosomes. This method although simple, has notable disadvantages. The chemicals are highly mutagenic, therefore, dangerous to untrained personal, the percentage of triploidy is typically less than 80%, and larval mortality rates are higher than in diploid-diploid crosses. In 1994, researchers from the Haskin Shellfish Research Laboratory at Rutgers University (Guo and Allen 1994a) developed a method to produce tetraploid oysters (4N), oysters with four sets of chromosomes, using oocytes from sexually mature triploid females. Production of large numbers of tetraploids enabled production of natural triploids by crossing tetraploid with diploid oysters, eliminating the use of noxious chemicals, increasing success of triploidy production to practically 100%, and reducing larval mortality

levels to those of diploid-diploid crosses. This technique, however, is dependent on sexually mature triploid females to start tetraploid broodstock lines. In the Pacific oyster, this was not a great concern because it is not uncommon to find triploid females with sufficient numbers of mature oocytes. However, for the eastern oyster, *C. virginica*, the concept of producing natural triploid oysters did not work well, because it is rare to find triploid females with mature oocytes or oocytes in sufficient numbers for producing tetraploids.

The requirement of sexually mature triploid eastern oysters to develop tetraploid oyster broodstocks provided impetus for the idea of enhancing gonadal development by the use of steroid hormones. Estradiol-17β is a likely candidate to stimulate oocyte maturation on eastern oysters based on the diversity of species that respond to high levels of estrogens during gametogenesis.

The purpose of this study was to evaluate the effect on enhance ovarian maturation of triploid eastern oysters by the use of the hormone E_2 to produce viable oocytes for use in production of tetraploid broodstocks. The objectives were to: 1) compare on quantitative and qualitative scales the effect on gonadal development of three dosages of E_2 in triploid and diploid eastern oysters, and 2) measure the concentration of E_2 in hemolymph of diploid and triploid oysters from the control groups, to establish its presence and correlate titers with the gonadal stage. The results of this study showed a positive correlation between the use of E_2 and gonadal maturation for female triploid oysters.

MATERIALS AND METHODS

The experiments were performed at the Sea Grant Grand Isle Bivalve Hatchery (29º15’12”N, 90º03’26”W) on Caminada Bay, Louisiana, in August of 2003, and were repeated in May and August of 2004. The diploid and triploid eastern oysters used in these studies were

produced and cultured at the Grand Isle Hatchery and were collected 2 weeks prior to each experiment.

Triploid Induction and Verification

The triploid oysters used in the three experiments were produced by chemical induction in June of 2002 following the methodology described by Supan *et al*. (2000). Briefly, newly fertilized eggs were treated with a solution containing 0.5mg of cytochalasin B (CB) dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) in 1 L of filtered sea water. The CB solution was added when 50% of the eggs showed polar body 1 and the duration of the treatment was 10 min.

The ploidy of each oyster was verified by flow cytometry a week before each experiment, and verified again after the experiment in the cases were triploid oysters were found in spawning condition. Briefly, the cells were prepared according to the method of Krishan (1975). One hundred microliters of oyster hemolymph were mixed with 35 µl of blood of male Nile tilapia (*Oreochromis nilotica*) diluted in phosphate buffered solution (1 X 10^6 cell/ml) (SOP-6) and stained with 1 ml of a solution containing 0.05mg/ml of propidium iodide (Sigma Chemical Co. St. Louis, Missouri), 38mM sodium citrate (EM Science Inc, Gibbstown, New Jersey), 0.1% of Triton X-100 (Mallinckrodt Specialty Chemical Co, Paris, Kentucky) (SOP-7), and 100 µl of RNase solution (Sigma Chemical) (SOP-8). Oysters tested as diploids were also used in the experiment to measure the effect of E_2 in female diploids.

Experimental Design

A total of three experiments were conducted during the summer of 2003 and 2004. In August of 2003 a total of 90 triploid oysters and 60 diploid oysters were used from the same cohort. This experiment also included 60 diploid oysters from a different cohort that were never exposed to CB for triploidy induction, with the purpose of evaluating differences in response to

the estrogen between diploid oysters exposed and not exposed to CB. The oysters from this experiment were under treatment for 12 d. In May of 2004 a total of 240 triploid and diploid oysters were used from the same cohort. One half of the population of oysters from this experiment was under treatment for 9 d and the rest for 12 d. In August of 2004 a total of 360 triploid and diploid oysters from the same cohort as in the previous experiments were used. One half of the population of oysters from this experiment was under treatment for 12 d and the rest for 15 d. In all of the experiments a sample of 20 diploid and triploid oysters was evaluated at the beginning of the experiment to determine the initial gonadal condition.

The treatments consisted of: 1) a control with no E_2; 2) a low dose with 37.5 ng of E_2 per g of wet weight; and, 3) a high dose with 75 ng of E_2 per g of wet weight. These dosages were chosen based on the response of oysters to E_2 in preliminary experiments conducted in August and September of 2001 (J. Lynn, LSU Department of Biological Sciences, unpublished data). The diploid and triploid oysters were divided equally among the treatments. The appropriate E_2 doses were applied every 3 d during the experiment by injection into the adductor muscle through a notch in the shell, using a 1-ml tuberculin syringe (Becton Dickinson, Franklin Lakes, New Jersey) with a 23-ga needle (Becton Dickinson). After injection, the oysters were held out of the water for 30 min to prevent flushing of the E_2 solution. Duration and dosage regime of the experiments are detailed in Table 5.1.

Calculation and Preparation of the Estradiol Dosages

The dosages were estimated on a body wet weight basis. Because there was no direct way to measure the body meat weight of an oyster without killing it, a linear regression of shell length to body weight was used to estimate the body weight, based on measurements of 750 triploid and diploid eastern oysters collected from 1994 to 1997 by Dr. Supan (Figure 5.1).

Table 4.1 Estradiol-17β dose injection and evaluation schedule for the experiments

Experiment	Injection and evaluation days					
	Day 0	**Day 3**	**Day 6**	**Day 9**	**Day 12**	**Day 15**
August 2003	1st dose	2nd dose	3rd dose	4th dose	Final evaluation	--------
May 2004	1st dose	2nd dose	3rd dose	4th dose and 1st evaluation	Final evaluation	--------
August 2004	1st dose	2nd dose	3rd dose	4th dose	5th dose and 1st evaluation	Final evaluation

The shell height values of triploid and diploid oysters were combined after covariant statistical testing showed no significant differences ($P > 0.05$) in the slope of the regression line of diploid and triploid oysters.

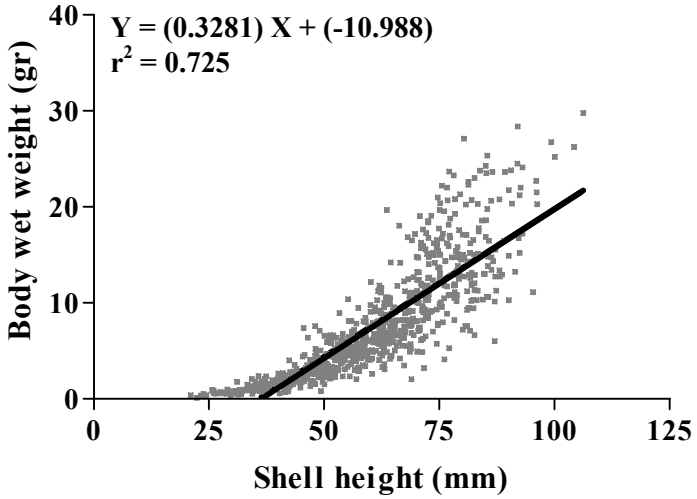

Figure 4.1 Linear regression of shell height by body wet weight from diploid and triploid eastern oysters

To simplify the application of the dosages, the oysters were divided into 8 shell height categories, from 70 mm to 150 mm, in intervals of 10 mm, and the dose for each category was calculated using the mean shell length of that category.

The solution for the control treatment consisted of filtered sea water with a concentration of 0.1% DMSO. The solution for the low dose treatment consisted of FSW with a concentration of 3125 ng/ml of β-Estradiol 98% (Sigma Chemical), and for the high dose treatment was FSW with a concentration of 6250 ng/ml of β-Estradiol (Sigma Chemical) the appropriate volume was injected based on the oyster shell height (SOP-10).

Description of Systems

The experiments were done in a 500-L rectangular fiberglass tank with continuous flow of filtered (100 μm) ambient sea water. The oysters were held in labeled plastic bins within an

8 x 5 array (Figure 5.2) to keep them in a known position throughout the experiment. Neither temperature nor food was regulated during the experiments.

Figure 4.2 Experimental units were plastic bins in an 8 x 5 array that separated individual oysters.

Histology

Sectioning of the oysters for histological comparisons was done according to Morales-Alamo and Mann (1989) (SOP-1). The oyster meats were removed from the shells and a 4-mm thick cross-section posterior to the gill-palp junction was dissected, placed in a tissue cassette (Omnisette, Fisher-Scientific, Pittsburg, Pennsylvania) and preserved in Davidson's fixative (Humason 1967) (SOP-2). Processing of the tissue samples was done on an automated tissue processor (Leica TP1050, Mayer Instruments, Houston, Texas). A 4-µm section was cut

approximately 1 mm from the gill-palp junction, mounted on a microscope slide, stained with hematoxylin (Anatech Ltd, Battle Creek, Michigan) and counterstained with eosin (Anatech).

Quantitative Analysis (Gonad-to-body ratio)

Gonad-to-body ratios were estimated from histological samples by comparison of gonad and body areas calculated by image analysis (SOP-4).

Qualitative Analysis (Gonad staging)

Complete gonad sections from each histological slide were microscopically examined to determine the gonadal development of each oyster. The stages used to categorize the gonadal development on each oyster were: indifferent (I), reduced development and early development (III), late development (IV), spawning (V), and spawned (VI). The description of each stage is given in Chapter 3. After determining that there was no statistical difference between the GBR values of Stages II and III (as explained in Chapter 3), Stage II was omitted from the gonadal classification and the oysters in this stage were reclassified as Stage III. This was done for the purpose of adding replication to the group of triploid oysters in Stage III, and to standarized the statistical comparison of treatments in the diploid and triploid groups.

Estradiol Assay

The levels of E_2 in hemolymph samples of all the oysters in the control groups of each experiment were measured using a colorimetric estradiol enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor Michigan) which was based on the competition between free estradiol (sample) and a known concentration of estradiol (constant) for a limited number of specific binding sites for each well of a 96-well assay plate. A 1-ml hemolymph sample was drawn from the adductor muscle using a 1-ml syringe (Becton Dickson) with a 23-ga needle (Becton Dickinson). The sample was transferred to a 1.5-ml cryogenic vial (Cryoware Nalge

Nunc Int. Corp., Rochester, New York) and immediately frozen in liquid nitrogen (SOP-5). The samples were stored at -80^{0}C until analysis.

Oocyte Area

The area of 50 randomly chosen oocytes from histological slides of diploid and triploid eastern oysters at Stage V were measured by image analysis (SOP-11). Comparison of the total oocyte mean area of each treatment was done to test for differences among treatments. Only gonads of oysters at Stage V were evaluated to ensure the same level of development. Only oocytes with a clear germinal vesicle and nucleolus were selected for measurement.

Statistical Analysis

Comparisons of the total GBR values of each treatment were conducted to test for differences among treatments within and among experiments in each ploidy using a two-way analysis of variance (Statistical Analysis Software system Version 9 for Windows®, SAS Institute Inc., Cary, North Carolina). The model included GBR as the dependent variable, treatment as the fixed effect, and experiment and its interaction with the treatment as random effects. Also, comparison of GBR values at each stage was conducted to test for differences among the treatments within a stage, using a two-way analysis of variance (SAS system). The model included GBR as the dependent variable, treatment as the fixed effect, and stage and its interaction with the treatment as random effects. A Tukey-Kramer test was used to compare treatment differences, and these were considered to be significant at $P < 0.05$. The GBR values met the assumption of normality and homogeneity of variance after square root transformation (Kutner *et al.* 2005).

The levels of E_2 in the hemolymph at different stages of gonadal maturation were compared using a one-way analysis of variance (SAS system). A Tukey-Kramer test was used to

compare concentrations differences among stages, and these were considered to be significant at $P < 0.05$.

Comparisons of the total mean oocyte area of each treatment were conducted to test for differences among treatments within and among experiments using a two-way analysis of variance (SAS system). The model included oocyte area as the dependent variable, treatment as the fixed effect, and experiment and its interaction with the treatment as random effects. Differences were considered to be significant at $P < 0.05$

RESULTS

August 2003

Triploid oysters (Initial evaluation)

Indifferent gonads (Stage I) were generally observed for the triploid oysters evaluated at the beginning of the experiment. The GBR values were never higher than 10%, and this consisted only of follicular or indifferent gametic cells (Figure 4.3). Only 25% of the oysters had reduced or early maturing gonads (Stage III) and the rest were indifferent.

Triploid oysters (Day 12)

At day 12 of the experiment, the control group was characterized by reduced or early developed gonads (Stage III) with GBR values below 13%. No oysters were found at later development or spawning condition (Stages IV and V), but three oysters showed signs of having spawned (Figure 4.4), characterized by follicular disorganization and matured oocytes free in the lumina.

The low dose group was characterized by oysters at Stages I and III, but oysters at Stages IV, V, and VI were also present. Of the 19 female oysters in this group, one presented a late maturation development, and another had a gonad at a spawning condition with a GBR of 35%

(Figure 4.5). The oocytes present in this gonad were mature, non pendant from the follicular walls, and had large visible germinal vesicles

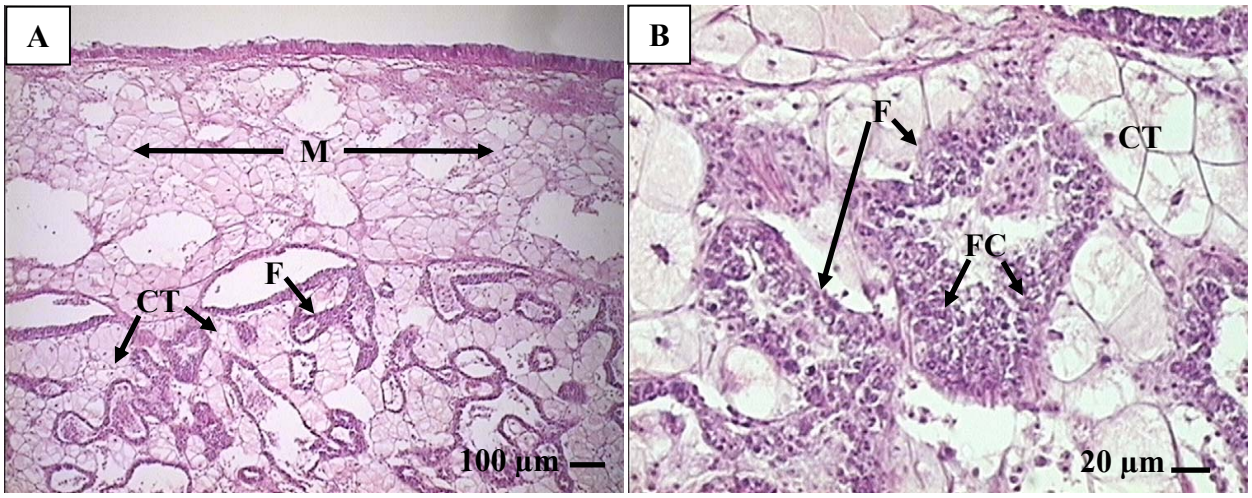

Figure 4.3 A & B Photomicrographs of a histological section showing the general pattern of gonadal development of triploid oysters at the beginning of the experiment (Stage I), August 2003. CT: connective tissue; F: follicles; FC: follicular cells; M: mantle.

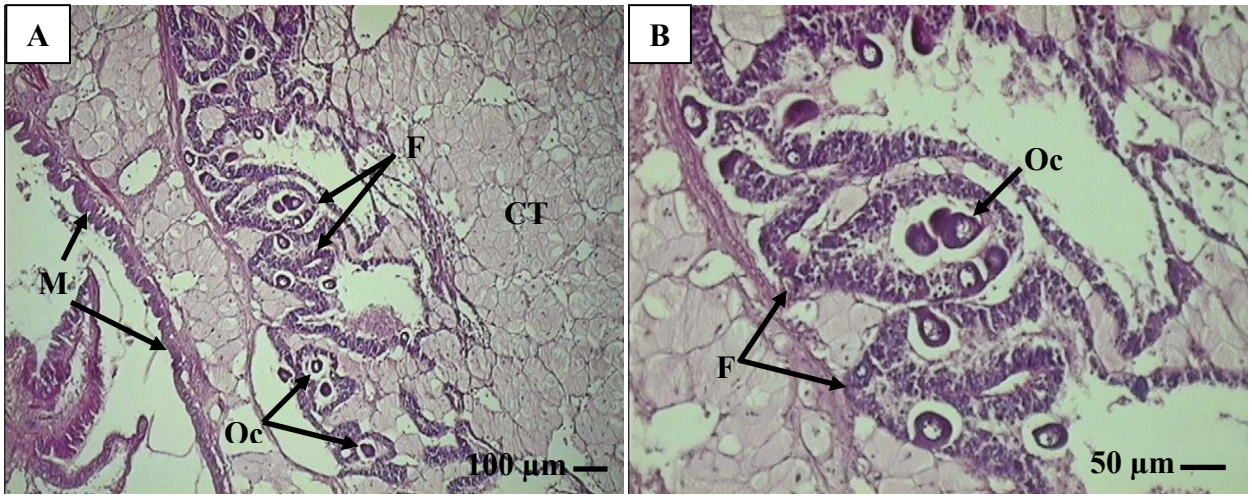

Figure 4.4 A & B Photomicrographs of a histological section showing a spawned gonad (Stage VI), of a female triploid eastern oyster in the control treatment in August 2003. CT: connective tissue; F: follicles; M: mantle; Oc: oocytes.

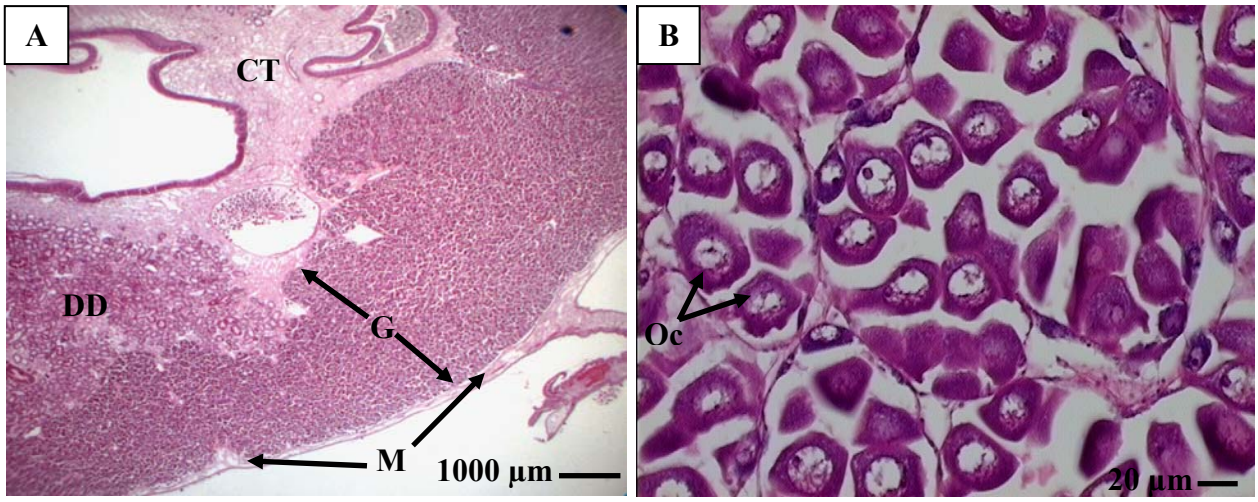

Figure 4.5 A & B Photomicrographs of a histological section showing a gonad in spawning condition (Stage V) from a female triploid eastern oyster in the low dose treatment in August 2003. Oocytes showed a prominent germinal vesicle. CT: connective tissue; DD: digestive diverticula; M: mantle; Oc: oocytes.

The high dose group was characterized by oysters with reduced and early development gonads (Stage III), 83% of the oysters in this group were at this stage. No oysters at spawning condition were found on this group, but an oyster at the later development (stage IV) with some mature oocytes was found (Figure 4.6). Figure 4.7 shows the average GBR values and the percentage of oysters at each stage for the three treatments.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage.

Diploid oysters (Initial evaluation)

Late development and matured gonads (Stages IV and V) were the general conditions of the diploid oysters from this group at the beginning of the experiment (Figure 4.8). No oysters were found at earlier stages of gonadal development, and the average GBR for the oysters at Stage V was 43%.

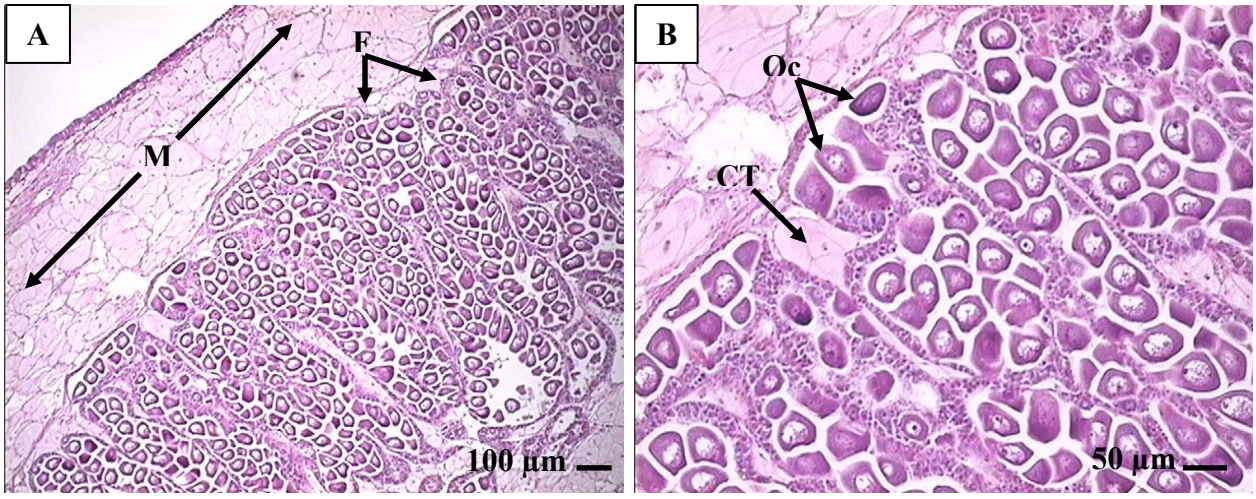

Figure 4.6 A & B Photomicrographs of a histological section showing a gonad in late development condition (Stage IV) from a female triploid eastern oyster in the high dose treatment in August 2003. CT: connective tissue; F: follicles; M: mantle; O: oocytes.

Diploid oysters (Day 12)

At day 12 of the experiment, the oysters from the control group were found to be at Stage V with an average GBR of 39%. In the low dose treatment, oysters were found at different stages of gonadal development, from Stage III to Stage VI (Figure 4.9), but again the highest percentage of oysters (68%) was found at Stage V. In the high dose treatment 76% of the oysters were found to be at Stage V; oysters at Stage VI were also found, and one oyster presented an indifferent gonad. Figure 4.10 shows the average GBR values and the percentage of oysters at each stage for the three treatments.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage.

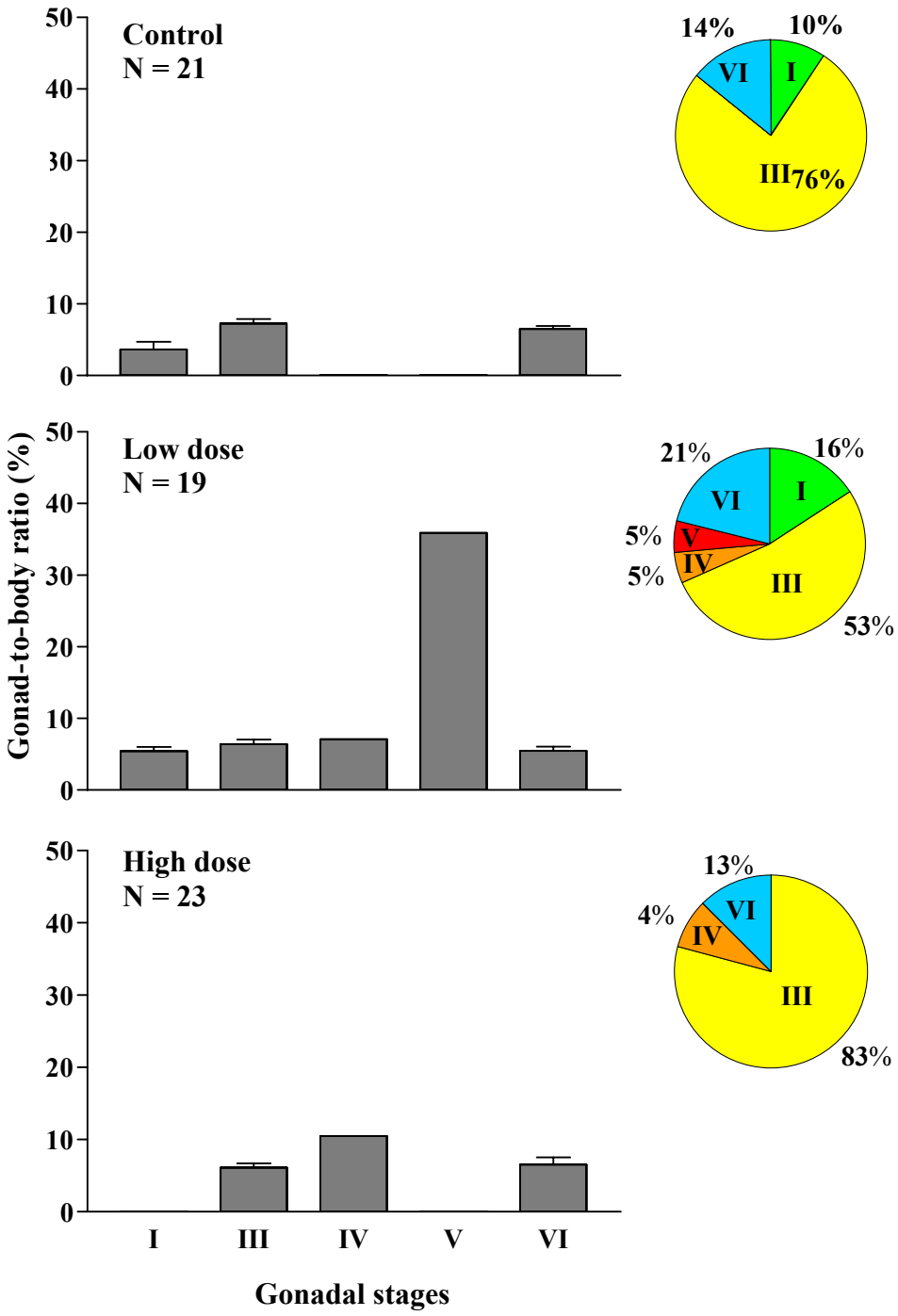

Figure 4.7 Gonadal assessment of the three experimental groups of triploid female eastern oysters evaluated in August 2003. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

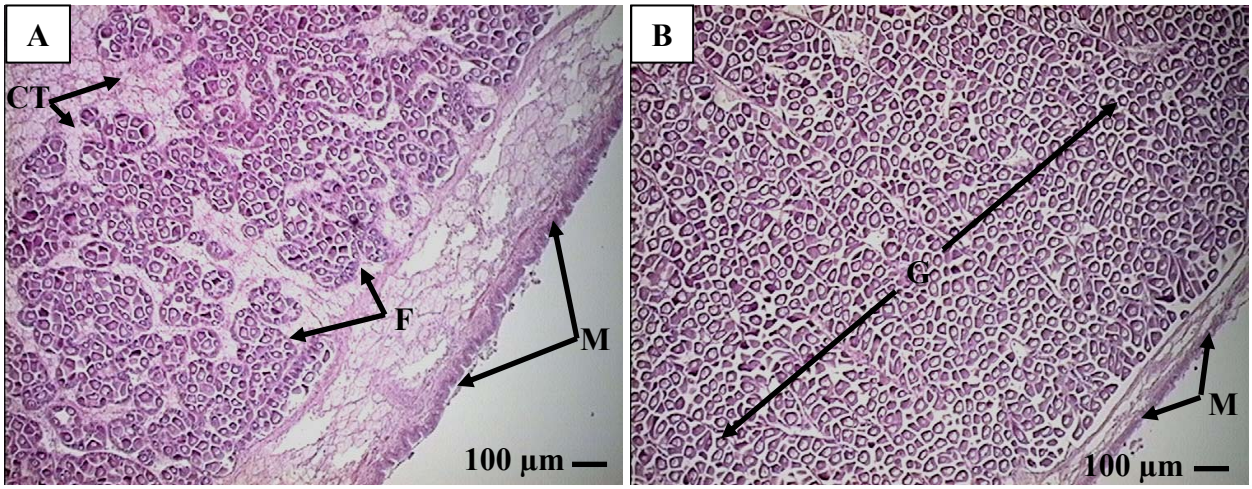

Figure 4.8 Photomicrographs of histological sections showing a gonad in: A) late development stage; B) spawning stage, from female diploid eastern oysters at the beginning of the experiment in August 2003. CT: connective tissue; F: follicles; G: gonad; M: mantle.

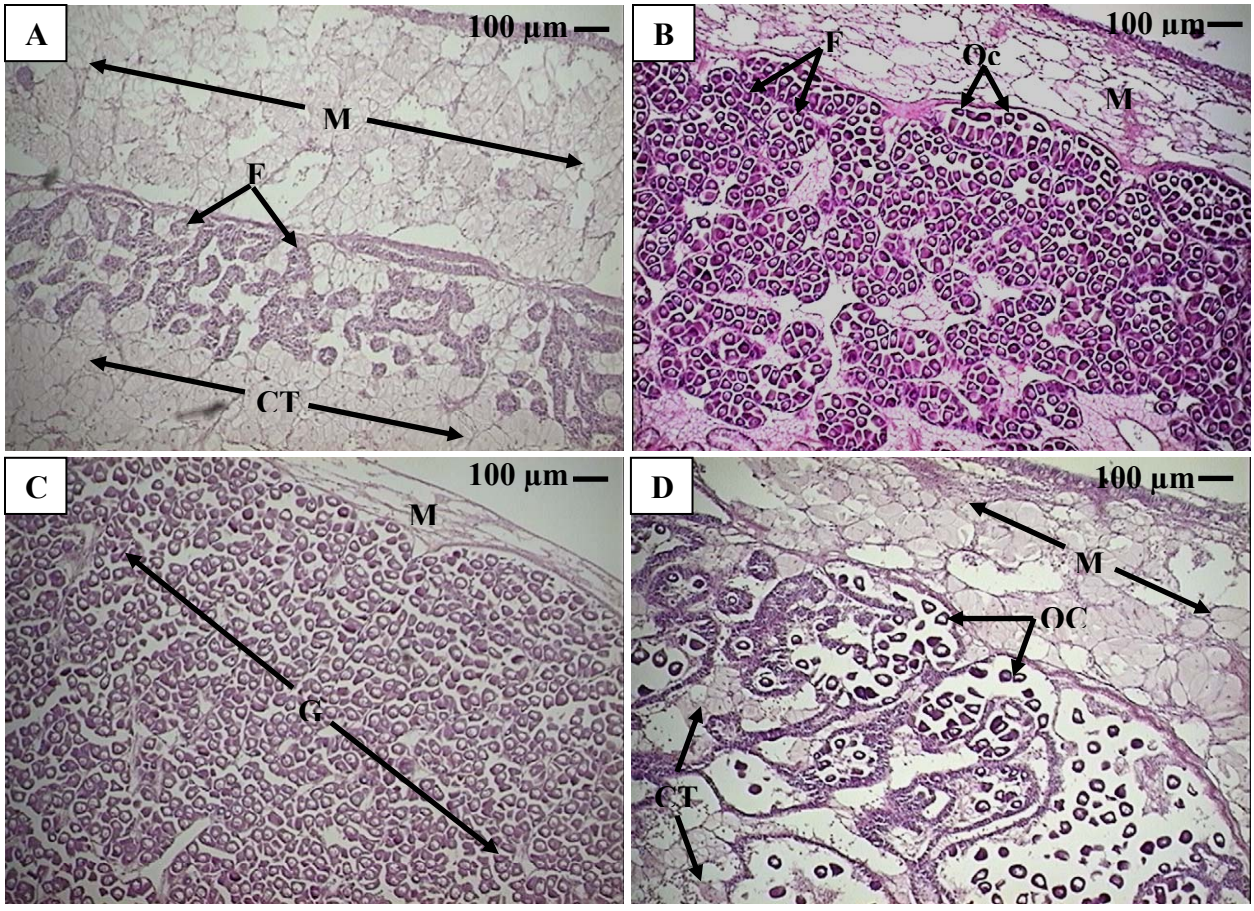

Figure 4.9 Photomicrographs of histological sections showing a gonad in: A) early development, B) late development, C) spawning condition, and D) spawned condition, from female diploid eastern oysters in the low dose treatment in August 2003. CT: connective tissue; F: follicles; G: gonad; M: mantle; Oc: oocytes.

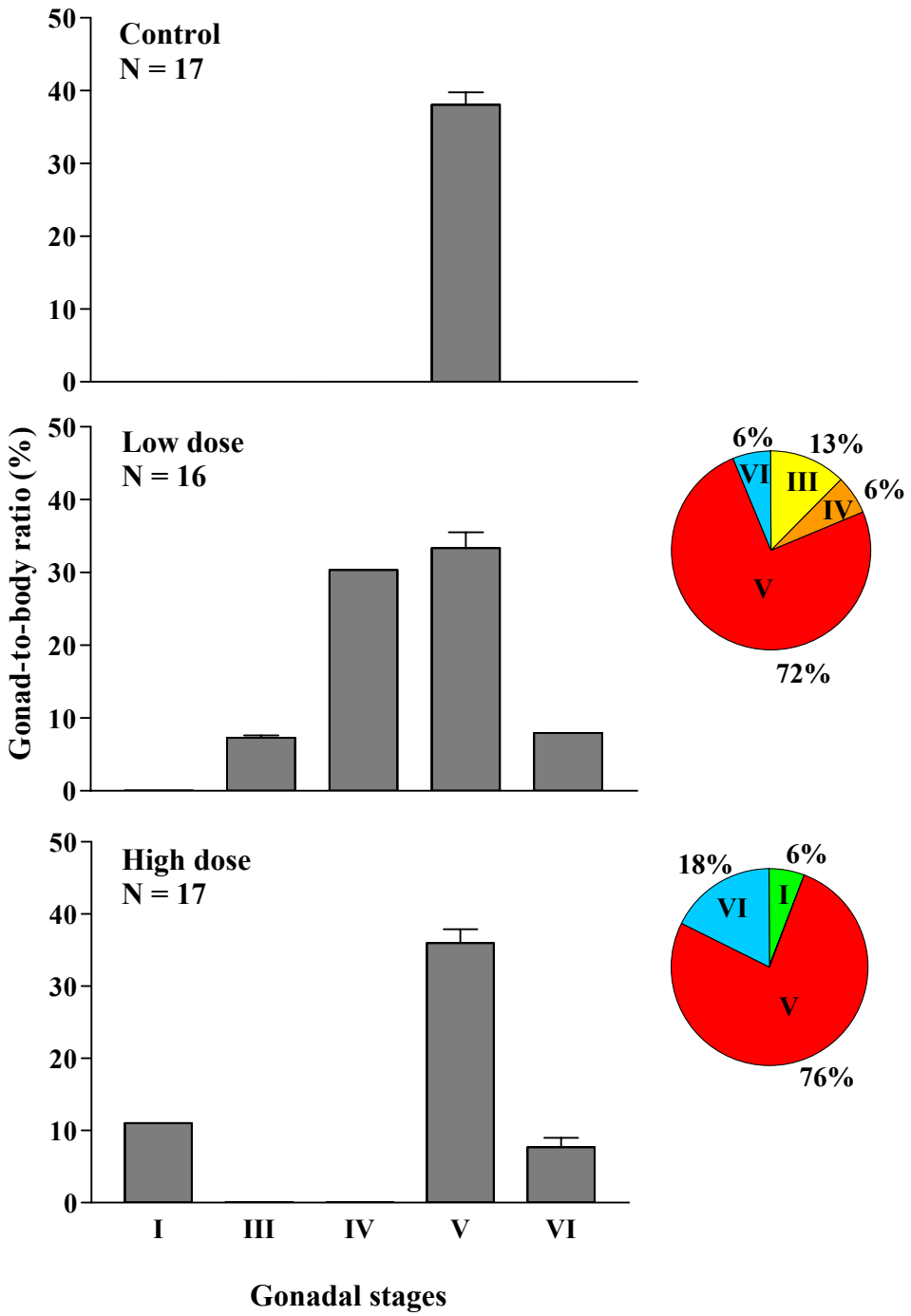

Figure 4.10 Gonadal assessment of the three experimental groups of diploid female eastern oysters evaluated in August, 2003. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

Diploid oysters not exposed to cytochalasin B (Initial evaluation)

Only matured and spawned gonads (Stages V and VI) were found for the diploid oysters of this group at the beginning of the experiment, 80% of the oysters were at Stage V with an average GBR of 31%.

Diploid oysters not exposed to CB (Day 12)

At day 12 of the experiment, 72% of oysters in the control group were at Stage V, but oysters were found with developing gonads in Stages III and IV, and one oyster was at Stage VI. In the low dose treatment, 63% of the oysters were found to be at Stage V, one oyster was found at Stage IV, and the proportion of oysters at Stage VI increased in comparison to the control treatment (Figure 4.11). In the high dose treatment, 64% of the oysters were found to be at Stage V; the proportion of oysters at Stage VI decreased to 18%, and one oyster at Stage I was found.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage.

The comparison between the GBR values of diploid oysters that were exposed to CB and those that were not, did not show any significant difference ($P > 0.05$). The gonad characteristics from both groups of oysters were the same at each stage of gonadal development, and the comparison of GBR within stages did not reveal any differences either.

May 2004

Triploid oysters (Initial evaluation)

Reduced or early developed gonads were the general conditions of triploid oysters evaluated at the beginning of the experiment, 86% of the oysters were at Stage III, and the rest of the oysters were at Stage I.

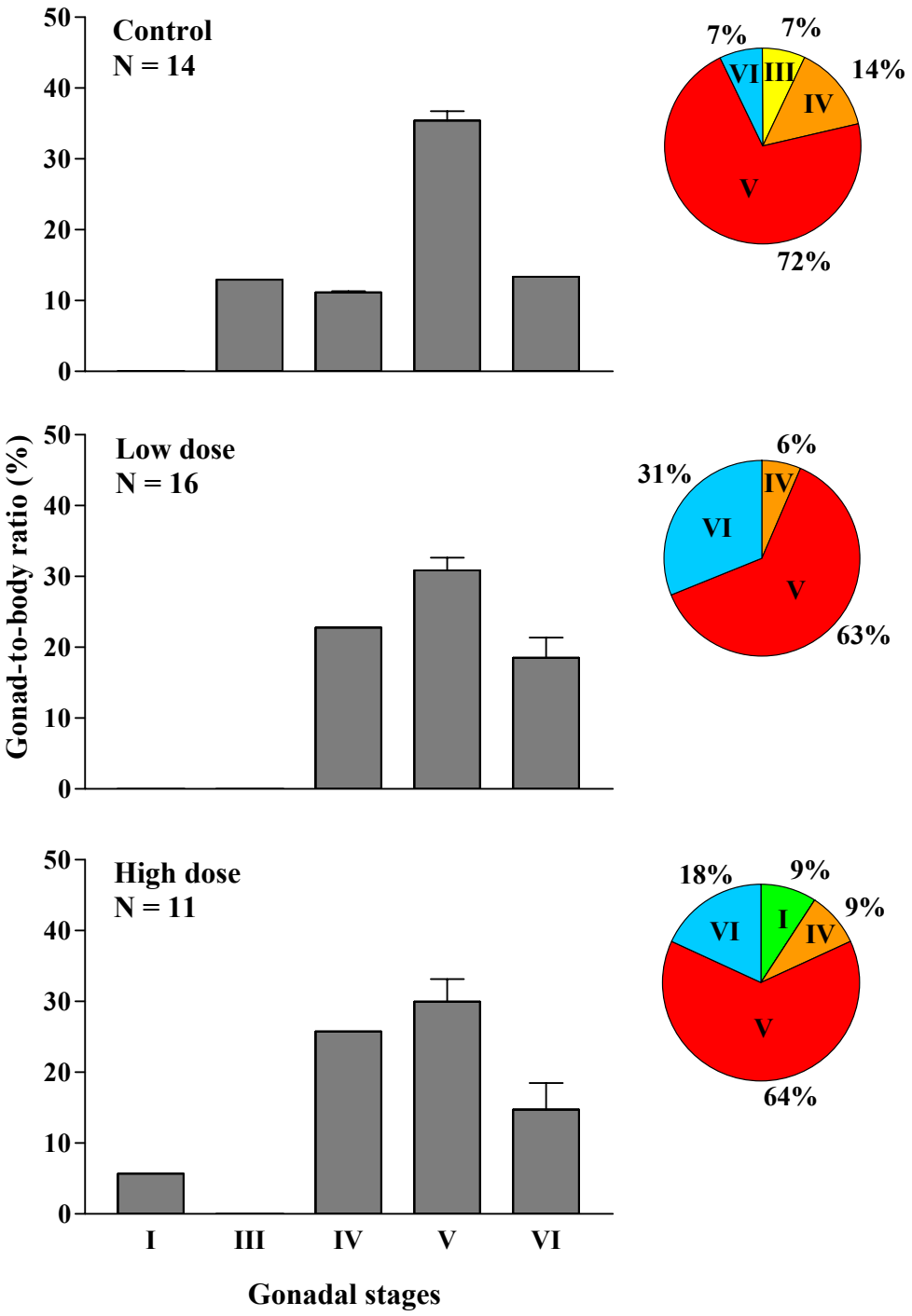

Figure 4.11 Gonadal assessment of the three experimental groups of the diploid female eastern oysters not exposed to CB evaluated in August 2003. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

Triploid oysters (Day 9)

At day 9 of the experiment, 84% of the oysters in the control group were characterized by reduced or early developed gonads (Stage III), with average GBR of 9% (Figure 4.12). One oyster was found at Stage IV and another had characteristics of a spawned gonad (Stage VI). The low dose treatment group was also characterized by oysters predominantly at Stage III with average GBR of 7%, but the proportion of oysters at this stage decreased to 73%. The proportion of oysters at Stage IV increased, and one oyster at Stage V with a GBR of 22% was found in this group (Figure 4.13). In the high dose treatment group the proportion of oysters at Stage III decreased to 69%, with average GBR of 12%. The rest of the oysters presented indifferent gonads.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage.

Triploid oysters (Day 12)

At day 12 of treatment, 87% of the oysters in the control group were characterized by reduced or early developed gonads (Stage III), with an average GBR of 7% (Figure 4.14). The low dose treatment group was also characterized by oysters at Stage III, with average GBR of 6%, but the proportion of oysters at this stage decreased to 77%. No oysters were found beyond Stage IV. In the high dose treatment, the proportion of oysters at stage III decreased to 75%, with an average GBR of 9%. The rest of the oysters presented indifferent gonads.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage. Also, the GBR comparison within treatment of the groups evaluated at day 9 showed no significant differences from the groups evaluated at day 12.

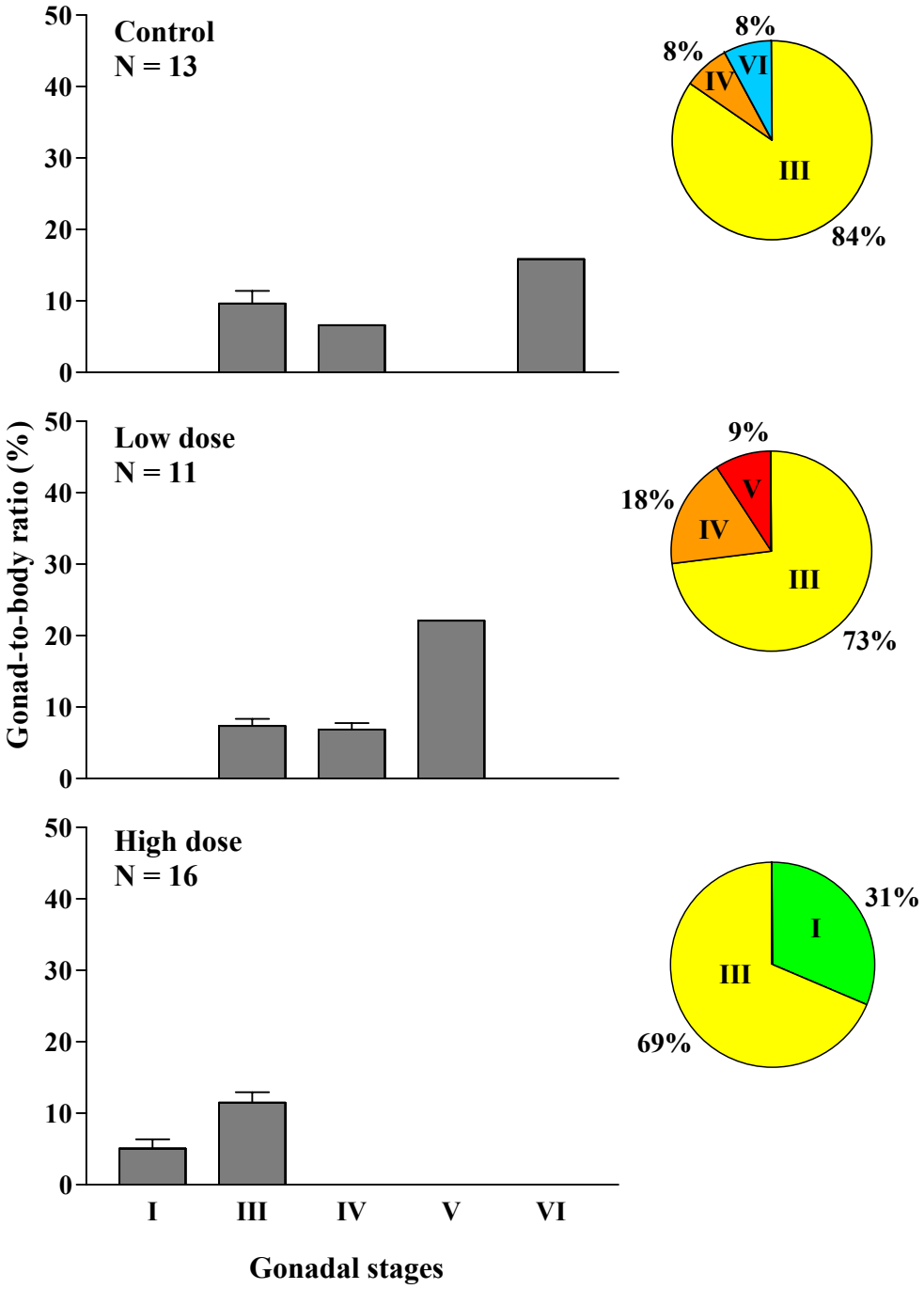

Figure 4.12 Gonadal assessment of the three experimental groups of triploid female eastern oysters evaluated in May 2004 at day 9 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

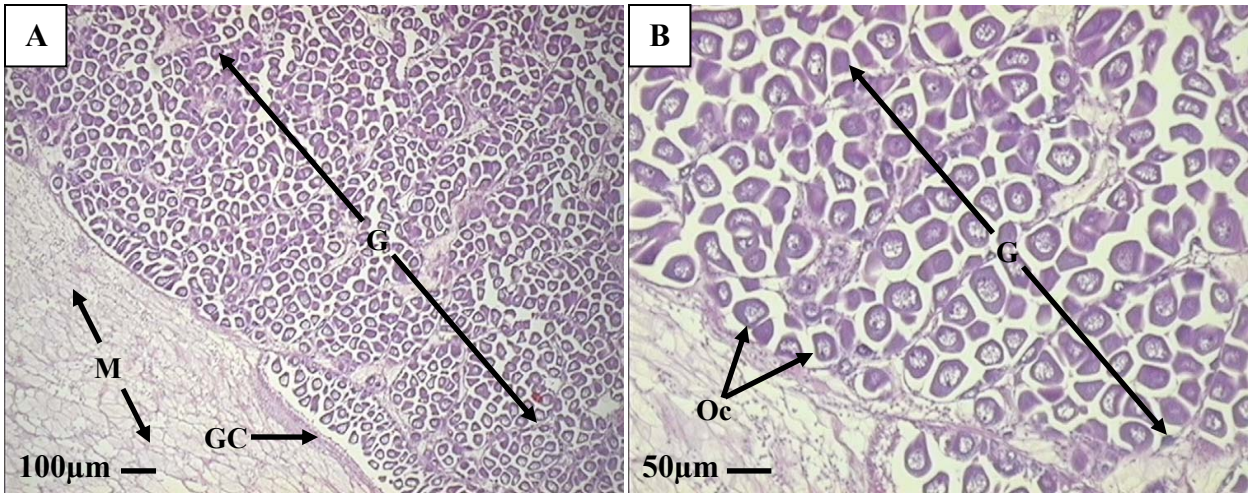

FIGURE 4.13 A & B Photomicrographs of a histological section showing a gonad in spawning condition (Stage V) from a female triploid eastern oyster in the low dose treatment at day 9th of experiment in May 2004. G: gonad; GC: genital canal; M: mantle; Oc: oocytes.

Diploid oysters (Initial evaluation)

Spawned gonads (Stage VI) was the general condition of the diploid oysters evaluated at the beginning of the experiment on May of 2004, 75% of the oysters were at this stage with an average GBR of 16%. The rest of the oysters were at Stage V, and one oyster was at Stage I.

Diploid oysters (Day 9)

At day 9 of the experiment, 59% of the oysters in the control group presented Stage V gonads with average GBR values of 34%. The proportion of gonads at Stage VI decreased to 27%, and one oyster was found at Stage III and another at Stage I. In the low dose treatment, 66% of the oysters were found to be at Stage V, with average GBR value of 29%; the rest of the oysters were at Stage VI, and one oyster was found to be at Stage IV. In the high dose treatment, the proportion of oysters with gonads at Stage V decreased to 47%, but the proportion of oysters at Stage IV increased to 32%. One oyster was found to be at Stage VI, and 16% of the oysters had indifferent gonads (Figure 4.15).

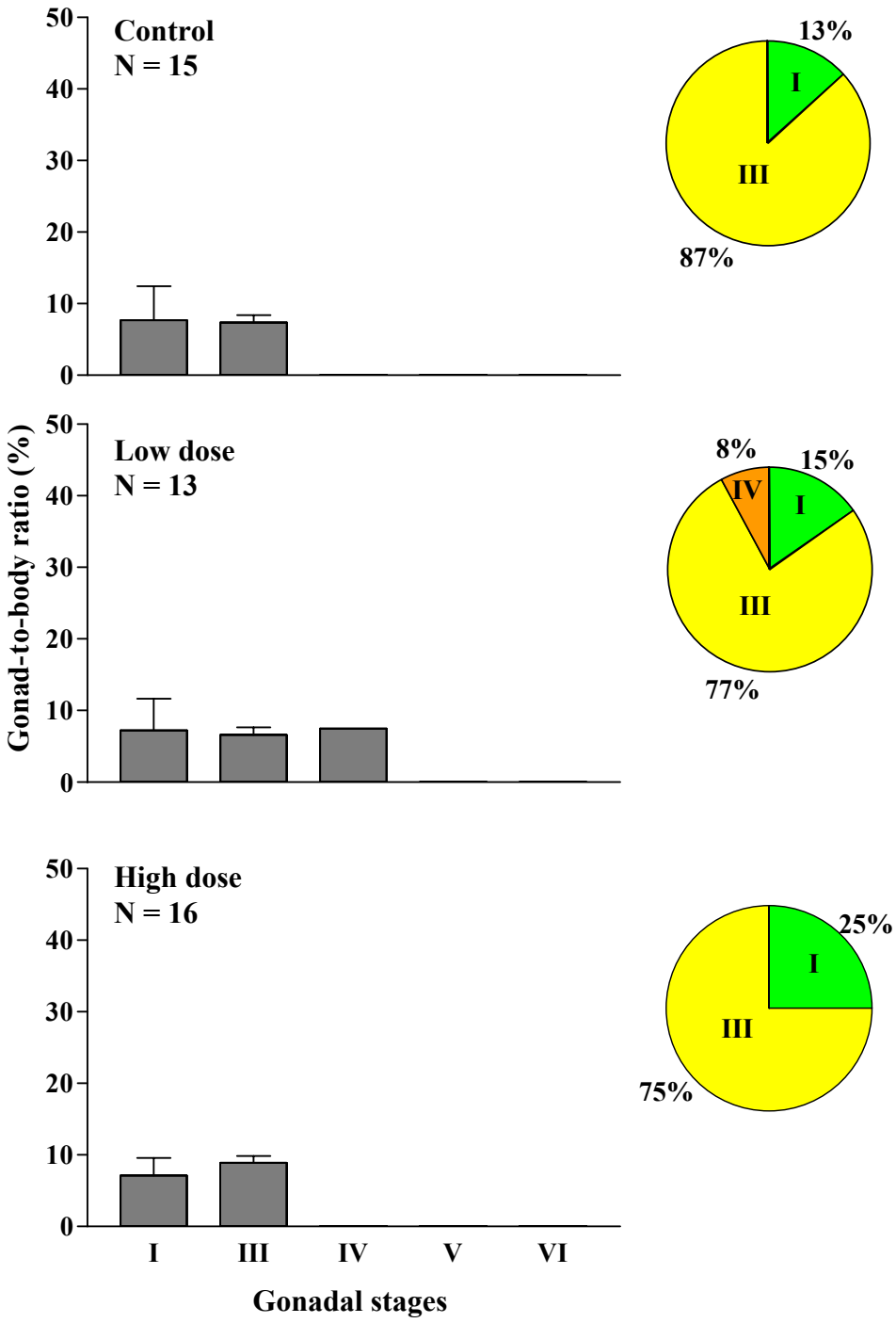

Figure 4.14 Gonadal assessment of the three experimental groups of triploid female eastern oysters evaluated in May 2004 at day 12 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

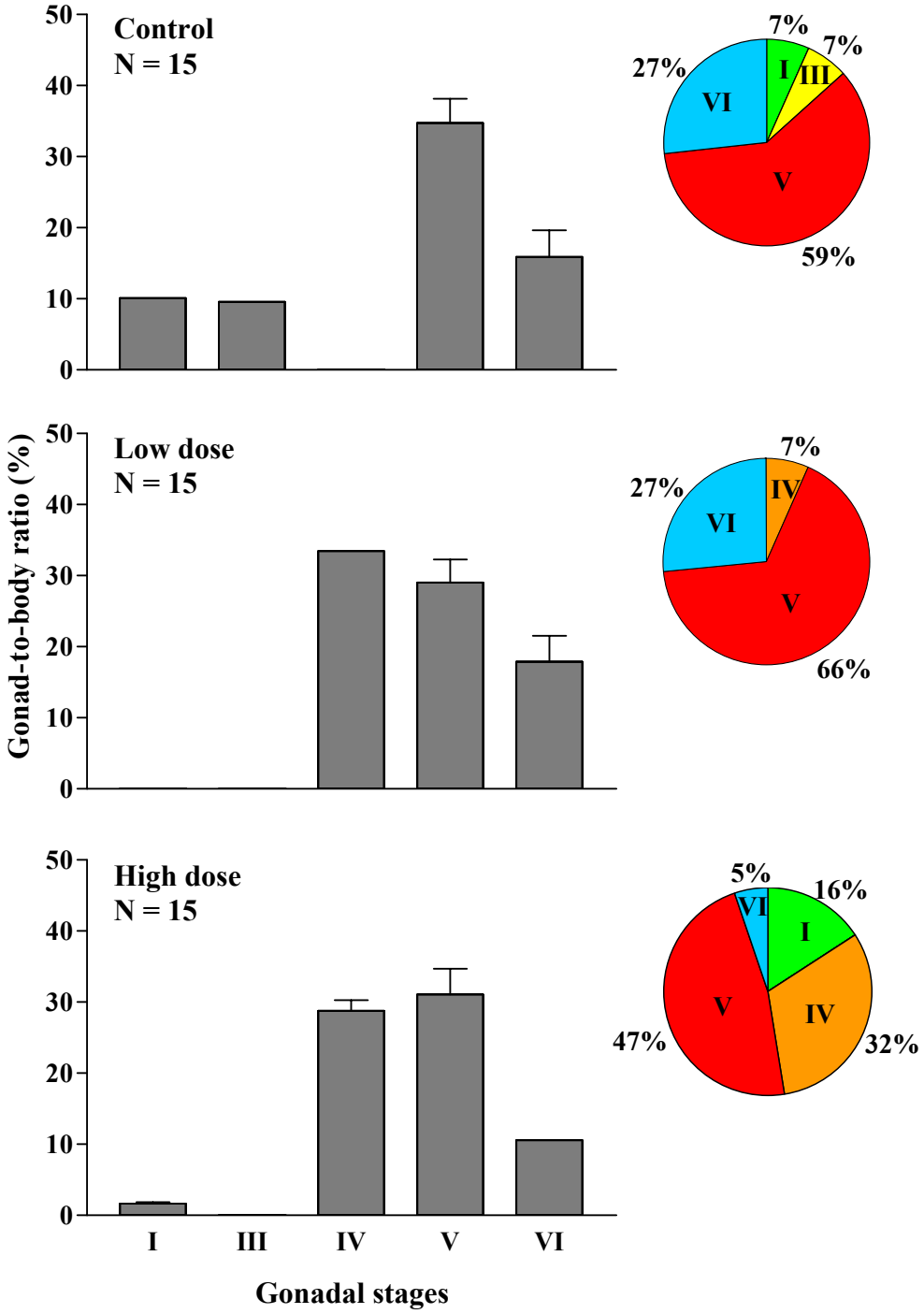

Figure 4.15 Gonadal assessment of the three experimental groups of diploid female eastern oysters evaluated in May 2004 at day 9 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage.

Diploid oysters (Day 12)

At day 12 of the experiment, the control group was characterized by oysters with developing gonads, 50% of the oysters in this group were at Stages IV or III, with an average GBR values of 23%. The rest of the oysters were in Stage V with an average GBR value of 35%, and one oyster had an indifferent gonad. In the low dose treatment, the proportion of oysters in Stage V was the same as in the control, the proportion of oysters at Stage IV decreased, and 21% of the oysters in this group were found to be at Stage VI. In the high dose treatment 70% of the oysters were found to be at Stage V with an average GBR value of 36%, and the proportion of oysters at Stages IV and VI decreased (Figure 4.16)

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage. Also, the GBR comparison within treatment of the groups evaluated at day 9 showed no significant differences from the groups evaluated at day 12.

August 2004

Triploid oysters (Initial evaluation)

Reduced and early developed gonads were the general condition of the triploid oysters evaluated at the beginning of the experiment; 52% of the oysters were at Stage III, but a distribution of oysters at all stages of gonadal development was observed (Figure 4.17)

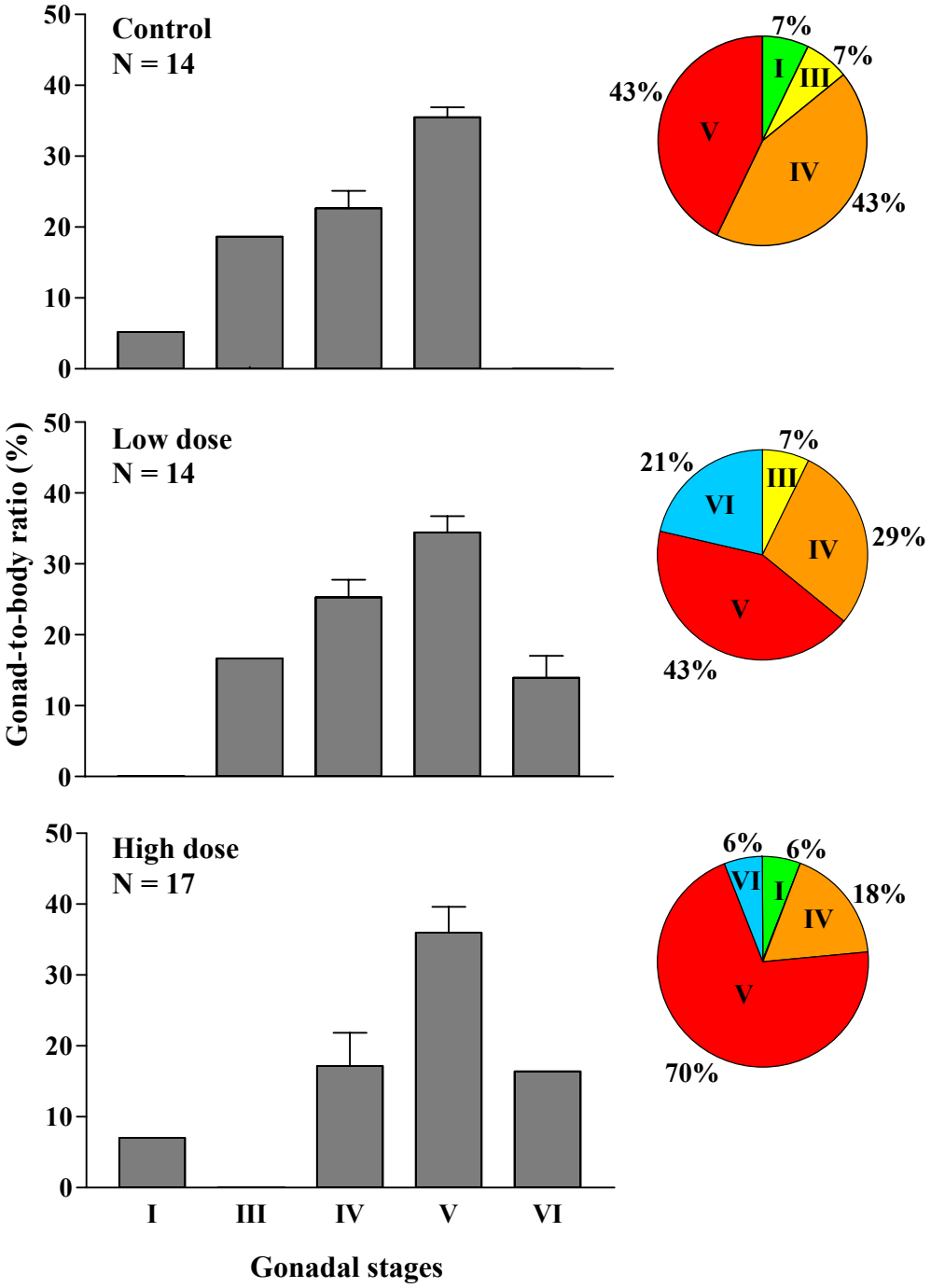

Figure 4.16 Gonadal assessment of the three experimental groups of diploid female eastern oysters evaluated in May 2004 at day 12 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

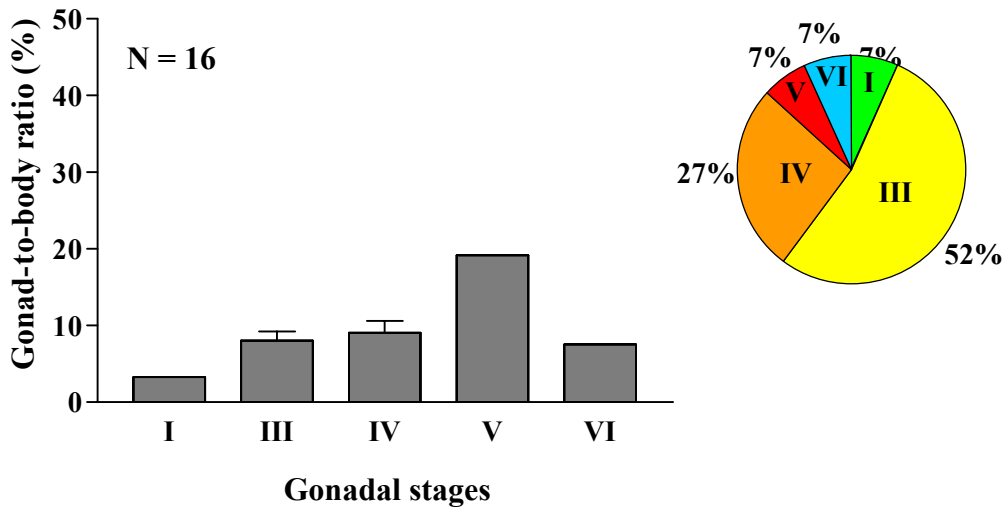

Figure 4.17 Gonadal assessment of female triploid eastern oysters evaluated at the beginning of the experiments in August 2004. Values represent average the percentage of gonad-to-body ratio ± SE by gonadal stage. The pie chart shows the percentage of oysters at each gonadal stage.

Triploid oysters (Day 12)

At day 12 of the experiment, 79% of the oysters in the control group were characterized by reduced or early developed gonads (Stage III), with an average GBR value of 8%. The low dose treatment group was also characterized by oysters in Stage III with an average GBR value of 7%, but the proportion of oysters at this stage decreased to 70%. Oysters at Stage IV were also observed in this group with an average GBR value of 9%, and oysters at Stages VI and I were also present. In the high dose treatment, the proportion of oysters at Stage III decreased to 55%, with an average GBR value of 8%. The proportion of oysters in Stage IV remained the same as in the low dose group, but the average GBR increased to 16%. From the 20 female oysters in this group, 3 (15%) had gonads at Stage V (Figure 4.18), with average GBR values of 23% (Figure 4.19).

There were no significant differences ($P > 0.05$) among any of the treatments when the average GBR values were compared from each treatment, or when the treatments GBR values were compared within stage.

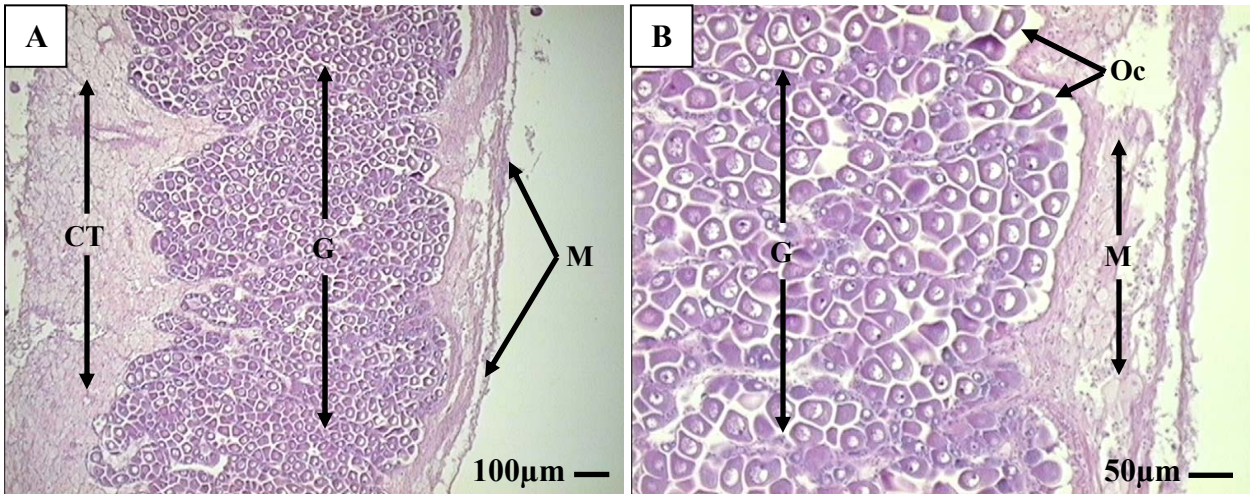

FIGURE 4.18 A & B Photomicrographs of a histological section showing a gonad in spawning condition (Stage V) from a female triploid eastern oyster in the high dose treatment at day 12 of experiment in August 2004. CT: connective tissue; G: gonad; M: mantle; OC: oocytes.

Triploid oysters (Day 15)

At day 15 of the experiment, 86% of the oysters in the control group were characterized by reduced or early developed gonads (Stage III), with average GBR values of 8%. The rest of the oysters were found at Stage IV. The low dose treatment was also characterized by oysters in Stage III with an average GBR value of 7%, but the proportion of oysters at this stage increased to 94%. In the high dose treatment, the proportion of oysters at Stage III decreased to 54%, with an average GBR value of 6%. Six oysters (40%) developed Stage V gonads (Figure 4.20) with an average GBR value of 27% (Figure 4.21). Compared to the day 12 evaluation, this proportion increased 25%.

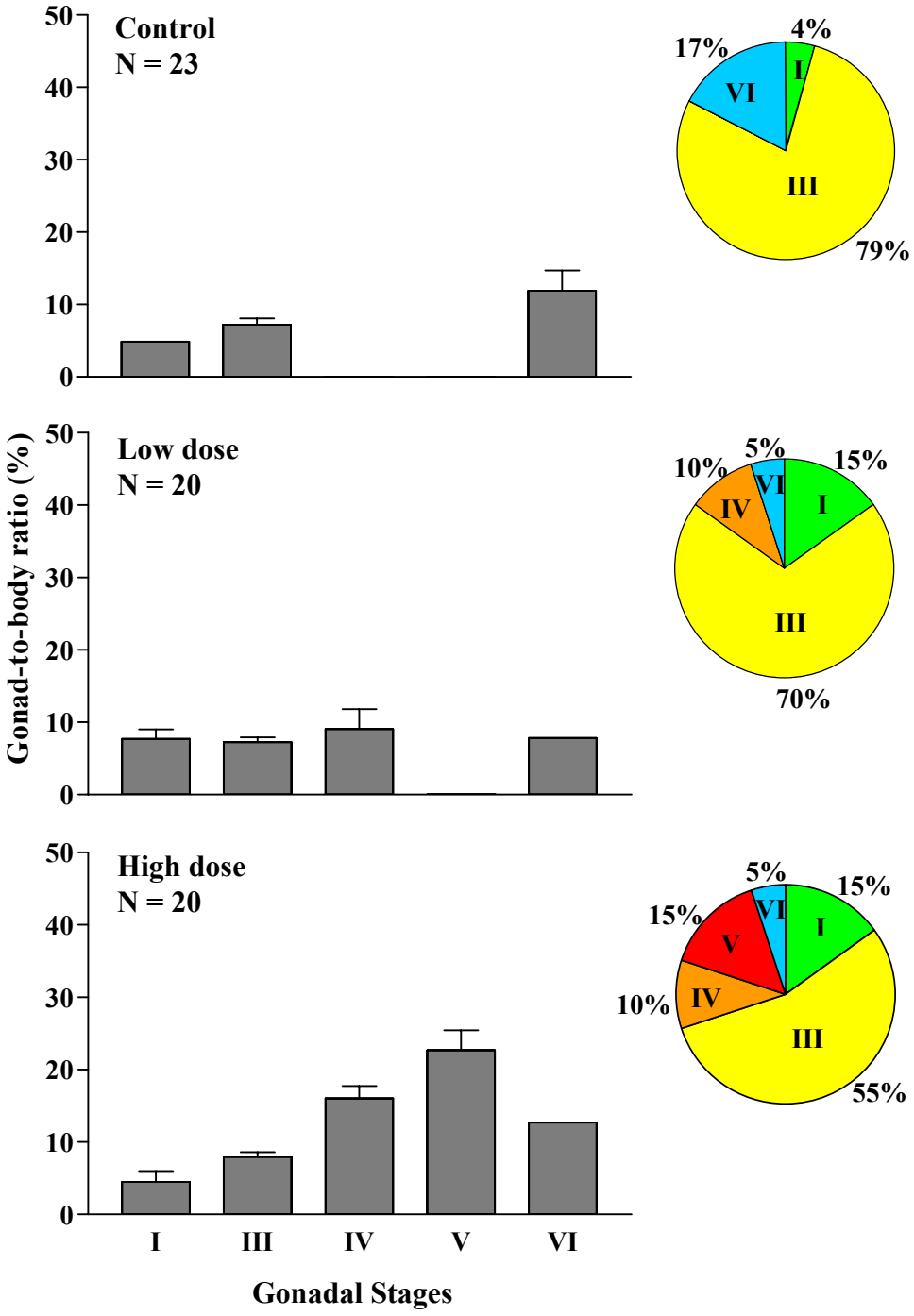

Figure 4.19 Gonadal assessment of the three experimental groups of triploid female eastern oysters evaluated in August 2004 at day 12 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatments GBR values were compared within stage. Also, the GBR comparison within treatment of the groups evaluated at day 12 showed no significant differences from the groups evaluated at day 15.

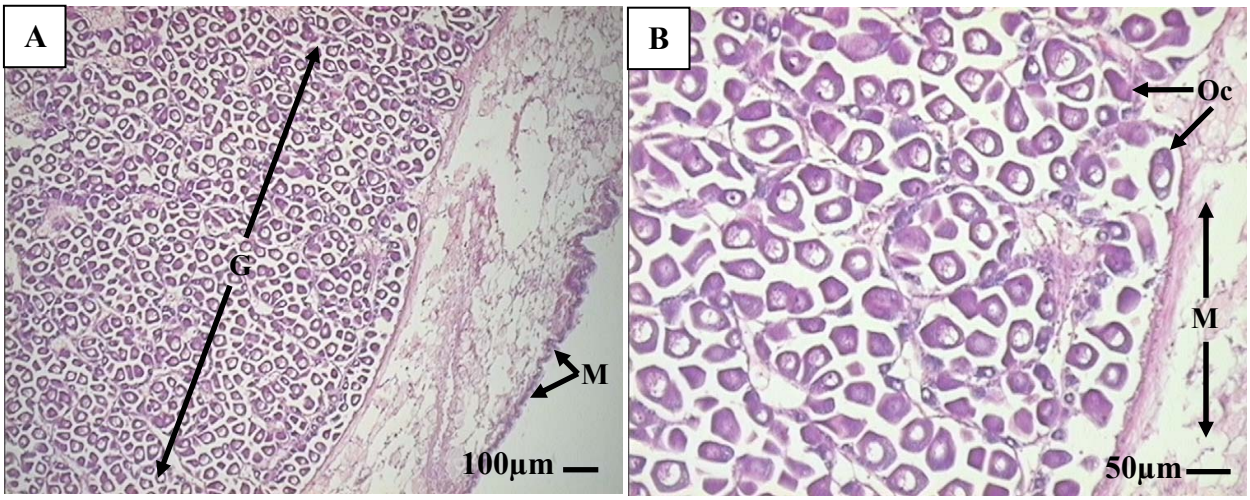

FIGURE 4.20 A & B Photomicrographs of a histological section showing a gonad in spawning condition (Stage V) from a female triploid eastern oyster in the high dose treatment at day 15 of experiment in August 2004. M: mantle; G: gonad; Oc: oocytes.

Diploid oysters (Initial evaluation)

All oysters evaluated at the beginning of the experiment had gonads at spawning condition (Stage V) with an average GBR value of 36%.

Diploid oysters (Day 12)

At day 12 of the experiment, 59% of the oysters in the control group presented gonads in Stage V with an average GBR value of 22%; 33% of the oysters were in Stage VI, and one oyster was in Stage IV. In the low dose treatment, the proportion of oysters in Stage V increased to 80% with an average GBR value of 23%, the rest of the oysters were in Stage IV with an average

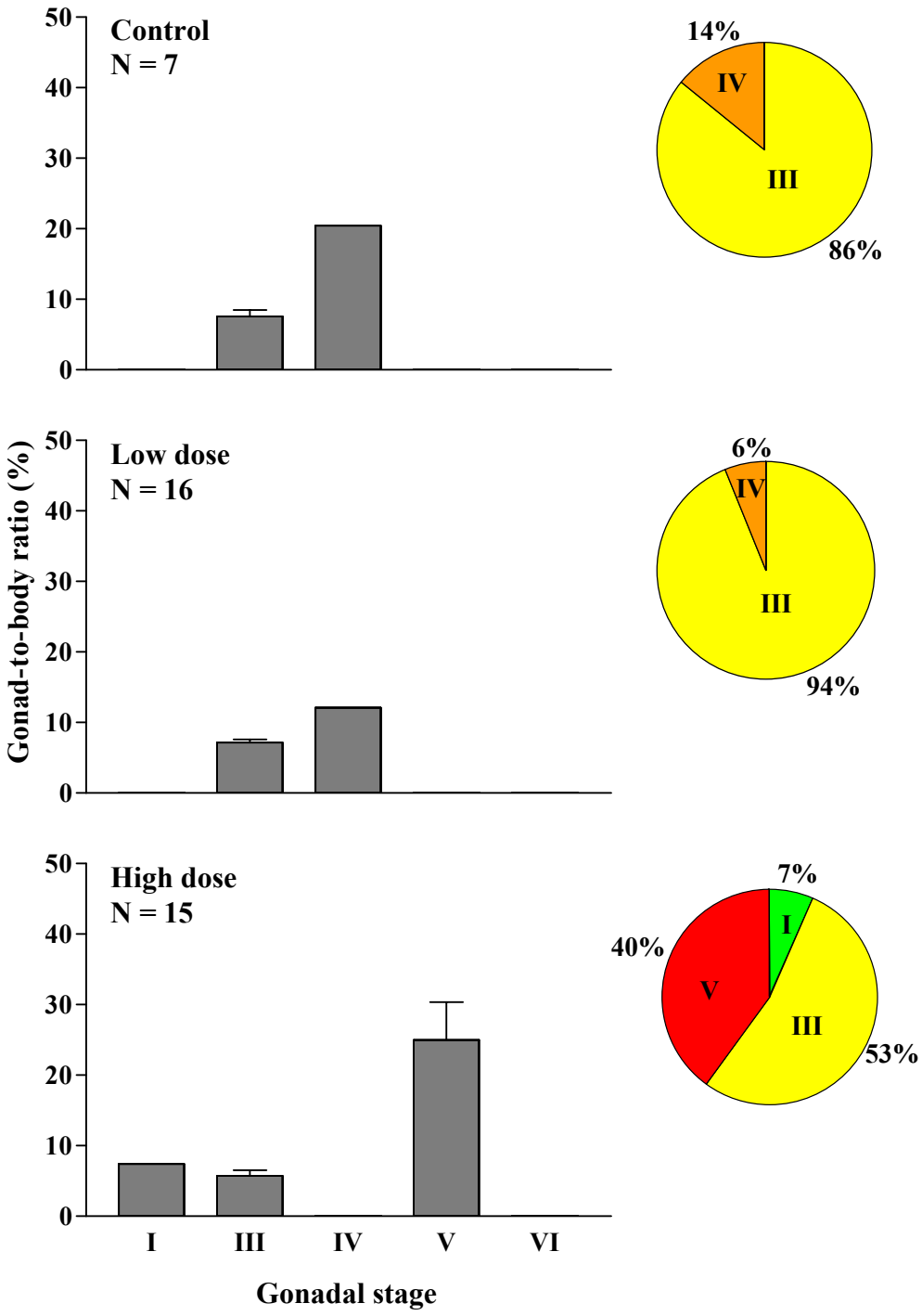

Figure 4.21 Gonadal assessment of the three experimental groups of triploid female eastern oysters evaluated in August 2004 at day 15 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

GBR value of 17%. In the high dose treatment, the proportion of oysters with Stage V gonads decreased to 73%, but the proportion of oysters in Stage IV increased to 27% with an average GBR value of 21% (Figure 4.22).

There were no significant differences ($P > 0.05$) among any of the treatments when the average GBR values from each treatment were compared, or when the treatment GBR values were compared within stages.

Diploid oysters (Day 15)

At day 15 of the experiment, 90% of the oysters in the control group were in Stage V with an average GBR value of 30%, and one oyster was in Stage VI. The low dose and high dose treatment groups presented only oysters with gonads at Stage V, although the high dose group had a 5% lower average GBR (Figure 4.23).

There were no significant differences ($P > 0.05$) among any of the treatments when average GBR values from each treatment were compared, or when the treatment GBR values were compared within stages. Also, the GBR comparison within treatment of the groups evaluated at day 12 showed no significant differences from the groups evaluated at day 15.

Estradiol Levels

In the diploid oysters, the concentrations of E_2 in general showed a gradual increase from indifferent to late developed gonads, and gradually decreased after that (Figure 4.24). In the triploid oysters the concentrations of E_2 in general showed a gradual increase from indifferent to early developed gonads. From that point, the concentrations decreased, reaching their lowest levels in the oysters with gonads in spawning condition (Figure 4.24). There were no significant differences ($P > 0.05$) among the concentrations of E_2 at the different gonadal stages for the triploid or diploid oysters.

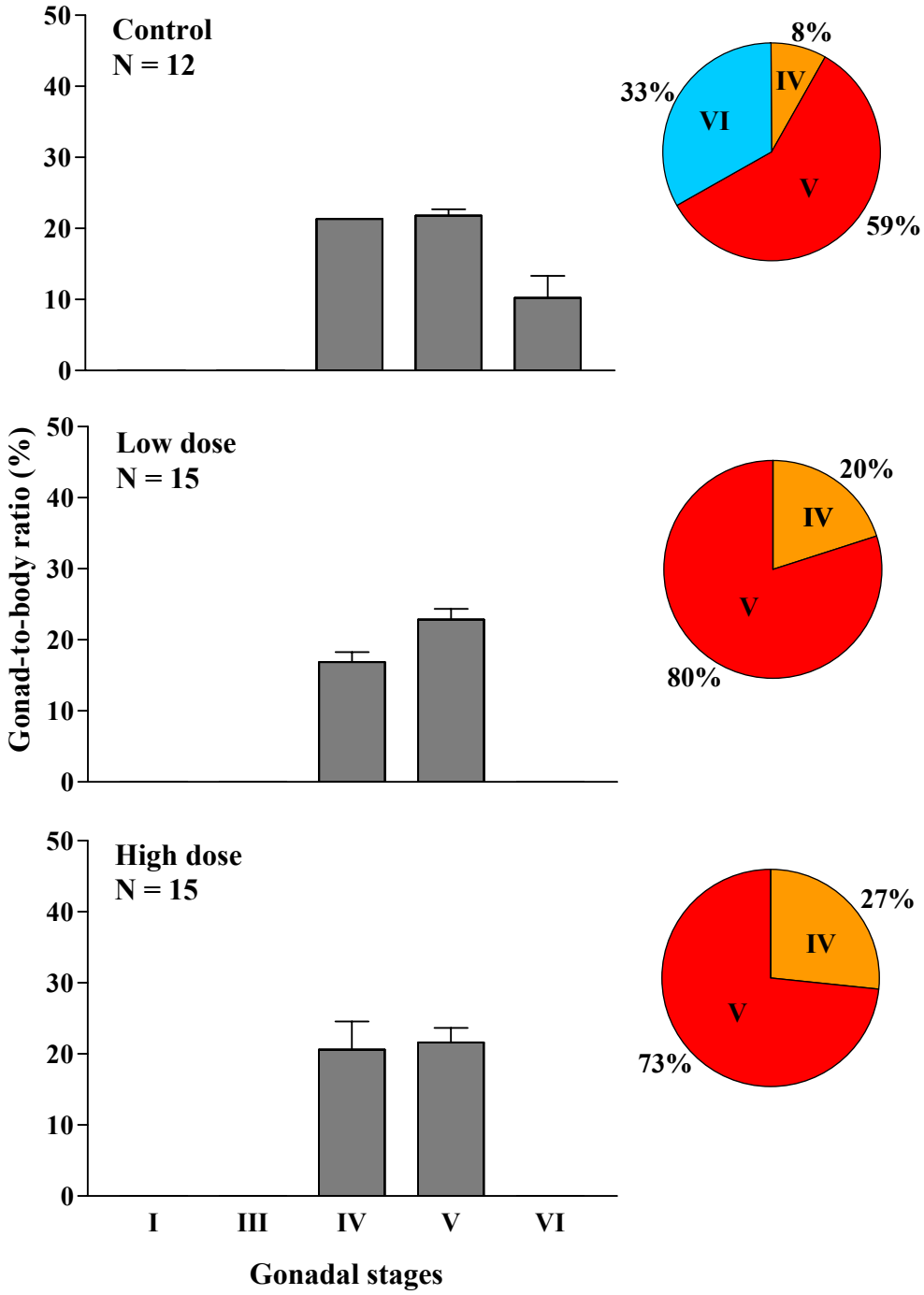

Figure 4.22 Gonadal assessment of the three experimental groups of diploid female eastern oysters evaluated in August 2004 at day 12 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. Pie charts show the percentage of oysters at each gonadal stage.

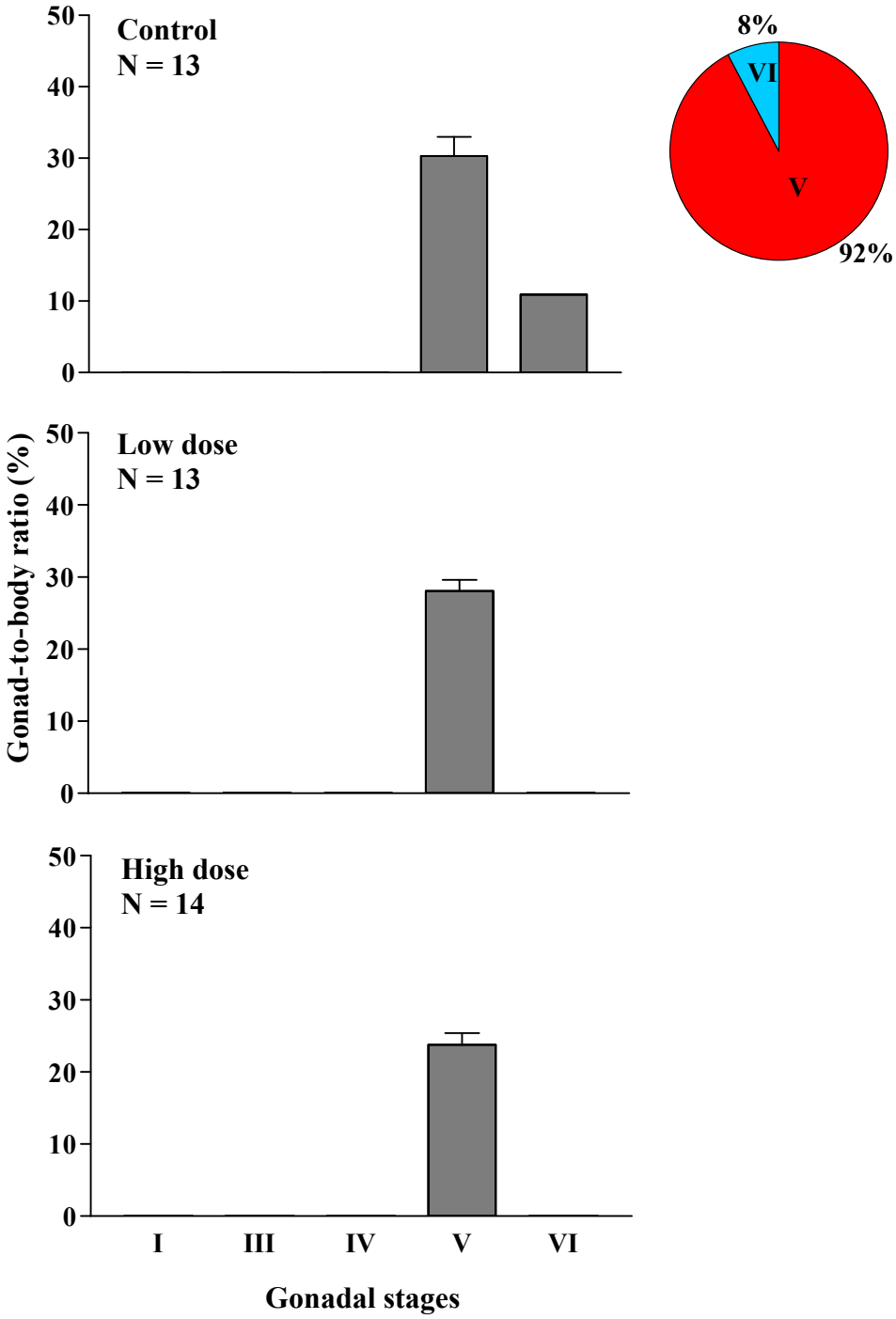

Figure 4.23 Gonadal assessment of the three experimental groups of diploid female eastern oysters evaluated in August 2004 at day 15 of the experiment. Values represent the average percentage of gonad-to-body ratio ± SE by gonadal stage. The pie chart shows the percentage of oysters at each gonadal stage.

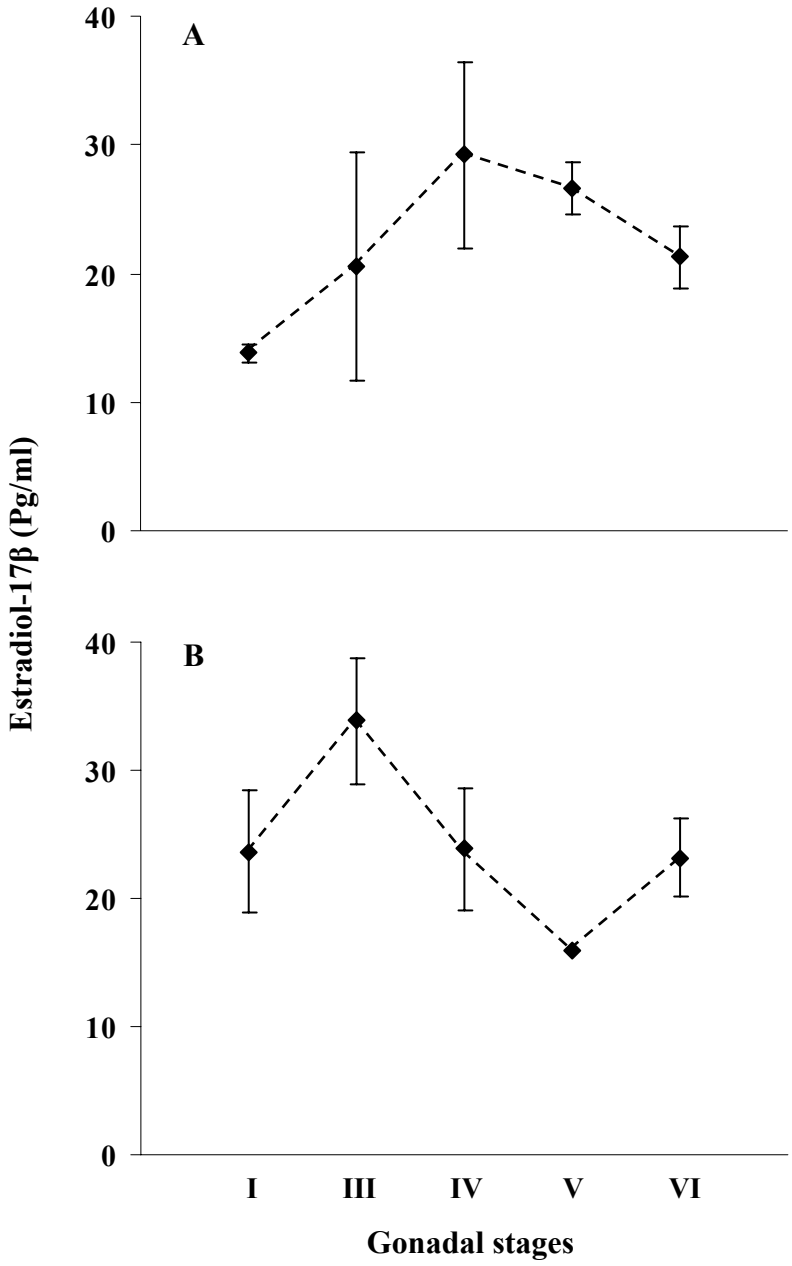

Figure 4.24 Changes in estradiol-17β at different stages of gonadal development of female eastern oysters. A: diploids (N = 99); B: triploids (N = 95). Values represent average concentration ± SE.

Oocyte Area

No significant differences ($P > 0.05$) among the treatments were found in August 2003 and 2004 for the oocyte area comparison (Table 4.2, 4.4). In May 2004 there were significant differences ($P < 0.05$) among the treatments at both days of evaluation (Table 4.3). In day 9 the control showed a higher oocyte area than the two E_2 treatments and at day 12 the low dose group showed the highest oocyte area. In the triploid oysters comparison of oocyte area was not possible because of lack of oysters at Stage V in every treatment.

Table 4.2 Statistical comparison of the oocyte area among treatments for diploid eastern oysters in August 2003, (N =40)

	Oocyte area (µm)		
Treatment	**Mean**	**SE***	**Comparison****
Control	948.16	16.87	A
Low dose	971.98	23.63	A
High dose	1025.33	26.68	A

*SE: Standard error, **Different letters indicate significant differences among treatment, as indicated by Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

Table 4.3 Statistical comparison of the oocyte area among treatments for diploid eastern oysters in May 2004 on day 9 and 12 of the experiment, (N =80).

	Oocyte area (µm)		
Treatment	**Mean**	**SE***	**Comparison****
Control (day 9)	820.63	28.1	A
Low dose (day 9)	670.36	13.95	B
High dose (day 9)	685.35	17.51	B
Control (day 12)	695.18	25	B
Low dose (day 12)	792.15	11.44	A
High dose (day 12)	741.94	13.21	Ab

*SE; Standard error. **Different letters indicate significant differences among treatment, as indicated by Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

Table 4.4 Statistical comparison of the oocyte area among treatments for diploid eastern oysters in August 2004 on day 12 and 15 of the experiment, (N =61)

Treatment	Oocyte area (µm)		Comparison**
	Mean	SE*	
Control (day 12)	679.78	20.9	A
Low dose (day 12)	650.42	16.34	A
High dose (day 12)	658.55	13.73	A
Control (day 15)	647.94	13.34	A
Low dose (day 15)	662.18	12.89	A
High dose (day 15)	662.39	13.12	A

*SE; Standard error. **Different letters indicate significant differences among treatment, as indicated by Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

Although none of the GBR values showed any significant differences among the treatments in the experiments, on average, the percentage of gonad-to-body ratio was higher in the oysters that were treated with E_2 compared to the controls. In the diploid group, except for the experiment of August 2003, the GBR percentage of the control treatments was always lower than at least one of the E_2 treatments (Figure 4.25 A). For the triploid oysters, except for the evaluation at day 9 of the experiment of May 2004, the GBR percentage of the control treatments was always lower than at least one of the E_2 treatments, and the triploid oysters appeared to have responded better to the high dose treatment (Figure 4.25 B).

DISCUSSION

The goal of this study was to enhance ovarian maturation in triploid eastern oysters, with the purpose of producing viable oocytes that could be used for the production of tetraploid broodstocks. Accordingly, three dosages of the steroid estradiol-17β (E_2) were tested in triploid and diploid eastern oysters and gonadal development was evaluated on qualitative and quantitative scales.

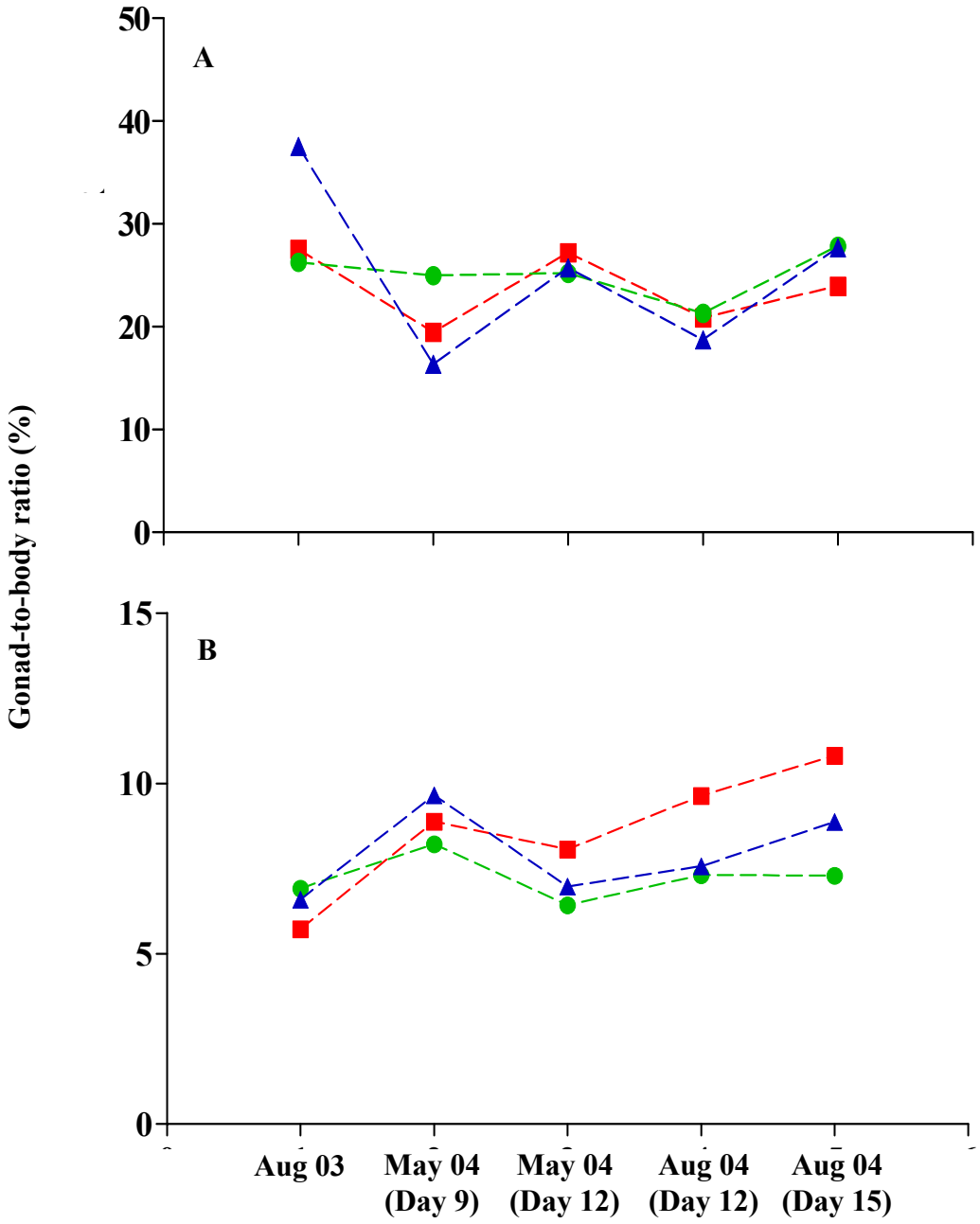

Figure 4.25 Average percentage of gonad-to-body ratio (GBR) of the eastern oysters for the three treatments in the three experiments. Control (triangles), low dose (circles), and high dose (squares). A: diploids, N = 99; B: triploids, (N = 95).

Although the use of E_2 to enhance gonadal maturation has been studied before in other bivalve species (Mori 1969), and the relation between E_2 levels and its fluctuations at different stages of sexual maturation have been documented, to our knowledge this is the first time that exogenous E_2 has been used to enhance gonadal development in diploid or triploid eastern oysters, and the first time the levels of E_2 have been measured in diploid eastern oysters or in triploid oysters of any species.

The use of E_2 in triploid oysters showed no significant effects in the GBR values among the different treatments, or when the treatments were compared within stages in any of the experiments, although the general tendency of the GBR values was to be higher in the groups treated with the high dose of E_2 than in the controls (Figure 4.25). While GBR may not be the best indicator to evaluate the effect of E_2, a positive effect of E_2 on gonadal maturation was observed when the percentages of oysters with gonads at Stages IV and V in the groups treated with E_2 were compared to the control groups.

In the experiment conducted in August of 2004, there was a stronger response of the triploid oysters to E_2, compared to the other experiments of August 2003 and May 2004; with 15% of the oysters found in Stage V at day 12 and 40% at day 15 at the high dose. The proportion of triploid oysters found in spawning condition (Stage V) in this experiment can be seen as relevant given that only 10 in 1600 (Supan *et al.* 2000a) was the proportion of mature triploid oysters that are expected to be found under normal conditions. The reason for the strong response of triploid oysters to E_2 in this experiment is not clear, but it is possible that: 1) the longer exposure to the E_2 had a greater effect on the gonadal maturation, and 2) the initial gonadal condition of the oysters, which had a higher percentage of oysters at Stage IV compared to the two previous experiments, favored a better response to the E_2. For example, it has been suggested that E_2 does not affect all oocytes and that a threshold size may be required before

oocyte growth can be stimulated by E_2 in the starfish *Asteria rubens* (Schoenmakers *et al.* 1981). Estradiol probably does not affect the production of oocytes, but rather the maturation of the primary oocytes already present in the gonad, because E_2 is one of the major factors that controls vitellogenesis (Li *et al*. 1998). In the eastern oyster, the threshold size of the oocytes for responsiveness to E_2 is more likely to be found between the early and late gonadal development stages where primary oocytes begin the accumulation of nutrients (vitellogenesis). For example, it has been reported that for *C. gigas* the highest concentrations of protein, as a result of vitellin accumulation by the oocytes, was found just before the beginning of the spawning season (Li *et al*. 2000).

The fact that triploid oysters with clear characteristics of having spawned were observed in all experimental groups indicates that regardless of the quality of the gonadal material or the amount of oocytes present in the gonad, the oysters followed a developmental sequence where the spawning of gametes in the gonads was the final step. This could be triggered by environmental and physiological factors that function independently of the gonadal condition. The same reproductive behavior was found in triploid Pacific oysters, where males and females showed characteristics of having spawned even when the gonads were not fully matured (Allen and Downing 1990). This is important to consider because although triploid oysters were found at Stage VI even in the control groups in percentages as high as 18%, this does not necessarily indicate that these oysters went through a process of gonadal maturation and subsequent spawning as is assumed in a normal cycle of reproduction for diploid oysters.

The exposure of oocytes to cytochalasin B at the time of the triploid induction had no effect on the process of subsequent gonadal maturation for the oysters that remained as diploids. Evidence of this was that the gonadal and gametic characteristics of the diploid oysters that were

exposed to CB during triploid induction were not different from the characteristics of the diploid oysters that were not exposed to CB in any of the three treatments or at the initial evaluation.

The use of E_2 in diploid oysters showed no significant effect on the gonadal maturation based on the GBR values for any of the experiments. This could have been as a result of the time of the year when the dosages were applied, when the diploid oysters were at a spawning condition or had already spawned. In either case the oocytes had already matured or there were not enough glycogen reserves to begin a new round of gonadal maturation. Regardless, the percentages of oysters with gonads at Stage V in the groups treated with E_2 were higher than in the control groups in the last two experiments, suggesting that the oysters had a positive response to E_2.

The occurrence of E_2 in the hemolymph of the triploid and diploid eastern oysters and fluctuations of titers with respect to the different stages of gonadal maturation, although were not significantly different, suggested that: 1) E_2 is synthesized by triploid and diploid eastern oysters, and 2) that the changes in titer correlate with oocyte maturation as a result of vitellogenesis. The profile of E_2 concentrations found for the diploid oysters in these experiments was similar to those reported for other species of bivalves (Matsumoto *et al*. 1997) where the concentration of E_2 starts to decrease when the animal enters the spawning stage. For the triploid oysters, the decline of E_2 concentrations started at an earlier stage (Stage III) compared to the diploid oysters (Figure 5.24). The reason for the decrease of E_2 in the triploid oysters at an earlier development stage (Stage III) than in the diploids is unclear, but may play a pivotal role in the reduced gonadal development observed in the triploid oysters.

The fact that no significant differences among treatments were found when the oocyte area of diploid oysters was compared could have been as the result of the initial gonadal condition of the diploid oysters, where the majority of oocytes were already at a mature stage

with fully developed oocytes, and therefore, E_2 had no effect on oocyte size. Another posible cause could have been the lack of sufficient nutrient reserves in the diploid oysters during the summer months, affecting the ability of the oocytes to incorporate vitellogenins to start a new round of vitellogenesis. Regardless, the use of oocyte areas to estimate the effect of E_2 on ovarian maturation of eastern oysters was not a useful measuring tool in the months tested.

In this study, diploid and triploid eastern oysters were capable of synthesizing estradiol-17β and the E_2 concentrations varied with gonadal stage. The use of E_2 in triploid oysters as a gonadal enhancer had no statistically significant effect on the GBR. The observation of oysters with matured gonads and in spawning condition was made only in the treated groups, and the percentage of oysters in later stages of maturation increased when the E_2concentration was higher and the treatment was longer, suggesting that E_2 had a positive effect on the oocyte maturation of triploid oysters, and thus merits further attention for use in the development of tetraploid broodstocks.

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CHAPTER 5 – SUMMARY AND CONCLUSIONS

The oyster industry in the state of Louisiana has traditionally been one of the most important in the United States and has consistently produced half the nation's oyster supply (GOIP 2003). Despite large annual landings, the profitability of this industry fluctuates each year due to seasonal variations of meat yields that are caused by the natural reproductive period. This has generated interest in the production of triploid eastern oysters. Triploid oysters have an increased growth rate and higher meat yield as a result of decreased fecundity because a large part of their energy reserves go to the production of meat rather than reproduction. The lack of a reliable and consistent method for the induction of triploidy in eastern oysters has been a major obstacle for the production of triploid eastern oysters on a commercial scale. The goal of this study was to evaluate ovarian maturation in triploid eastern oysters in response to the use of the hormone estradiol-17β (E_2) to produce viable oocytes for the production of tetraploid broodstocks that can be used to produce natural triploid oysters.

The results of this study showed that E_2 was involved in ovarian maturation in triploid eastern oysters. Evidence of this was the incidence of oysters with mature gonads (Stage V) only in the group of oysters treated with E_2. Although no significant differences were found when the gonad-to-body ratio (GBR) values of the different treatments were compared, the average GBR values of the E_2 treatment groups was higher in 4 of the 5 evaluations conducted in the three experiments. Higher percentages of oysters at Stage V and greater GBR values were found when the treatments were longer (12 and 15 days) and when the E_2 dosage was higher (75 ng/g wet weight). The use of E_2 in diploid oysters showed no significant effect on gonadal maturation based on the GBR values for any of the experiments, but the percentages of oysters with gonads at Stages V and VI in the groups treated with E_2 were greater than in the control groups, indicating a direct effect on maturation and spawning.

One of the most interesting results of this work was the observation that diploid and triploid eastern oysters were each capable of synthesizing E_2, and that the levels of this hormone in the hemolymph varied in general with the stage of gonadal maturation, where the highest concentrations were found before the onset of spawning. This observation suggests that E_2 plays an important role in the process of vitellogenesis and oocyte maturation in the eastern oyster. Previous to this, no published study had documented the occurrence of E_2 in the eastern oyster.

The use of appropriate methodologies to estimate reproductive condition in diploid and triploid eastern oysters was crucial to evaluate the effect of E_2 on reproductive development. In this study, a methodology to estimate gonad-to-body ratio by image analysis was developed. The use of image analysis to quantitatively estimate gonadal development proved to be fast and reliable, and yield values comparable to existing comparable methodologies. In addition, a descriptive scale to stage reproductive development based on gonadal characteristics was created. The combination of these two measuring parameters facilitated the interpretation of the results in this study.

The overall response of triploid eastern oysters to E_2 suggests that the use of this hormone had a positive effect on ovarian maturation. Expansion of the methodology to determine better dose response curves, dose frequency, and appropriate physicochemical parameters, would provide techniques directly applicable by oyster hatchery industries for the development of tetraploid broodstocks in the eastern oyster and could facilitate production of 100% triploid eastern oysters, as a direct benefit for the Gulf oyster industry.

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APPENDIX A
STANDARD OPERATING PROCEDURES

List of standard operating procedures (SOP)

SOP - 1 Tissue collection

Materials

- Dissecting knife blade with handle (Pathco, Palo Alto, California)
- Commercial oyster shucking knife
- Tissue cassette (Omnisette, Fisher Scientific, Pittsburg, PA.)
- 1-L plastic bottle with cap
- Davidson solution (SOP-2)

Procedures

1. Open oyster carefully with a shucking knife and sever the adductor muscle from both valves.
2. Make a perpendicular cut into the gill-labial palp junction; make a second cut 4 mm posterior (towards the adductor muscle) to the first cut (Figure A.1).
3. Remove a 4-mm section and place into a tissue cassette with the junction of the labial palp-gill junction side facing down.
4. Place cassettes in a plastic bottle containing 750-ml of Davidson's solution.
5. Fixed sections can be taken to the LSU School of Veterinary Medicine for sectioning, staining, and mounting. Sections should be 5-µm thick, approximately 1 mm from the gill-labial palp junction, and stained with Gill's hematoxylin and counter stained with eosin, and mounted on a glass slide.

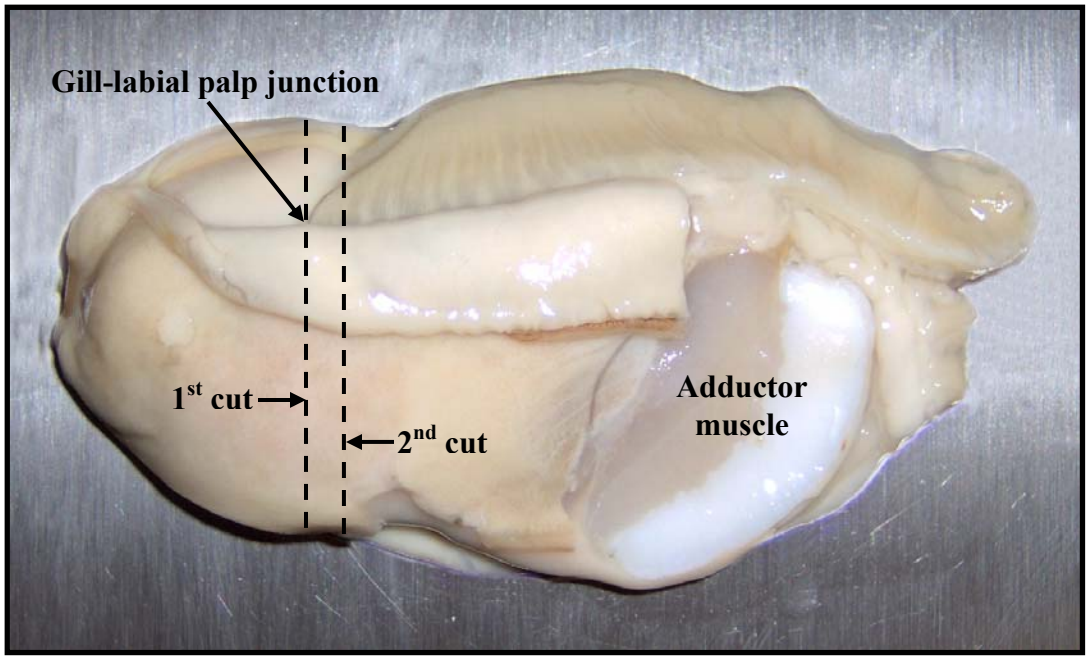

Figure A.1 Photograph of an oyster showing the location of the cuts for the histological section

SOP - 2 Davidson's fixative

Materials

- 220 ml (w/w) formalin 37% [FW = 30.03], (Fisher Scientific, Fair Lawn, New Jersey)
- 330 ml ethanol 95%, (AAPER Alcohol, Shelbyville, Kentucky)
- 115 ml acetic acid glacial [FW = 60.05], (Mallinckrodt Baker Inc. Phillipsburg, New Jersey)
- 335 ml filtered sea water (same salinity from where the oysters were collected)
- Glass bottle

Procedures

1. Mix all the reagents to produce a stock solution that can be stored for 6 months at room temperature.
2. When ready to use the fixative, mix two parts of the stock solution with one part of filtered sea water.

SOP - 3 Gonad-to-body ratio (transect method)

Materials

- Histological slide of an oyster transverse section
- Compound microscope with vertical and horizontal scale

Procedures

1. Using the horizontal scale of the microscope, divide the histological section into ten equidistant transects from ventral to dorsal side of the section avoiding the curvature of the gonad (Figure A.1)
2. Move the microscope pointer or crosshairs to transect 3 and position it at the outer membrane of the mantle, position M1 (Figure A.2). Record the position using the vertical scale
3. Move downward (right to left) the gonad along the transect and with the vertical scale record the positions (Figure A.3)
4. Determine the GBR value of that transect with the following formula:

$$GBR = [(G1 - MG1) + (MG2 - G2)] / (M2 - M1)$$

5. Average the GBR values of transects 3 to 8 to calculate the total GBR

$$\text{Oyster Total GBR} = \Sigma \text{ GBR values transect}_{3\text{-}8} / 6$$

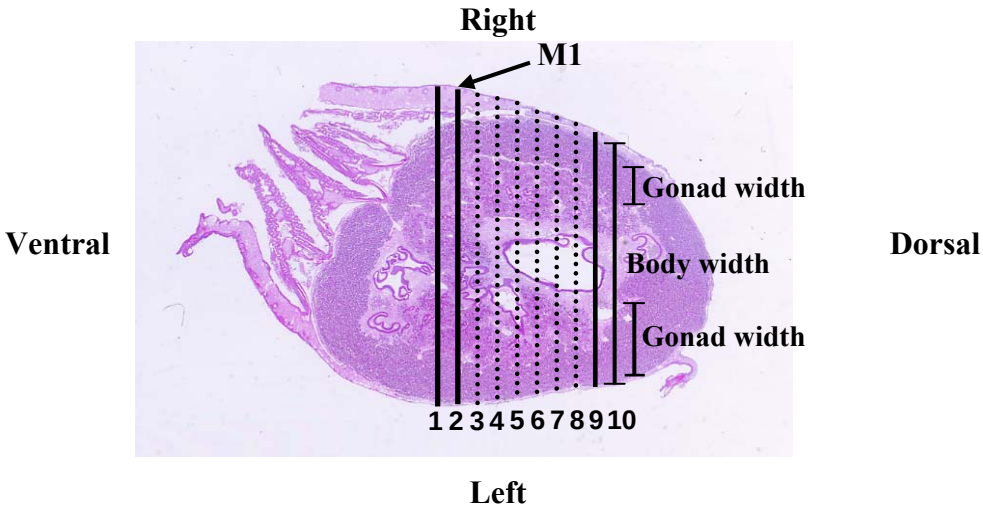

Figure A.2 Photomicrograph of a transverse histological section of an eastern oyster showing ten equidistant transects, M1: outer membrane of the mantle

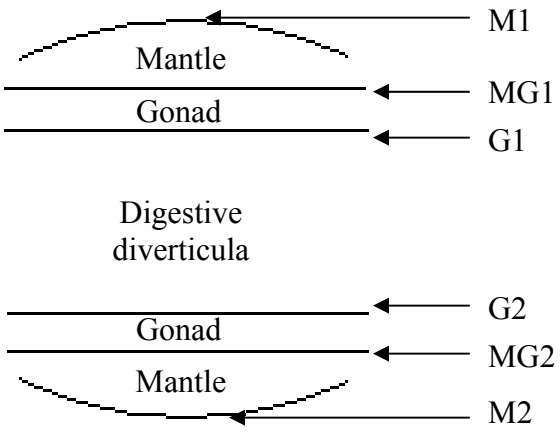

Figure A.3 Division of layers of a transverse section through the mid-body region of an oyster. Each boundary has been labeled to simplify the calculation of the GBR.

SOP - 4 Gonad-to-body ratio (Image analysis methods)

Materials

- Digital scanner (Epson Perfection 1640SU, Epson America Inc. Long Beach, California)
- Histological slide of an oyster transverse section
- Software Adobe Photoshop 7.0
- Digital pen and a drawing tablet (Hyper Pen 12000U, Aiptek Inc. Irvine, California)
- Personal computer (Dell Precision workstation 360, Dell Inc. Austin, Texas)
- Image analysis software Metaview 6.1

Procedures

1. Open the scanner software program (Epson smart panel) and select "scan to OCR application". Inside that window select "A4" for paper size and "color" (300 dpi standard resolution) for scan mode and scan the histological slides. When finish, the scanner applications will automatically give the option of exporting the files to several programs. Choose the software Adobe Photoshop and save the images as a TIF file.
2. Open the program Metaview 6.1 and open the file with the scanned image.
3. Magnify the image twice using the magnifying glass icon in the toolbar options.
4. From the toolbar open the "measurements" menu.
5. From the measurements menu select "region measurements".
6. From the "region measurements" window select the "region" tab and choose "area".
7. From the toolbar select the "free trace tool" option (lasso icon).
8. Go to the image, position the digital pen pointer on any place of the outer margin of the mantle and trace it following the outline of the body; this will be body area (B), (Figure A.4).
9. Position the digital pen pointer at any place of the outer margin of the gonad and trace it following the inner and outer outline of the gonad; this will be gonad area (G), (Figure A.4).
10. Calculate the GBR by dividing B by G.

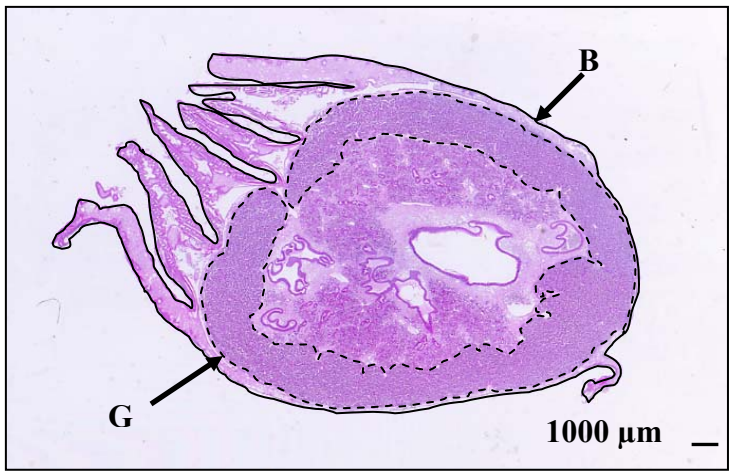

Figure A.4 Diagrammatic representation of the method used to determine the gonad-to-body ratio by area. Solid lines outline the body area; dashed lines outline the gonadal area, G: gonad area, B: body area.

SOP - 5 Hemolymph collection and storage

Materials

- Angle grinder (DeWalt 4-1/2 in, model DW402K)
- Masonry cut-off wheel (115 x 2 mm)
- Dissecting needle
- 1-ml disposable syringe (Becton Dickinson Co. Franklin Lakes, New Jersey)
- 23-ga needle (Becton Dickinson Co.)
- 1.5-ml cryovials (Dot Scientific Inc. Lippincott Burton, Michigan)
- Crushed ice
- Liquid nitrogen

Procedures

1. With the angle grinder make a 5-mm notch on the shell, on the dorsal part of the oyster toward the posterior end next to the adductor muscle (Figure A.5).
2. Through the notch, insert the syringe, when you encounter the adductor muscle, there will be resistance. Insert the needle 5-mm to 7-mm more, pull on the plunger to create suction and rotate the syringe until hemolymph starts to come out. Hemolymph is colorless and cloudy.
3. Immediately transfer the hemolymph to a cryovial and put it in a ziplock bag on ice if the sample is to be used for ploidy analysis or put the criovial directly in liquid nitrogen if it is to be used for estradiol assay. Samples for ploidy analysis should be analyzed within 24 h; samples for estradiol assay can be stored at -80 C^0 until analysis.

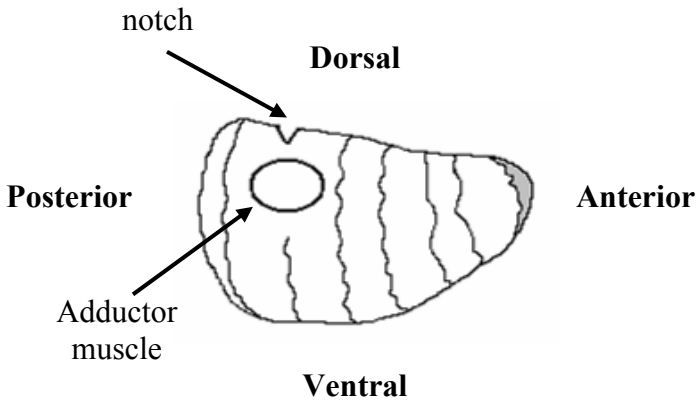

Figure A.5 Diagram showing location of the shell notch

SOP - 6 Nile Tilapia and channel catfish blood collection and storage

Materials

- Tricane methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, Washington)
- 20-L bucket
- 1-ml disposable syringe (Becton Dickinson Co. Franklin Lakes, New Jersey)
- 23-ga needle(Becton Dickinson Co.)
- Paper towels
- Tank water
- 1.5-ml plastic cryovial (Dot Scientific Inc. Lippincott Burton, Michigan)
- Phosphate buffered solution Ca and Mg free (PBS) (Gibco, Grand Island, New York)
- Dimethyl sulfoxide (DMSO) [FW = 294.11] (Fisher Scientific, Fair Lawn, New Jersey)
- 35-µm filter screen
- Anticoagulant solution (ACD) (Becton Dickinson, Franklin Lake, New Jersey)

Procedure

1. In a plastic bucket add 8 L of tank water and 200 mg/L for tilapia, *Oreochromis nilotica*, or 300 mg/L for channel catfish, *Ictalurus punctatus*, of MS-222 and mix.
2. Put the fish in the plastic bucket and wait 30 seconds for the fish to be anesthetized, if the fish is not anesthetized within 30 seconds, add 100 mg of MS-222. Continue doing this until the fish is anesthetized.
3. Take the fish out of the water and lay it on paper towels.
4. Collect the blood by caudal puncture with a syringe containing 10% of ACD (Figure A.6).
5. Dilute the blood to a final concentration of $1x10^6$ cell/ml with PBS containing 8% DMSO.
6. Filter the diluted blood through a 35µm screen and aliquoted to cryovials.
7. The blood can be store at -80^0C until analysis.

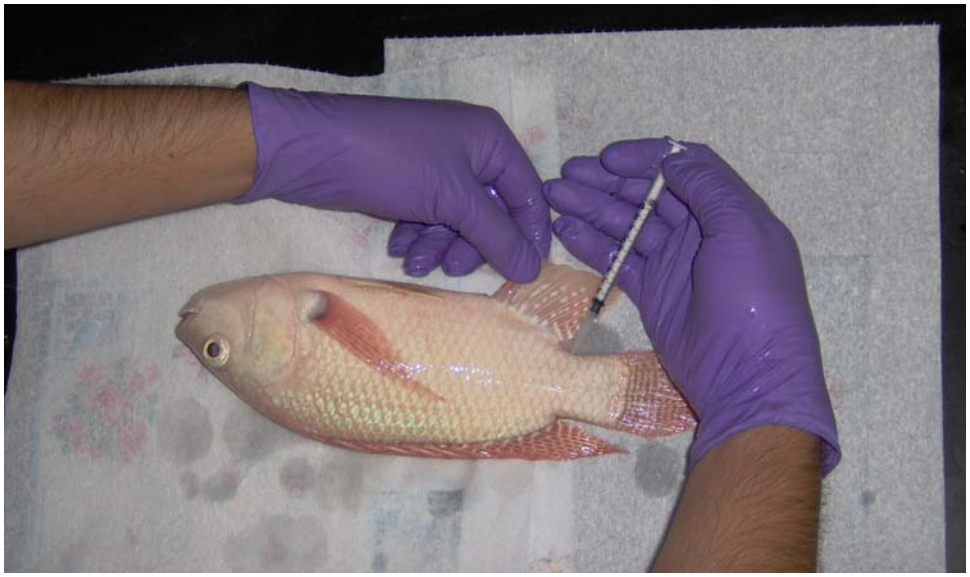

Figure A.6 Extraction of blood by caudal puncture to a Nile tilapia.

SOP - 7 Propidium iodide solution

Materials

- Propidium iodide (PI) [FW = 668.41] (Sigma Chemical Co. St. Louis, Missouri)
- Sodium citrate [FW = 294.11] (EM Science Inc., Gibbstown, New Jersey)
- Triton X-100 (Mallinckrodt Specialty Chemicals Co., Paris, Kentucky)
- RNase solution (SOP - 7)
- Deionized water
- 200-ml glass bottle
- 1-ml disposable syringe (Becton Dickinson Co. Franklin Lakes, New Jersey)
- 18-ga needle(Becton Dickinson Co.)
- Aluminum foil
- 35 µl Male Nile tilapia blood (SOP-6)

Procedures

1. Wrap the glass bottle with aluminum foil.
2. Mix in the bottle 100 ml of deionized water, 5 mg of PI, 112 mg of sodium citrate, 0.1 ml of Triton X-100, and 100 µl of RNase solution. (Triton X-100 is viscous, so dispense with the syringe).
3. The solution can be stored for 24 h with refrigeration and in the dark.
4. For the sample preparation, mix 100 µl of oyster hemolymph, 35 µl of male Nile tilapia blood (SOP-6) and 500 µl of PI solution.
5. Store samples in the dark for 15 min before analysis by flow cytometry.

SOP - 8 RNase solution

Materials

- Tris (hydroxymethyl) aminomethane HCL [FW = 157.6] (Life Technologies, Grand Island, New York)
- Sodium acetate [FW = 82.026] (Sigma Chemical Co. St. Louis, Missouri)
- RNase from bovine pancrease (Sigma Chemical Co.)
- Deionized water
- 1.5-ml microcentrifuge plastic vials (Dot Scientific Inc. Lippincott Burton, Michigan)

Procedures

1. Dissolve 1.58 g of Tris in 10 ml of deionized water.
2. Dissolve 0.082 g of sodium acetate into 100 ml of deionized water.
3. Dissolve 12.5 mg of RNase into 100 ml of the sodium acetate solution.
4. Placed the RNase-sodium mixture into a heated water bath at 100^0C for 15 min. Time the boiling from when the contents begin to bubble.
5. Cool to room temperature.
6. Adjust the pH to 7.4 by adding small volumes (~1.6 ml) of Tris.
7. Dispense into aliquots of 10 µl into sterile microcentrifuge tubes.
8. Store at -20^0C.

SOP - 9 Operation of the flowcytometer

Materials

- Flow cytometer (FACSCalibur, BD Biosciences, San Jose, California)
- Sheath fluid (BD FACSFlow, BD Biosciences, San Jose, California)
- CellQuest software version 1.0 (BD Biosciences, San Jose, California)
- Personal computer (Power Mac G4, Apple Computer Inc. Cuperino, California)
- Commercial Bleach
- 5-ml Polystyrene round-bottom test tubes (BD Biosciences, Bedford, Massachusetts)

Procedures

Startup of the equipment

1. In this order, turn on the flow cytometer and computer.
2. Depressurized the waste and sheath tanks.
3. Empty the waste reservoir tank and add ~200 ml of bleach and replace in the instrument.
4. Fill the sheath tank (1/2 in below the upper limit) and replace in the instrument.
5. Pressurized the waste and sheath tanks.
6. Purge air bubbles from the sheet filter.
7. Placed a test tube with 1 ml of fresh DI water on the sip tube and prime the flow cytometer.
8. Allow the flow cytometer to warm up for 5 min.

Operating the software

1. Click on the apple icon in the upper left corner of the desktop and launch CellQuest.
2. Go to “acquire” menu in the toolbar and select “connect to cytometer”.
3. Go to “acquire” menu and select “parameter description”
4. Go to “acquire” menu and select “counters”
5. In the parameter description window select or create the file and folder were the data is going to be stored.
6. Go to “plot’ menu in the toolbar and select “histogram plot”.
7. In the “plot” menu go to “plot source” and select “acquisition analysis”.
8. In the “plot” menu go to “parameter” and select “FL2A” (for DNA fluorescence in red spectrum).
9. Go to “cytometer” menu in the toolbar and select “detector/amps”
10. In the “detector/amps” window go to “mode” and select “LIN” (Mode = LIN).
11. In the “detector/amps” window go to “DDM” and select parameter “FL2”.
12. In the “detector/amps” and set up the voltage for FL2 at 511, this will produced a peak for the tilapia sample at the 400 channel and for a diploid oyster at the 200 channel.
13. Put the sample in the machine.
14. Click the acquire button in the “acquisition control” window to analyze the sample, make sure the setup box is unclick in order to save the data.

SOP - 10 Estradiol stock solutions

Materials

- β-estradiol 98% [FW = 272.39] (Sigma Chemical Co. St. Louis, Missouri)
- Dimethyl sulfoxide (DMSO) [FW = 78.13] (Fisher Scientific, Fair Lawn, New Jersey)
- Filtered sea water (0.45 μm)
- 25-ml glass bottle with cap

Procedure

Control

1. Filtered sea water with a concentration of 0.1 % of DMSO.

Low dose solution

1. Weigh 5 mg of β-estradiol and add to a glass bottle.
2. Add 1.6 ml of DMSO.
3. Dilute 1:1000 with 0.45-μm filtered sea water to obtained final concentration of 3125 ng/ml.

High dose solution

1. Weigh 10 mg of β-estradiol and add to a glass bottle.
2. Add 1.6 ml of DMSO.
3. Dilute 1:1000 with 0.45-μm filtered sea water to obtained final concentration of 6250 ng/ml.

Table A.1 Injection volumes of estradiol-17β

Shell height (mm)	Estimated body average weight* (g)	Volume injected (ml)
70-80	13.6	0.15
81-90	17.0	0.20
91-100	20.4	0.25
101-110	23.6	0.30
111-120	26.9	0.35
121-130	30.2	0.40
131-140	33.5	0.45
141-150	36.8	0.50

*Regression shown in Chapter 4

SOP – 11 Oocyte area measurements

Materials

- Digital scanner (Epson Perfection 1640SU, Epson America Inc. Long Beach, California)
- Histological slide of an oyster transverse section
- Personal computer (Dell Precision workstation 360, Dell Inc. Austin, Texas)
- Image analysis software Scion Image Beta 4.02
- Compound microscope (Nikon Photoshot, Tokyo, Japan)
- Microscope digital camera (Costar CV 730, Southern Imaging Inc. Carrollton, Texas)

Procedures

1. Place the histological slide in the microscope; take a photomicrograph of a gonad section at 100x magnification. Save the images as a TIF file.
2. Open the program Scion Image and import the scanned image.
3. Set scale to 1.115 pixels/micrometers.
4. From the toolbar open the "map" option and adjust the contrast using the sliding scale with B or C on it. Adjust until the oocytes appear darker and more crisp then the surroundings (work with the black and white picture while comparing to original picture).
5. Go to the "analyze" menu and select "options".
6. Select "measurement of area", include interior holes, and auto-measure.
7. From the toolbar menu select the "wand" icon and start selecting the oocytes to be measure by clicking them. Make sure a measurement number appears in the middle of the oocyte to ensure that you measured only the oocyte (measurements should be around 600-1400 μm).
8. Select 50 oocytes from the whole picture. The criteria to select oocytes are circular oocyte shape, clear germinal vesicle, and a visible nucleolus.

APPENDIX B
UNANALYZED DATA

Table B.1 Gonad-to-body ratio (GBR) and time spend by the different methods (Chapter 2).

Transect method GBR	Time (min)	Total area GBR	Time (min)	Area w/o gills GBR	Time (min)	Area w/o curvature GBR	Time (min)	*Gonad stage
0.32707	13.13	0.39687	2.65	0.41910	2.10	0.34794	1.73	V
0.33084	14.80	0.37277	3.45	0.39906	2.92	0.33189	2.08	V
0.12717	18.28	0.20688	4.33	0.21268	3.67	0.14455	2.25	V
0.05255	17.93	0.04670	4.03	0.09839	3.92	0.06585	2.98	III
0.12235	17.78	0.16954	3.25	0.18931	2.65	0.13939	1.92	VI
0.34323	17.70	0.42721	3.42	0.43395	3.03	0.33269	2.07	V
0.17497	17.42	0.23538	3.10	0.25238	2.92	0.18537	2.15	V
0.24746	13.03	0.20437	2.13	0.21253	1.98	0.20041	1.02	V
0.21854	17.33	0.27090	1.73	0.29351	1.62	0.21525	0.88	V
0.20463	16.47	0.19539	2.05	0.21210	2.12	0.22651	0.85	V
0.28585	14.65	0.33620	3.03	0.34936	2.88	0.28540	1.75	V
0.29713	13.48	0.27124	2.67	0.31567	2.02	0.27385	0.88	V
0.15792	15.52	0.20105	3.95	0.21233	3.17	0.19979	2.58	VI
0.20547	17.58	0.23896	2.38	0.24756	1.83	0.20547	0.73	V
0.21742	17.47	0.30759	3.28	0.32371	3.05	0.22672	2.25	V
0.19011	17.82	0.23807	3.62	0.24804	3.60	0.24211	1.63	V
0.26281	14.27	0.35842	1.47	0.37983	1.38	0.34676	0.60	V
0.20988	19.27	0.18563	1.35	0.20128	1.18	0.15847	0.63	IV
0.31618	15.07	0.35764	1.67	0.38909	1.48	0.36488	0.60	V
0.38128	13.30	0.41484	1.20	0.45036	0.97	0.36493	0.45	V
0.38558	13.45	0.41603	0.95	0.43944	0.90	0.36250	0.47	V
0.22801	14.47	0.28766	3.75	0.30829	3.40	0.21231	1.38	V
0.19435	15.28	0.23028	3.25	0.25142	2.95	0.18616	1.62	V
0.28214	13.47	0.34622	3.50	0.36391	3.12	0.25170	1.78	V
0.11891	17.72	0.08882	5.22	0.09196	4.83	0.06683	2.20	III
0.32114	15.07	0.30315	3.68	0.33282	3.88	0.34131	2.23	V
0.29337	14.72	0.23128	5.82	0.25306	4.92	0.23971	2.95	V
0.18577	17.70	0.17816	3.55	0.17874	3.48	0.17259	2.28	V
0.27717	14.33	0.37791	3.88	0.41760	3.35	0.28044	1.97	V
0.22673	16.48	0.29356	3.32	0.30501	3.20	0.23278	1.98	V
0.12286	17.27	0.18312	3.78	0.18988	2.45	0.13939	1.45	IV
0.27079	13.27	0.32560	4.40	0.32131	3.25	0.30944	1.47	V
0.07157	13.88	0.09903	4.08	0.10423	3.48	0.07805	1.87	III
0.17012	16.98	0.17476	4.10	0.18559	3.47	0.15796	1.85	VI
0.19770	13.75	0.23757	3.92	0.27919	3.27	0.25424	1.48	V
0.12003	12.00	0.08141	2.17	0.09081	1.85	0.08481	0.90	III
0.14462	11.47	0.20756	4.27	0.22436	3.55	0.16835	1.20	V
0.24728	11.12	0.28528	1.68	0.29357	1.72	0.29273	1.03	V
0.25887	13.72	0.24227	3.15	0.26553	2.72	0.26090	2.17	IV
0.08217	12.00	0.10345	1.35	0.11068	1.18	0.11170	0.60	VI
0.18790	11.42	0.16127	2.05	0.20988	1.70	0.17014	1.03	VI
0.04780	13.88	0.04866	4.38	0.06328	3.92	0.05012	3.00	III
0.10200	12.85	0.13473	3.15	0.16932	2.73	0.09645	2.02	IV

Table B.1 continued

0.12510	11.45	0.10880	2.73	0.15370	2.28	0.11436	1.85	IV
0.22146	14.27	0.24873	3.68	0.27551	3.08	0.21453	2.62	V
0.16987	12.78	0.16662	3.37	0.21330	2.98	0.17153	2.20	VI
0.23015	11.90	0.25300	2.73	0.27335	2.27	0.22438	1.78	V
0.19485	13.37	0.20037	3.52	0.22096	2.92	0.19531	2.38	IV
0.21037	11.75	0.19520	2.33	0.22291	1.93	0.21317	1.62	IV
0.21632	10.00	0.20510	2.07	0.24688	1.55	0.21938	0.63	V

III: early development, IV: late development, V: spawning, VI: advanced spawning and regression

Table B.2 Unanalyzed data from diploid oysters evaluated in August 2003 and May and August 2004 (Chapter 3).

Oyster No	Ploidy	Stage*	GBR**	Oyster No	Ploidy	Stage*	GBR**
1	2N	I	0.057	43	2N	V	0.22214
2	2N	I	0.1009	44	2N	V	0.23715
3	2N	I	0.1411	45	2N	V	0.24189
4	2N	I	0.0187	46	2N	V	0.24366
5	2N	I	0.0519	47	2N	V	0.24620
6	2N	I	0.0701	48	2N	V	0.26969
7	2N	III	0.06941	49	2N	V	0.27353
8	2N	III	0.07599	50	2N	V	0.28000
9	2N	III	0.0956	51	2N	V	0.28205
10	2N	III	0.1665	52	2N	V	0.28640
11	2N	III	0.1864	53	2N	V	0.29681
12	2N	IV	0.10938	54	2N	V	0.29934
13	2N	IV	0.30354	55	2N	V	0.30023
14	2N	IV	0.25777	56	2N	V	0.30413
15	2N	IV	0.22772	57	2N	V	0.30970
16	2N	IV	0.11324	58	2N	V	0.31452
17	2N	IV	0.2727	59	2N	V	0.31165
18	2N	IV	0.3025	60	2N	V	0.31855
19	2N	IV	0.1143	61	2N	V	0.31922
20	2N	IV	0.1349	62	2N	V	0.32094
21	2N	IV	0.1511	63	2N	V	0.32385
22	2N	IV	0.2111	64	2N	V	0.32719
23	2N	IV	0.2204	65	2N	V	0.33063
24	2N	IV	0.2480	66	2N	V	0.33202
25	2N	IV	0.2568	67	2N	V	0.33465
26	2N	IV	0.2588	68	2N	V	0.33907
27	2N	IV	0.2652	69	2N	V	0.34306
28	2N	IV	0.2874	70	2N	V	0.34735
29	2N	IV	0.3211	71	2N	V	0.34869
30	2N	IV	0.134439	72	2N	V	0.34987
31	2N	IV	0.134883	73	2N	V	0.35232
32	2N	IV	0.167395	74	2N	V	0.35451
33	2N	IV	0.181063	75	2N	V	0.35595
34	2N	IV	0.234926	76	2N	V	0.35773
35	2N	IV	0.150976	77	2N	V	0.35954
36	2N	IV	0.140386	78	2N	V	0.36108
37	2N	IV	0.184481	79	2N	V	0.36498
38	2N	IV	0.213272	80	2N	V	0.36700

Table B.2 Continued

39	2N	IV	0.303985	81	2N	V	0.36927
40	2N	IV	0.302075	82	2N	V	0.37163
41	2N	IV	0.190676	83	2N	V	0.37386
85	2N	V	0.38154	130	2N	V	0.34474
86	2N	V	0.38227	131	2N	V	0.36474
87	2N	V	0.38739	132	2N	V	0.36502
88	2N	V	0.38770	133	2N	V	0.36780
89	2N	V	0.39244	134	2N	V	0.36956
90	2N	V	0.39569	135	2N	V	0.40724
91	2N	V	0.39990	136	2N	V	0.42736
92	2N	V	0.41301	137	2N	V	0.49471
93	2N	V	0.41373	138	2N	V	0.50900
94	2N	V	0.41447	139	2N	V	0.53622
95	2N	V	0.41617	140	2N	V	0.22832
96	2N	V	0.42579	141	2N	V	0.21328
97	2N	V	0.42680	142	2N	V	0.21226
98	2N	V	0.43081	143	2N	V	0.23658
99	2N	V	0.43646	144	2N	V	0.23435
100	2N	V	0.43654	145	2N	V	0.21883
101	2N	V	0.44513	146	2N	V	0.20509
102	2N	V	0.46094	147	2N	V	0.20421
103	2N	V	0.47324	148	2N	V	0.21640
104	2N	V	0.47365	149	2N	V	0.26257
105	2N	V	0.20415	150	2N	V	0.28310
106	2N	V	0.28770	151	2N	V	0.27333
107	2N	V	0.33549	152	2N	V	0.29648
108	2N	V	0.32097	153	2N	V	0.25535
109	2N	V	0.27292	154	2N	V	0.30080
110	2N	V	0.44507	155	2N	V	0.33657
111	2N	V	0.27485	156	2N	V	0.26017
112	2N	V	0.35748	157	2N	V	0.33453
113	2N	V	0.23768	158	2N	V	0.23652
114	2N	V	0.36314	159	2N	V	0.38796
115	2N	V	0.29069	160	2N	V	0.25226
116	2N	V	0.44201	161	2N	V	0.36704
117	2N	V	0.32083	162	2N	V	0.25219
118	2N	V	0.44538	163	2N	V	0.26176
119	2N	V	0.21499	164	2N	V	0.28867
120	2N	V	0.24487	165	2N	V	0.39924
121	2N	V	0.25621	166	2N	V	0.29847
122	2N	V	0.28083	167	2N	V	0.32776
123	2N	V	0.30104	168	2N	V	0.21709
124	2N	V	0.30280	169	2N	V	0.22176
125	2N	V	0.32069	170	2N	V	0.28545
126	2N	V	0.32671	171	2N	V	0.20266
127	2N	V	0.33129	172	2N	V	0.33864
128	2N	V	0.34171	173	2N	V	0.25092
129	2N	V	0.34239	174	2N	V	0.29890
175	2N	V	0.22100	195	2N	VI	0.19509
176	2N	V	0.34171	196	2N	VI	0.16618
177	2N	V	0.38154	197	2N	VI	0.19801
178	2N	V	0.25092	198	2N	VI	0.19920
179	2N	V	0.34401	199	2N	VI	0.27137
180	2N	V	0.25795	200	2N	VI	0.05633

Table B.2 Continued

181	2N	V	0.28650	201	2N	VI	0.07074
182	2N	V	0.25999	202	2N	VI	0.10570
183	2N	V	0.27197	203	2N	VI	0.20845
184	2N	V	0.25742	204	2N	VI	0.22065
185	2N	V	0.31553	205	2N	VI	0.20966
186	2N	V	0.29290	206	2N	VI	0.21149
187	2N	V	0.44135	207	2N	VI	0.22452
188	2N	VI	0.05128	208	2N	VI	0.10276
189	2N	VI	0.07953	209	2N	VI	0.11440
190	2N	VI	0.08720	210	2N	VI	0.16387
191	2N	VI	0.09193	211	2N	VI	0.20063
192	2N	VI	0.09541	212	2N	VI	0.07130
193	2N	VI	0.10966	213	2N	VI	0.13331
194	2N	VI	0.18462	214	2N	VI	0.18139

*Gonadal stage, **Gonad-to-Body Ratio

Table B.3 Unanalyzed data from triploid oysters evaluated in August 2003 and May and August 2004 (Chapter 3).

Oyster No	Ploidy	*Stage	**GBR	Oyster No	Ploidy	*Stage	**GBR
1	3N	I	0.025051	43	3N	II	0.051636
2	3N	I	0.020314	44	3N	II	0.051638
3	3N	I	0.029374	45	3N	II	0.052265
4	3N	I	0.042325	46	3N	II	0.052301
5	3N	I	0.043852	47	3N	II	0.052301
6	3N	I	0.044111	48	3N	II	0.052754
7	3N	I	0.04745	49	3N	II	0.053064
8	3N	I	0.048612	50	3N	II	0.053286
9	3N	I	0.05662	51	3N	II	0.053538
10	3N	I	0.062612	52	3N	II	0.053575
11	3N	I	0.063107	53	3N	II	0.05376
12	3N	I	0.063456	54	3N	II	0.053798
13	3N	I	0.067978	55	3N	II	0.05432
14	3N	I	0.071697	56	3N	II	0.055111
15	3N	I	0.073855	57	3N	II	0.055653
16	3N	I	0.074918	58	3N	II	0.055691
17	3N	I	0.100562	59	3N	II	0.055941
18	3N	I	0.104087	60	3N	II	0.056488
19	3N	I	0.116591	61	3N	II	0.057242
20	3N	I	0.124176	62	3N	II	0.057334
21	3N	I	0.140743	63	3N	II	0.057539
22	3N	II	0.015798	64	3N	II	0.057572
23	3N	II	0.031618	65	3N	II	0.057621
24	3N	II	0.032131	66	3N	II	0.057809
25	3N	II	0.034425	67	3N	II	0.057838
26	3N	II	0.034721	68	3N	II	0.058411
27	3N	II	0.036009	69	3N	II	0.05869
28	3N	II	0.036581	70	3N	II	0.058811
29	3N	II	0.036962	71	3N	II	0.059192
30	3N	II	0.037264	72	3N	II	0.059209
31	3N	II	0.037271	73	3N	II	0.059686
32	3N	II	0.037419	74	3N	II	0.060214
33	3N	II	0.037604	75	3N	II	0.060829

Table B.3 continued

34	3N	II	0.037636	76	3N	II	0.061026
35	3N	II	0.038392	77	3N	II	0.061191
36	3N	II	0.038967	78	3N	II	0.061248
37	3N	II	0.04036	79	3N	II	0.061316
38	3N	II	0.042356	80	3N	II	0.061837
39	3N	II	0.044167	81	3N	II	0.062297
40	3N	II	0.045133	82	3N	II	0.062594
41	3N	II	0.045697	83	3N	II	0.062881
42	3N	II	0.046754	84	3N	II	0.063413
85	3N	II	0.065225	129	3N	II	0.098545
86	3N	II	0.065715	130	3N	II	0.099146
87	3N	II	0.066373	131	3N	II	0.100146
88	3N	II	0.066809	132	3N	II	0.100166
89	3N	II	0.067539	133	3N	II	0.101227
90	3N	II	0.067914	134	3N	II	0.103184
91	3N	II	0.068182	135	3N	II	0.105282
92	3N	II	0.069724	136	3N	II	0.110816
93	3N	II	0.070049	137	3N	II	0.111294
94	3N	II	0.072877	138	3N	II	0.112359
95	3N	II	0.07434	139	3N	II	0.116661
96	3N	II	0.074359	140	3N	II	0.117851
97	3N	II	0.075784	141	3N	II	0.126032
98	3N	II	0.075844	142	3N	II	0.128878
99	3N	II	0.076021	143	3N	II	0.132016
100	3N	II	0.076248	144	3N	II	0.132355
101	3N	II	0.076657	145	3N	II	0.13247
102	3N	II	0.077361	146	3N	II	0.133313
103	3N	II	0.077597	147	3N	II	0.14409
104	3N	II	0.077921	148	3N	II	0.147425
105	3N	II	0.079141	149	3N	II	0.164098
106	3N	II	0.079261	150	3N	II	0.172512
107	3N	II	0.081028	151	3N	II	0.173714
108	3N	II	0.081416	152	3N	III	0.070565
109	3N	II	0.081452	153	3N	III	0.037327
110	3N	II	0.08267	154	3N	III	0.040641
111	3N	II	0.084714	155	3N	III	0.045375
112	3N	II	0.08484	156	3N	III	0.049265
113	3N	II	0.085273	157	3N	III	0.053193
114	3N	II	0.085944	158	3N	III	0.056274
115	3N	II	0.086717	159	3N	III	0.058425
116	3N	II	0.086886	160	3N	III	0.068509
117	3N	II	0.086941	161	3N	III	0.074274
118	3N	II	0.087862	162	3N	III	0.074741
119	3N	II	0.088357	163	3N	III	0.076952
120	3N	II	0.0889	164	3N	III	0.080504
121	3N	II	0.090999	165	3N	III	0.085895
122	3N	II	0.091317	166	3N	III	0.085922
123	3N	II	0.091346	167	3N	III	0.09406
124	3N	II	0.093255	168	3N	III	0.104879
125	3N	II	0.093778	169	3N	III	0.107642
126	3N	II	0.094047	170	3N	III	0.109398
127	3N	II	0.094124	171	3N	III	0.120121
128	3N	II	0.09454	172	3N	III	0.14185

Table B.4 Unanalyzed data from initial evaluation of the experiment conducted in August 2003 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
1	Initial	3N	113	16.23	I	0.0690	I	N/D
2	Initial	3N	95.2	14.73	I	0.0744	I	N/D
3	Initial	3N	98	11.78	F	0.0772	III	N/D
4	Initial	3N	99	12.4	I	0.0845	I	N/D
1	Initial	2N	87.5	8.96	F	0.1904	IV	N/D
2	Initial	2N	94	12.07	F	0.4462	V	N/D
3	Initial	2N	75	5.56	F	0.1621	VI	N/D
4	Initial	2N	87.2	11.23	F	0.4061	V	N/D
5	Initial	2N	92.5	7.35	F	0.1721	IV	N/D
1	Initial	2N*	95	13.24	F	0.2466	V	N/D
2	Initial	2N*	91	13.27	F	0.4109	V	N/D
3	Initial	2N*	96.2	7.47	F	0.1891	VI	N/D
4	Initial	2N*	90	8.65	F	0.2593	V	N/D
5	Initial	2N*	72.1	9.83	F	0.3221	V	N/D

*Diploid oysters not exposed to cytochalasin B

Table B.5 Unanalyzed data from triploid oysters evaluated in August 2003 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
A1	Control	3N	97.2	8.28	F	0.12887	III	116.268
A5	Control	3N	92.2	10.89	F	0.06229	III	37.171
A7	Control	3N	101.2	13.66	F	0.08671	III	33.663
A8	Control	3N	73	10.89	F	0.05841	III	16.327
A9	Control	3N	92.4	10.85	F	0.05808	VI	13.988
A10	Control	3N	101	9.38	F	0.07287	III	30.142
A15	Control	3N	92	21.25	F	0.05920	III	24.607
A16	Control	3N	98	14.73	F	0.08589	III	24.969
A18	Control	3N	85.6	14.8	F	0.05226	III	28.042
A21	Control	3N	112.4	15.63	F	0.07178	VI	25.062
A22	Control	3N	87.2	9.88	F	0.03161	III	27.939
A24	Control	3N	109.1	16.61	F	0.07665	III	32.647
A26	Control	3N	93.5	10.15	F	0.07759	III	14.786
A28	Control	3N	85.7	10.13	F	0.12012	III	17.162
A32	Control	3N	95.5	14.93	F	0.05724	III	20.928
A33	Control	3N	83.2	11.39	F	0.09406	III	21.309
A34	Control	3N	91.4	12.37	F	0.05511	III	23.714
A36	Control	3N	92	17.42	F	0.04235	III	20.142
A37	Control	3N	83	11.6	F	0.06588	VI	21.568
A39	Control	3N	100	6.5	I	0.02505	I	19.258
B4	Control	3N	93	12.77	I	0.04745	I	21.854
B8	Low	3N	88	8.57	F	0.10487	III	N/D
B9	Low	3N	96	19.77	F	0.05662	I	N/D
B11	Low	3N	89.6	12.93	F	0.07434	III	N/D
B13	Low	3N	80	7.74	F	0.05753	III	N/D

Table B.5 Continued

B14	Low	3N	88.6	11.39	F	0.08484	III	N/D
B19	Low	3N	84.7	10.4	F	0.07101	IV	N/D
B20	Low	3N	82.4	13.58	F	0.06962	VI	N/D
B21	Low	3N	94.6	NW	F	0.05641	VI	N/D
B23	Low	3N	86	13.53	F	0.04775	III	N/D
B24	Low	3N	99	15.14	F	0.05033	VI	N/D
B29	Low	3N	91.2	9.19	I	0.06310	I	N/D
B34	Low	3N	106.1	9.99	I	0.04385	I	N/D
B35	Low	3N	98.5	15.78	F	0.04416	III	N/D
B38	Low	3N	115.2	13.96	F	0.04234	VI	N/D
C3	Low	3N	83.5	8.4	F	0.05115	III	N/D
C5	Low	3N	95.5	17.08	F	0.07792	III	N/D
C8	Low	3N	88.7	12.31	F	0.05919	III	N/D
C13	Low	3N	87	8.79	F	0.35885	V	N/D
C14	Low	3N	92	13.32	F	0.03760	III	N/D
C15	High	3N	90	7	F	0.09099	III	N/D
C16	High	3N	84.8	15.36	F	0.08102	III	N/D
C17	High	3N	110	16.59	F	0.06424	VI	N/D
C18	High	3N	80	10.75	F	0.05757	III	N/D
C21	High	3N	96.5	11.86	F	0.04794	VI	N/D
C23	High	3N	91.4	10.74	F	0.04569	III	N/D
C25	High	3N	89.6	9.76	F	0.05306	III	N/D
C27	High	3N	91.4	8.76	F	0.13235	III	N/D
C28	High	3N	109.3	9.97	F	0.01579	III	N/D
C32	High	3N	91.6	11.67	F	0.06637	III	N/D
C37	High	3N	93	11.01	F	0.05968	III	N/D
C38	High	3N	88.5	17.28	F	0.06522	III	N/D
C39	High	3N	84.9	10.37	F	0.00043	III	N/D
C40	High	3N	85.7	14.91	F	0.05869	III	N/D
D1	High	3N	83.2	9.89	F	0.07056	III	N/D
D3	High	3N	90	11.04	F	0.03896	III	N/D
D5	High	3N	92.4	15.91	F	0.05230	III	N/D
D6	High	3N	101.3	12.66	F	0.05353	III	N/D
D7	High	3N	92.4	10.4	F	0.05163	III	N/D
D9	High	3N	93	18.56	F	0.03600	III	N/D
D11	High	3N	91.6	12.97	F	0.08786	III	N/D
D12	High	3N	106.5	16.97	F	0.10460	IV	N/D
D13	High	3N	82	12.57	F	0.08319	VI	N/D

Table B.6 Unanalyzed data from diploid oysters evaluated in August 2003 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
A2	Control	2N	85.5	9.38	F	0.43081	V	35.641
A12	Control	2N	90	9.85	F	0.45543	V	22.764
A13	Control	2N	96	9.49	F	0.33907	V	77.961
A14	Control	2N	92.5	7.58	F	0.43646	V	20.177
A19	Control	2N	74.8	7.63	F	N/D	V	44.05

Table B.6 Continued

A23	Control	2N	88.8	8.27	F	0.44512	V	25.441
A29	Control	2N	90	11.35	F	0.36927	V	10.476
A31	Control	2N	97.6	2.3	F	0.34869	V	41.491
A38	Control	2N	78.6	7.93	F	0.47365	V	28.401
B1	Control	2N	78.2	8.52	F	0.36497	V	16.918
B3	Control	2N	98.1	11.47	F	0.35451	V	20.548
B6	Control	2N	86.4	6.5	F	0.30412	V	20.412
B15	Control	2N	112.5	10.98	F	0.23727	V	14.68
B17	Control	2N	119	7.79	F	0.31922	V	22.191
B18	Control	2N	89.1	8.74	F	0.39243	V	31.649
B25	Control	2N	85.1	10.99	F	0.43654	V	13.94
B31	Control	2N	88	8.22	F	0.34305	V	36.039
B32	Low	2N	88.2	11.52	F	0.19919	V	N/D
B33	Low	2N	85.5	12.12	F	0.33063	V	N/D
B37	Low	2N	95.2	13.58	F	0.39569	V	N/D
B39	Low	2N	92	13.85	F	0.37163	V	N/D
C6	Low	2N	78	11.59	F	0.41446	V	N/D
C9	Low	2N	88.3	6.08	F	0.30353	IV	N/D
C10	Low	2N	92.4	8.88	F	N/D	V	N/D
C12	Low	2N	100.7	14.36	F	0.07953	VI	N/D
C19	Low	2N	88.2	8.3	F	0.06941	III	N/D
C34	Low	2N	85.9	13.55	F	0.33202	V	N/D
C35	Low	2N	100	10.65	F	0.32385	V	N/D
D4	Low	2N	106.1	12.25	F	0.24189	V	N/D
D10	Low	2N	105.1	18.3	F	0.07598	III	N/D
D16	Low	2N	92.3	9.92	F	0.42579	V	N/D
D17	Low	2N	92.8	9.96	F	0.36108	V	N/D
D19	Low	2N	93.1	13.66	F	0.27352	V	N/D
D23	High	2N	91.3	13.04	F	0.26968	V	N/D
D26	High	2N	91	10.65	F	0.47324	V	N/D
D31	High	2N	95.7	17.21	F	0.08719	VI	N/D
D32	High	2N	100.7	9.2	F	0.09193	VI	N/D
D34	High	2N	100.7	9.27	F	0.46094	V	N/D
E1	High	2N	99.4	9.91	F	0.31451	V	N/D
E2	High	2N	105.4	10.53	F	0.37453	V	N/D
E4	High	2N	105.5	11.53	F	0.31165	V	N/D
E9	High	2N	93	8.64	F	0.35595	V	N/D
E10	High	2N	90	8.77	F	0.32093	V	N/D
E11	High	2N	91.5	9.76	F	0.34987	V	N/D
E12	High	2N	82	5.8	F	0.11016	I	N/D
E14	High	2N	108.4	10.83	F	0.38738	V	N/D
E17	High	2N	102.3	8.32	F	0.05128	VI	N/D
E23	High	2N	84.2	11.22	F	0.24619	V	N/D
E33	High	2N	85.1	9.91	F	0.38769	V	N/D
E34	High	2N	105	11.25	F	0.42680	V	N/D

Table B.7 Unanalyzed data from diploid oysters not exposed to cytochalasin B, evaluated in August 2003 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
G1	Control	2N	92	8.42	F	0.13350	VI	N/D
G2	Control	2N	98	14.64	F	0.11323	IV	N/D
G4	Control	2N	87	13.07	F	0.33465	V	N/D
G5	Control	2N	107	20.07	F	0.12928	III	N/D
G6	Control	2N	92	14.72	F	0.10937	IV	N/D
G7	Control	2N	90	9.96	F	0.35953	V	N/D
G9	Control	2N	86	8.12	F	0.32718	V	N/D
G11	Control	2N	98	14.21	F	0.38227	V	N/D
G12	Control	2N	89	12.19	F	0.29681	V	N/D
G14	Control	2N	109	15.74	F	0.37385	V	N/D
G17	Control	2N	106	10.19	F	0.36699	V	N/D
G18	Control	2N	88	10.13	F	0.41301	V	N/D
G20	Control	2N	106	19.04	F	0.39990	V	N/D
G21	Control	2N	90	9.24	F	0.28640	V	N/D
G31	Low	2N	92	10.71	F	0.30022	V	N/D
G33	Low	2N	82	11.9	F	0.34735	V	N/D
G34	Low	2N	110	21.39	F	0.23715	V	N/D
G35	Low	2N	94	15.77	F	0.29933	V	N/D
G36	Low	2N	101	8.81	F	0.30970	V	N/D
G39	Low	2N	86	10.18	F	0.35773	V	N/D
G40	Low	2N	87	13.9	F	0.22772	IV	N/D
G41	Low	2N	98	13.35	F	0.27137	VI	N/D
G42	Low	2N	99	11.26	F	0.19801	VI	N/D
G43	Low	2N	89	10.22	F	0.31855	V	N/D
G44	Low	2N	108	16.73	F	0.19508	VI	N/D
G46	Low	2N	100	14.94	F	0.22100	V	N/D
G47	Low	2N	106	17.34	F	0.09540	VI	N/D
G48	Low	2N	97	13.8	F	0.27999	V	N/D
G51	Low	2N	103	14.59	F	0.41372	V	N/D
G52	Low	2N	99	12.32	F	0.16618	VI	N/D
H5	High	2N	82	14.3	I	0.05680	I	N/D
H6	High	2N	111	17.62	F	0.22214	V	N/D
H7	High	2N	98	15.6	F	0.25776	IV	N/D
H10	High	2N	107	18.8	F	0.18462	VI	N/D
H13	High	2N	82	7.33	F	0.20047	V	N/D
H14	High	2N	111	12.87	F	0.35232	V	N/D
H15	High	2N	95	8.16	F	0.38154	V	N/D
H16	High	2N	101	17.67	F	0.41616	V	N/D
H17	High	2N	90	6.02	F	0.10965	VI	N/D
H19	High	2N	96	13.09	F	0.28205	V	N/D
H27	High	2N	87	11.5	F	0.24366	V	N/D

Table B.8 Unanalyzed data from initial evaluation of the experiment conducted in May 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
1	Initial	3N	110	23.05	F	0.08978	III	16.545
2	Initial	3N	96	26.39	F	0.17188	III	20.563
4	Initial	3N	99	15.64	F	0.04427	III	15.583
5	Initial	3N	113	22.47	F	0.08318	III	19.542
6	Initial	3N	107	18.18	F	0.04424	III	10.641
9	Initial	3N	105	22.54	I	0.03009	I	12.928
10	Initial	3N	126	30.5	F	0.09166	III	23.806
12	Initial	3N	116	20.53	F	0.07954	III	26.193
13	Initial	3N	88	13.7	F	0.08847	III	10.915
14	Initial	3N	112	26.22	F	0.07544	III	N/D
15	Initial	3N	121	30	F	0.08470	III	14.692
17	Initial	3N	95	16.24	F	0.11432	III	16849
18	Initial	3N	112	17.1	F	0.16830	III	13.581
20	Initial	3N	116	15.01	I	N/D	I	16.583
1	Initial	2N	95	11.23	F	0.07548	III	11.911
2	Initial	2N	83	12.96	F	0.18083	VI	12.091
3	Initial	2N	89	14.64	F	0.21936	VI	12.091
5	Initial	2N	103	14.66	F	0.16854	VI	18.783
6	Initial	2N	91	16.12	F	0.35018	VI	22.523
7	Initial	2N	94	11.4	I	0.03430	I	14.474
8	Initial	2N	107	14.04	F	0.16397	VI	20.263
10	Initial	2N	122	28.83	F	0.33311	VI	14.996
11	Initial	2N	93	14.07	F	0.46815	V	25.495
12	Initial	2N	99	18.44	F	0.12192	VI	13.448
14	Initial	2N	100	13.66	F	0.30932	VI	12.815
15	Initial	2N	111	25.34	F	0.38954	V	12.383
16	Initial	2N	87	17.32	F	N/D	VI	13.838
17	Initial	2N	101	15.48	F	0.32174	VI	14.277
18	Initial	2N	99	14.5	F	0.37371	VI	13.567
20	Initial	2N	104	12.38	F	0.19794	VI	19.338

Table B.9 Unanalyzed data from triploid oysters evaluated at day 9 of experiment in May 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
F2	Control	3N	115	26.02	F	0.10123	III	27.715
F4	Control	3N	106	28.98	F	0.15868	VI	14.824
F6	Control	3N	118	22.64	F	0.06595	IV	16.104
F8	Control	3N	99	18.81	F	0.06851	III	17.955
F13	Control	3N	112	27.93	F	N/D	III	22.039
F15	Control	3N	115	27.88	F	0.06132	III	16.821
E46	Control	3N	108	29.8	F	N/D	III	42.461
E47	Control	3N	101	19.04	F	0.17251	III	20.018

Table B.9 Continued

E49	Control	3N	119	24.76	F	N/D	III	45.755
E50	Control	3N	99	21.22	F	0.06341	III	17.742
E53	Control	3N	114	14.06	F	N/D	III	239.95
E55	Control	3N	112	23.82	F	N/D	III	36.52
E56	Control	3N	107	25.32	F	0.11236	III	18.475
G1	Low	3N	124	27.92	F	N/D	IV	N/D
G5	Low	3N	106	19.43	F	0.06184	III	N/D
G8	Low	3N	113	20.75	F	0.05936	IV	N/D
G13	Low	3N	112	24.16	F	0.0805	III	N/D
F28	Low	3N	107	24.8	F	N/D	V	N/D
F29	Low	3N	116	29.13	F	0.04064	III	N/D
F31	Low	3N	104	21.23	F	N/D	III	N/D
F39	Low	3N	90	17.9	F	0.06398	III	N/D
F45	Low	3N	89	16.61	F	0.11785	III	N/D
F48	Low	3N	110	24.03	F	0.09405	III	N/D
F54	Low	3N	92	15.43	F	0.05627	III	N/D
A1	High	3N	109	15.7	F	0.09454	III	N/D
A2	High	3N	124	18.83	F	0.14409	III	N/D
A3	High	3N	131	26.27	F	N/D	III	N/D
A4	High	3N	108	12.16	I	0.07492	I	N/D
A5	High	3N	80	15.24	I	N/D	I	N/D
A6	High	3N	133	20.26	I	0.07056	I	N/D
A7	High	3N	118	24.29	I	0.02301	I	N/D
A9	High	3N	118	22.06	F	0.07625	III	N/D
A10	High	3N	109	18.54	I	0.03386	I	N/D
A11	High	3N	109	16.27	F	0.17431	III	N/D
A12	High	3N	96	21.84	F	0.13331	III	N/D
A13	High	3N	104	24.27	F	0.17371	III	N/D
A14	High	3N	122	27.01	F	0.09412	III	N/D
A16	High	3N	126	31.89	F	0.04926	III	N/D
H53	High	3N	137	31.5	F	0.09342	III	N/D
H56	High	3N	119	21.81	F	N/D	III	N/D

Table B.10 Unanalyzed data from triploid oysters evaluated at day 12 of experiment in May 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
E1	Control	3N	121	26.66	I	0.02937	I	16.393
E2	Control	3N	109	21.92	F	0.03764	III	21.059
E6	Control	3N	116	29.29	F	0.05164	III	21.666
E8	Control	3N	124	26.26	F	0.09268	III	16.746
E9	Control	3N	114	28.88	F	0.08592	III	20.916
E12	Control	3N	112	32.19	F	0.03472	III	N/D
E13	Control	3N	106	21.41	F	0.06495	III	12.243
E14	Control	3N	89	14.99	F	0.14743	III	9.613
E17	Control	3N	103	34.43	F	0.13202	III	25.413

Table B.10 Continued

E18	Control	3N	101	16.11	I	0.12418	I	55.302
E21	Control	3N	104	15.85	F	0.04671	III	23.225
E29	Control	3N	115	20.17	F	0.05781	III	334.99
E30	Control	3N	119	25.71	F	N/D	III	42.1
E32	Control	3N	124	24.75	F	0.07695	III	31.076
E37	Control	3N	132	21.44	F	0.05358	III	27.179
G17	Low	3N	88	17.9	F	0.09762	III	N/D
G23	Low	3N	101	20.21	F	0.05784	III	N/D
G24	Low	3N	103	24.89	F	0.05319	III	N/D
G27	Low	3N	116	29.51	F	0.03014	III	N/D
G30	Low	3N	109	18.25	F	0.07427	III	N/D
G35	Low	3N	116	32.09	F	N/D	III	N/D
G44	Low	3N	120	14.75	I	0.11659	I	N/D
G47	Low	3N	108	19.92	F	0.07467	IV	N/D
G51	Low	3N	112	25.29	F	0.03733	III	N/D
G52	Low	3N	107	13.86	I	0.02786	I	N/D
G56	Low	3N	99	21.24	F	0.0538	III	N/D
H4	Low	3N	127	23.04	F	0.05881	III	N/D
H5	Low	3N	119	18.97	F	0.13023	III	N/D
H7	High	3N	112	30.51	F	0.13247	III	N/D
H8	High	3N	99	16.21	I	0.06798	I	N/D
H10	High	3N	111	18.93	F	0.07914	III	N/D
H14	High	3N	132	33.56	F	0.09855	III	N/D
H27	High	3N	118	24.1	F	0.14185	III	N/D
H30	High	3N	110	23.59	F	0.03442	III	N/D
H31	High	3N	111	29.63	F	0.07474	III	N/D
H32	High	3N	113	26.19	I	0.04411	I	N/D
H33	High	3N	123	26.16	I	0.14074	I	N/D
H34	High	3N	125	23.39	F	0.08594	III	N/D
H37	High	3N	113	14.49	F	0.08267	III	N/D
H39	High	3N	97	19.19	F	0.1094	III	N/D
H40	High	3N	93	15.64	I	0.03169	I	N/D
H42	High	3N	127	22.57	F	0.05594	III	N/D
H46	High	3N	117	23.15	F	0.08471	III	N/D
H48	High	3N	116	16.84	F	N/D	III	N/D

Table B.11 Unanalyzed data from diploid oysters evaluated at day 9of the experiment in May 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
F3	Control	2N	83	15.83	F	0.2877	V	52.357
F5	Control	2N	96	25.4	F	N/D	V	34.298
F7	Control	2N	112	17.72	F	N/D	V	31.911
F9	Control	2N	90	19.92	F	0.33549	V	15.779
F10	Control	2N	98	18.52	F	0.44538	V	34.98
F11	Control	2N	88	9.59	I	0.10089	I	13.095
F16	Control	2N	103	27.38	F	N/D	V	30.612

Table B.11 Continued

F17	Control	2N	105	15.82	F	0.32097	V	15.711
F20	Control	2N	93	13.62	F	0.0956	III	38.327
F21	Control	2N	92	10.3	F	0.20845	VI	12.074
E42	Control	2N	101	17.02	F	N/D	V	10.226
E43	Control	2N	100	14.8	F	N/D	V	29.25
E44	Control	2N	110	8.33	F	0.22065	VI	18.853
E45	Control	2N	93	9.01	F	0.05633	VI	28.38
E48	Control	2N	97	17.78	F	0.15015	VI	42.739
G2	Low	2N	105	18.28	F	0.33425	IV	N/D
G3	Low	2N	83	19.9	F	0.27292	V	N/D
F30	Low	2N	87	14.18	F	0.22452	VI	N/D
F34	Low	2N	114	22.96	F	0.16636	V	N/D
F35	Low	2N	96	21.97	F	0.44507	V	N/D
F38	Low	2N	132	26.23	F	0.27485	V	N/D
F40	Low	2N	78	10.33	F	0.35748	V	N/D
F41	Low	2N	117	16.35	F	0.23768	V	N/D
F42	Low	2N	79	9.74	F	0.20966	VI	N/D
F43	Low	2N	83	14.95	F	N/D	V	N/D
F46	Low	2N	121	13.08	F	0.21149	VI	N/D
F47	Low	2N	102	18.15	F	0.07074	VI	N/D
F50	Low	2N	111	27.49	F	N/D	V	N/D
F51	Low	2N	104	24.56	F	0.20415	V	N/D
F55	Low	2N	108	21.58	F	0.36314	V	N/D
H18	High	2N	101	17.42	F	N/D	IV	N/D
H19	High	2N	111	14.24	F	0.1057	VI	N/D
H21	High	2N	104	16.17	F	0.29069	V	N/D
H22	High	2N	109	18.98	F	N/D	IV	N/D
H23	High	2N	103	22.13	F	N/D	IV	N/D
H24	High	2N	97	17.13	F	N/D	V	N/D
H25	High	2N	97	11.79	I	0.01873	I	N/D
H28	High	2N	117	21.93	F	N/D	V	N/D
H29	High	2N	102	14.7	F	0.44201	V	N/D
H36	High	2N	103	23.97	F	0.30253	IV	N/D
H38	High	2N	92	18.84	F	N/D	V	N/D
H41	High	2N	98	16.61	F	0.32083	V	N/D
H44	High	2N	101	18.99	F	0.26995	V	N/D
H45	High	2N	109	18.88	F	0.22972	V	N/D
H47	High	2N	110	21.96	F	N/D	V	N/D
H50	High	2N	132	28.7	I	N/D	I	N/D
H51	High	2N	94	11.6	I	0.01411	I	N/D
H52	High	2N	100	14.74	F	0.27266	IV	N/D
H55	High	2N	102	15.16	F	N/D	IV	N/D

Table B.12 Unanalyzed data from diploid oysters evaluated at day of the experiment 12 in May 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
E3	Control	2N	105	18.27	F	0.34239	V	12.843
E4	Control	2N	97	17.01	F	0.36956	V	10.22
E11	Control	2N	128	27.28	F	0.34171	V	33.931
E15	Control	2N	102	20.26	F	0.28735	IV	17.147
E19	Control	2N	107	25.21	I	0.05188	I	N/D
E22	Control	2N	98	15.65	F	0.25683	IV	22.44
E23	Control	2N	124	14.08	F	0.3028	V	27.546
E24	Control	2N	97	13.5	F	0.18852	IV	21.983
E25	Control	2N	91	11.57	F	0.15115	IV	27.108
E26	Control	2N	117	15.92	F	N/D	IV	116.08
E28	Control	2N	107	20.79	F	0.24802	IV	N/D
E34	Control	2N	100	15.77	F	0.36502	V	16.535
E35	Control	2N	107	11.92	F	0.40724	V	14.253
E41	Control	2N	110	17.8	F	0.1864	III	11.483
G4	Low	2N	99	32.32	F	0.32114	IV	N/D
G6	Low	2N	94	11.03	F	0.34401	V	N/D
G9	Low	2N	94	16.28	F	0.32671	V	N/D
G11	Low	2N	92	15.56	F	0.42736	V	N/D
G14	Low	2N	93	15.34	F	0.34474	V	N/D
G16	Low	2N	119	19.55	F	0.16655	III	N/D
G18	Low	2N	98	14.99	F	0.1144	VI	N/D
G22	Low	2N	106	15.75	F	0.3678	V	N/D
G28	Low	2N	104	17.43	F	0.25884	IV	N/D
G31	Low	2N	100	20.44	F	0.2111	IV	N/D
G33	Low	2N	112	15.44	F	0.10276	VI	N/D
G34	Low	2N	99	18.12	F	0.20063	VI	N/D
G36	Low	2N	89	15.56	F	0.25621	V	N/D
G37	Low	2N	103	15.25	F	0.22036	IV	N/D
G38	High	2N	105	27.16	F	0.21499	V	N/D
G41	High	2N	114	17.76	F	0.49471	V	N/D
G42	High	2N	98	15.41	F	0.16387	VI	N/D
G45	High	2N	96	17.8	F	N/D	V	N/D
G46	High	2N	115	28.72	F	0.32069	V	N/D
G48	High	2N	94	13.21	F	0.13486	IV	N/D
G49	High	2N	110	19.4	F	0.24487	V	N/D
G50	High	2N	113	21.14	F	0.28083	V	N/D
G53	High	2N	110	11.79	I	0.07009	I	N/D
G55	High	2N	114	23.44	F	N/D	V	N/D
H1	High	2N	83	11.74	F	0.509	V	N/D
H3	High	2N	95	15.43	F	0.11435	IV	N/D
H6	High	2N	93	14.28	F	0.36474	V	N/D
H9	High	2N	91	9.83	F	0.53622	V	N/D
H11	High	2N	119	22.77	F	0.33129	V	N/D
H15	High	2N	114	15.29	F	0.30104	V	N/D
H17	High	2N	95	18.83	F	0.26525	IV	N/D

Table B.13 Unanalyzed data from initial evaluation of the experiment conducted in August 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
1	Initial	3N	122.7	16.84	F	0.086746	III	29.82
2	Initial	3N	105	27.12	F	0.075101	VI	41.39
3	Initial	3N	113	13.45	F	0.084174	III	16.47
5	Initial	3N	102.2	17.52	F	0.14683	III	17.86
6	Initial	3N	106.6	16.81	F	0.068608	III	27.100
7	Initial	3N	126.6	31.87	F	0.049154	IV	18.53
8	Initial	3N	109.5	13.19	F	0.094739	IV	26.4
10	Initial	3N	103.5	20.05	F	0.077739	III	39.96
11	Initial	3N	86.5	14.25	F	0.055253	III	15.16
13	Initial	3N	128.6	18.9	F	0.028676	III	21.14
14	Initial	3N	92.9	8.89	F	0.09467	III	20.71
15	Initial	3N	117.5	27.5	F	0.123435	IV	15.18
18	Initial	3N	119.7	26.85	F	0.095893	IV	20.72
19	Initial	3N	124.5	16.83	I	0.032854	I	18.83
20	Initial	3N	120	14.77	F	0.191343	V	16.01
1	Initial	2N	120	14.75	F	0.227953	V	16.07
3	Initial	2N	98	11.28	F	0.357312	V	19.67
5	Initial	2N	110	16.34	F	0.441621	V	19.44
6	Initial	2N	107.2	12.02	F	0.415728	V	15.11
7	Initial	2N	117.6	15.99	F	0.3328	V	14.25
9	Initial	2N	100.7	16.38	F	0.27722	V	16.65
11	Initial	2N	110.6	12.46	F	0.477376	V	17.5
12	Initial	2N	106	11.74	F	0.458601	V	N/D
13	Initial	2N	112	23.1	F	0.320888	V	24.65
14	Initial	2N	100.5	14.96	F	0.361932	V	35.12
15	Initial	2N	108	18.11	F	0.258204	V	21.69
16	Initial	2N	118.4	24.96	F	N/D	V	20.09
17	Initial	2N	112.6	15.07	F	0.335911	V	24.68
18	Initial	2N	121.2	10.92	F	0.44444	V	28.37
19	Initial	2N	107.7	17.45	F	0.259858	V	20.04
20	Initial	2N	102	15.93	F	0.396016	V	18.31

Table B.14 Unanalyzed data from triploid oysters evaluated at day 12 of the experiment in August 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
E5	Control	3N	121	24.98	F	0.06395	III	N/D
E6	Control	3N	130.5	24.32	F	0.037264	III	18.36
E7	Control	3N	113.7	22.74	F	0.052301	III	69.45
E8	Control	3N	94.5	16.07	F	0.099146	III	29.48
E9	Control	3N	129	18.29	F	0.067914	III	N/D
E10	Control	3N	117.7	29.7	I	0.048612	I	28.21

Table B.14 Continued

E11	Control	3N	99	22.66	F	0.077206	VI	33.8
E13	Control	3N	112	10.11	F	N/D	III	48.83
E16	Control	3N	93.1	9.77	F	0.185338	VI	16.12
E19	Control	3N	117.8	12	F	0.164098	III	37.35
E20	Control	3N	115	16.87	F	0.093255	III	14.3
E21	Control	3N	86.8	12.98	F	0.037419	III	24.25
E22	Control	3N	125.5	20.71	F	0.036581	III	24.72
E24	Control	3N	84.1	16.95	F	0.060214	III	59.45
E25	Control	3N	100	8.36	F	0.068182	III	36.850
E26	Control	3N	108.9	10.87	F	N/D	III	71.73
E27	Control	3N	106.1	18.06	F	0.046754	III	28.2
E28	Control	3N	115.5	15.55	F	0.075844	III	19.51
E29	Control	3N	119.2	17.29	F	0.144695	VI	20.58
E30	Control	3N	97.5	22.07	F	0.107642	III	45.07
E32	Control	3N	101.7	13.9	F	0.06904	VI	21.01
E33	Control	3N	111.1	13.11	F	0.065715	III	21.23
E34	Control	3N	95.9	7.82	F	N/D	III	32.94
J1	Low	3N	118	21.65	F	0.036962	III	N/D
J2	Low	3N	98.5	14.59	F	0.096583	III	N/D
J3	Low	3N	95.5	12.62	F	0.117935	IV	N/D
J4	Low	3N	103.5	7.84	F	0.061248	III	N/D
J5	Low	3N	141	18.77	F	0.126032	III	N/D
J7	Low	3N	119	24.87	F	0.066809	III	N/D
J8	Low	3N	102	17.11	F	0.074359	III	N/D
J10	Low	3N	113	10.2	F	0.056488	III	N/D
J17	Low	3N	104.5	18.9	F	0.091346	III	N/D
J18	Low	3N	107.5	23.57	F	0.078295	VI	N/D
J19	Low	3N	141.2	23.08	I	0.104087	I	N/D
J21	Low	3N	106.1	12.31	F	0.063585	IV	N/D
J22	Low	3N	129.5	23.09	I	0.063456	I	N/D
J23	Low	3N	126.8	22.12	F	0.081452	III	N/D
J24	Low	3N	122	25.53	F	0.05376	III	N/D
J25	Low	3N	115	13.31	F	0.075784	III	N/D
J26	Low	3N	107.7	9.86	I	0.062612	I	N/D
J27	Low	3N	111.5	9.3	F	0.100166	III	N/D
J28	Low	3N	83.6	14.29	F	0.048376	III	N/D
J29	Low	3N	121.5	21.75	F	0.04036	III	N/D
J31	High	3N	110	22.03	F	0.05432	III	N/D
J33	High	3N	108	17.55	F	N/D	VI	N/D
J35	High	3N	123	20.38	F	0.057621	III	N/D
J36	High	3N	119	8.25	F	0.081416	III	N/D
J38	High	3N	86	15.87	F	0.067539	III	N/D
J39	High	3N	105	20.21	F	0.061191	III	N/D
J40	High	3N	98	11.1	F	0.116661	III	N/D
J41	High	3N	118	27.4	F	N/D	IV	N/D
J42	High	3N	150	36.62	I	0.020314	I	N/D
J43	High	3N	107	9.47	F	0.086941	III	N/D
J45	High	3N	122	14.08	F	0.240466	V	N/D
J46	High	3N	122	22.18	F	0.079261	III	N/D

Table B.14 Continued

J47	High	3N	101	15.16	F	0.052754	III	N/D
J50	High	3N	122	21.5	F	N/D	IV	N/D
J53	High	3N	102	16.26	I	0.071697	I	N/D
J55	High	3N	96	12.56	F	0.103184	III	N/D
J56	High	3N	100	13.25	F	0.111294	III	N/D
E2	High	3N	125	19.88	F	N/D	V	N/D
E3	High	3N	116.8	22.41	F	0.266438	V	N/D
E4	High	3N	111	21.77	I	0.042325	I	N/D

Table B.15 Unanalyzed data from triploid oysters evaluated at day 15 of the experiment in August 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
H31	Control	3N	96	18.2	F	0.045133	III	24.370
H33	Control	3N	128.9	16.52	F	0.060829	III	16.840
H35	Control	3N	112.5	14.89	F	0.085273	III	20.020
H48	Control	3N	109.2	22.62	F	0.093778	III	44.690
H49	Control	3N	101	14.06	F	0.20449	IV	46.040
H50	Control	3N	114	22.09	F	0.062594	III	36.000
H55	Control	3N	111	15.04	F	0.105282	III	25.880
H2	Low	3N	104	17.22	F	0.053286	III	N/D
H4	Low	3N	115	24.46	F	0.055691	III	N/D
H5	Low	3N	101	19.38	F	0.121115	IV	N/D
H6	Low	3N	112	14.24	F	0.070049	III	N/D
H7	Low	3N	121	24.44	F	0.045375	III	N/D
H8	Low	3N	124	20.13	F	0.0889	III	N/D
H11	Low	3N	110	18.22	F	0.077361	III	N/D
H12	Low	3N	134.5	24013	F	0.110816	III	N/D
H15	Low	3N	108.7	19.19	F	0.091317	III	N/D
H17	Low	3N	106.4	14.52	F	0.088357	III	N/D
H18	Low	3N	121.8	25.2	F	0.047049	III	N/D
H19	Low	3N	100	15.78	F	0.062881	III	N/D
H20	Low	3N	91.5	11.25	F	0.06408	III	N/D
H23	Low	3N	99	9.71	F	0.086886	III	N/D
H24	Low	3N	109.2	15.48	F	0.057334	III	N/D
H25	Low	3N	102	11.64	F	0.069724	III	N/D
G1	High	3N	104	16.57	F	0.061026	III	N/D
G3	High	3N	100	16.45	F	0.201841	V	N/D
G4	High	3N	97	11.93	F	N/D	V	N/D
G5	High	3N	106	14.05	I	0.073855	I	N/D
G6	High	3N	92	18.54	F	N/D	V	N/D
G10	High	3N	94.8	10.05	F	0.439279	V	N/D
G11	High	3N	121	17.85	F	0.285424	V	N/D
G15	High	3N	130	16.24	F	0.037271	III	N/D
G17	High	3N	101	16.27	F	0.100146	III	N/D
G20	High	3N	136.2	18.06	F	0.076021	III	N/D
G21	High	3N	117	14.82	F	0.126868	V	N/D

Table B.15 Continued

G22	High	3N	118	19.52	F	0.055653	III	N/D
G23	High	3N	140	25.29	F	0.038392	III	N/D
G24	High	3N	132	24.76	F	0.032131	III	N/D
G25	High	3N	100	16.28	F	0.058425	III	N/D

Table B.16 Unanalyzed data from diploid oysters evaluated at day 12 of the experiment in August 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
I2	Control	2N	115	19.84	F	0.213272	IV	57.25
I3	Control	2N	108	11.88	F	0.228321	V	25.12
I4	Control	2N	142	15.51	F	0.071301	VI	22.36
I5	Control	2N	122	19.02	F	0.180984	V	25.08
I8	Control	2N	143	17.19	F	N/D	V	23.43
I10	Control	2N	99	11.45	F	N/D	VI	46.42
I11	Control	2N	111.9	13.97	F	N/D	VI	25.7
I13	Control	2N	99	13.34	F	0.237695	V	27.15
I16	Control	2N	121	22.23	F	0.213281	V	26.74
I17	Control	2N	116	17.13	F	0.212259	V	36.65
I20	Control	2N	100	9.38	F	0.133306	VI	46.45
I21	Control	2N	104	10.37	F	0.236582	V	12.71
I33	Low	2N	105	15.38	F	0.234348	V	N/D
I34	Low	2N	116	21.18	F	0.218829	V	N/D
I35	Low	2N	98	10.87	F	0.181063	IV	N/D
I36	Low	2N	87	7.09	F	N/D	V	N/D
I37	Low	2N	99	9.89	F	0.205092	V	N/D
I38	Low	2N	112	15.28	F	0.204213	V	N/D
I39	Low	2N	94	12.4	F	0.216402	V	N/D
I40	Low	2N	110	16.62	F	0.181135	V	N/D
C1	Low	2N	134	12.28	F	0.134883	V	N/D
C4	Low	2N	144	16.97	F	0.184481	IV	N/D
C5	Low	2N	115.8	12.31	F	0.262574	V	N/D
C6	Low	2N	93	10.03	F	0.283097	V	N/D
C7	Low	2N	105.8	13.89	F	0.140386	IV	N/D
C8	Low	2N	111	11.05	F	0.302075	V	N/D
C9	Low	2N	104	11.27	F	0.273326	V	N/D
I41	High	2N	124	14.53	F	0.168677	V	N/D
I42	High	2N	97	9.91	F	N/D	V	N/D
I44	High	2N	110	11.93	F	N/D	V	N/D
I47	High	2N	105	17.46	F	0.303985	IV	N/D
I49	High	2N	92	13.31	F	N/D	V	N/D
I50	High	2N	111	12.76	F	0.166264	V	N/D
C21	High	2N	105	11.78	F	0.134439	IV	N/D
C25	High	2N	124	13.28	F	0.150976	IV	N/D
C26	High	2N	139	16.85	F	0.296482	V	N/D
C28	High	2N	104	12.46	F	0.167395	V	N/D
C29	High	2N	92	10.36	F	0.190676	V	N/D

Table B.16 Continued

C30	High	2N	111	14.34	F	0.300796	V	N/D
C31	High	2N	125	19.8	F	0.234926	IV	N/D
C35	High	2N	101	10.49	F	0.255348	V	N/D
C38	High	2N	122	18.17	F	0.181387	V	N/D

Table B.17 Unanalyzed data from diploid oysters evaluated at day 15 in August 2004 (Chapter 4).

Oyster No	Treatment	Ploidy	Shell height (mm)	Weight (g)	Sex	GBR	Gonad Stage	Estradiol (pg/ml)
G28	Control	2N	99	9.83	F	N/D	VI	16.090
G29	Control	2N	123	20.54	F	0.336573	V	21.390
G30	Control	2N	122	21.97	F	N/D	V	23.750
G31	Control	2N	129	16.16	F	0.164247	V	41.900
G32	Control	2N	117	19.67	F	0.260167	V	126.300
G35	Control	2N	111	23.39	F	0.334527	V	20.290
G36	Control	2N	108	29.34	F	0.236516	V	40.520
G37	Control	2N	102	11.43	F	0.387955	V	16.710
G38	Control	2N	91.8	9.99	F	N/D	V	21.620
G39	Control	2N	117	15.48	F	0.252258	V	23.690
G43	Control	2N	113	22.07	F	0.441348	V	41.840
G44	Control	2N	105	16.5	F	0.367044	V	16.660
G46	Control	2N	114	18.13	F	0.252189	V	31.920
B11	Low	2N	111.7	24.99	F	0.261757	V	N/D
B14	Low	2N	119	19.46	F	0.288667	V	N/D
B15	Low	2N	113	10.76	F	0.399239	V	N/D
B18	Low	2N	110	16.49	F	0.298472	V	N/D
B19	Low	2N	115	20	F	0.327758	V	N/D
B20	Low	2N	112	20.15	F	0.217094	V	N/D
B21	Low	2N	116	19.16	F	0.221758	V	N/D
B22	Low	2N	117	17.4	F	0.285447	V	N/D
B23	Low	2N	122	17.49	F	0.202661	V	N/D
B24	Low	2N	110	20.43	F	0.338644	V	N/D
B25	Low	2N	104	21.1	F	0.250921	V	N/D
B27	Low	2N	108.7	24.53	F	0.298905	V	N/D
B28	Low	2N	123	17.76	F	0.257946	V	N/D
D12	High	2N	100	16.76	F	0.199923	V	N/D
D14	High	2N	121	25.99	F	0.286505	V	N/D
D15	High	2N	125	19.05	F	0.259994	V	N/D
D17	High	2N	93.5	15.82	F	0.160245	V	N/D
D18	High	2N	150	24.78	F	0.298345	V	N/D
D20	High	2N	107	10.72	F	0.18361	V	N/D
D21	High	2N	107	14.81	F	0.27197	V	N/D
D22	High	2N	98	16.71	F	0.200349	V	N/D
D24	High	2N	121	23.61	F	0.179151	V	N/D
D25	High	2N	133	15.13	F	N/D	V	N/D
D26	High	2N	94	13.88	F	0.257419	V	N/D

Table B.18 Unanalyzed data of oocyte area from diploid oysters evaluated in August 2003 and May and August 2004 (Chapter 4).

Oyster No	Experiment	Treatment	Oocyte Area (µm)
A2	August 2003	Control	1072.08
A13	August 2003	Control	1006.22
A19	August 2003	Control	916.60
A29	August 2003	Control	948.83
A38	August 2003	Control	953.79
B3	August 2003	Control	931.31
B15	August 2003	Control	997.45
B18	August 2003	Control	827.43
B31	August 2003	Control	878.98
A12	August 2003	Control	930.15
A14	August 2003	Control	950.79
A23	August 2003	Control	1047.55
A31	August 2003	Control	864.89
B1	August 2003	Control	873.57
B6	August 2003	Control	925.98
B17	August 2003	Control	934.57
B25	August 2003	Control	1058.58
D17	August 2003	Low	974.27
B32	August 2003	Low	1070.77
B33	August 2003	Low	989.81
B39	August 2003	Low	912.94
C10	August 2003	Low	1014.89
C35	August 2003	Low	1055.82
D16	August 2003	Low	869.93
D19	August 2003	Low	1111.32
B37	August 2003	Low	840.76
C6	August 2003	Low	963.56
C34	August 2003	Low	912.44
D17	August 2003	Low	889.69
D4	August 2003	Low	947.37
E4	August 2003	High	1116.42
E10	August 2003	High	1117.75
E23	August 2003	High	1068.58
E34	August 2003	High	967.35
D26	August 2003	High	833.12
E1	August 2003	High	1033.54
E9	August 2003	High	1147.11
E11	August 2003	High	973.42
E33	August 2003	High	997.20
D23	August 2003	High	995.47
D34	August 2003	High	1028.75
E42	May 2004 (day 9)	Control	802.38
E44	May 2004 (day 9)	Control	869.90
F5	May 2004 (day 9)	Control	923.08
F9	May 2004 (day 9)	Control	822.36
F16	May 2004 (day 9)	Control	766.95
F20	May 2004 (day 9)	Control	706.86

Table B.18 continued

E43	May 2004 (day 9)	Control	968.96
F3	May 2004 (day 9)	Control	870.48
F7	May 2004 (day 9)	Control	876.72
F10	May 2004 (day 9)	Control	821.84
F17	May 2004 (day 9)	Control	815.28
F21	May 2004 (day 9)	Control	602.79
F30	May 2004 (day 9)	Low	702.01
F35	May 2004 (day 9)	Low	629.49
F40	May 2004 (day 9)	Low	681.90
F42	May 2004 (day 9)	Low	679.04
F46	May 2004 (day 9)	Low	595.42
F50	May 2004 (day 9)	Low	603.48
F55	May 2004 (day 9)	Low	745.93
G3	May 2004 (day 9)	Low	688.76
F34	May 2004 (day 9)	Low	644.00
F38	May 2004 (day 9)	Low	675.18
F41	May 2004 (day 9)	Low	642.06
F43	May 2004 (day 9)	Low	607.95
F47	May 2004 (day 9)	Low	646.01
F51	May 2004 (day 9)	Low	736.97
G2	May 2004 (day 9)	Low	777.20
H18	May 2004 (day 9)	High	760.85
H21	May 2004 (day 9)	High	706.07
H23	May 2004 (day 9)	High	641.43
H28	May 2004 (day 9)	High	597.30
H36	May 2004 (day 9)	High	790.77
H41	May 2004 (day 9)	High	708.05
H52	May 2004 (day 9)	High	682.37
H19	May 2004 (day 9)	High	792.58
H22	May 2004 (day 9)	High	607.97
H24	May 2004 (day 9)	High	596.06
H29	May 2004 (day 9)	High	639.93
H38	May 2004 (day 9)	High	673.01
H47	May 2004 (day 9)	High	686.67
H55	May 2004 (day 9)	High	711.88
E3	May 2004 (day12)	Control	645.62
E11	May 2004 (day12)	Control	649.75
E22	May 2004 (day12)	Control	706.68
E25	May 2004 (day12)	Control	747.90
E28	May 2004 (day12)	Control	775.92
E35	May 2004 (day12)	Control	765.67
E4	May 2004 (day12)	Control	684.22
E15	May 2004 (day12)	Control	716.48
E23	May 2004 (day12)	Control	726.13
E26	May 2004 (day12)	Control	792.38
E34	May 2004 (day12)	Control	663.44
E41	May 2004 (day12)	Control	468.02
G4	May 2004 (day12)	Low	795.46
G9	May 2004 (day12)	Low	766.96
G14	May 2004 (day12)	Low	787.21

Table B.18 Continued

G22	May 2004 (day12)	Low	807.96
G31	May 2004 (day12)	Low	723.44
G36	May 2004 (day12)	Low	737.07
G6	May 2004 (day12)	Low	867.78
G11	May 2004 (day12)	Low	835.86
G16	May 2004 (day12)	Low	819.58
G28	May 2004 (day12)	Low	797.94
G34	May 2004 (day12)	Low	780.33
G37	May 2004 (day12)	Low	786.34
G38	May 2004 (day12)	High	671.05
G42	May 2004 (day12)	High	756.95
G46	May 2004 (day12)	High	750.15
G50	May 2004 (day12)	High	752.93
H1	May 2004 (day12)	High	812.76
H6	May 2004 (day12)	High	717.78
H11	May 2004 (day12)	High	646.50
H17	May 2004 (day12)	High	748.23
G41	May 2004 (day12)	High	712.95
G45	May 2004 (day12)	High	802.80
G49	May 2004 (day12)	High	736.68
G55	May 2004 (day12)	High	683.64
H3	May 2004 (day12)	High	765.03
H9	May 2004 (day12)	High	831.16
H15	May 2004 (day12)	High	740.62
I3	August2004 (day12)	Control	622.72
I13	August2004 (day12)	Control	654.99
I17	August2004 (day12)	Control	658.69
I5	August2004 (day12)	Control	773.28
I6	August2004 (day12)	Control	685.19
I21	August2004 (day12)	Control	683.84
C1	August2004 (day12)	Low	596.96
I33	August2004 (day12)	Low	674.36
I36	August2004 (day12)	Low	689.22
I38	August2004 (day12)	Low	647.43
I40	August2004 (day12)	Low	558.98
C5	August2004 (day12)	Low	692.55
I34	August2004 (day12)	Low	616.43
I37	August2004 (day12)	Low	691.54
I39	August2004 (day12)	Low	686.37
C26	August2004 (day12)	High	634.03
C30	August2004 (day12)	High	712.05
I41	August2004 (day12)	High	620.77
I44	August2004 (day12)	High	659.45
C28	August2004 (day12)	High	704.06
C35	August2004 (day12)	High	684.22
I42	August2004 (day12)	High	648.60
I49	August2004 (day12)	High	605.23
G29	August2004 (day15)	Control	646.62
G31	August2004 (day15)	Control	672.59
G35	August2004 (day15)	Control	621.72

Table B.18 Continued

G37	August2004 (day15)	Control	661.23
G39	August2004 (day15)	Control	712.80
G44	August2004 (day15)	Control	680.05
G30	August2004 (day15)	Control	621.69
G32	August2004 (day15)	Control	651.52
G36	August2004 (day15)	Control	590.00
G38	August2004 (day15)	Control	729.59
G43	August2004 (day15)	Control	584.05
G46	August2004 (day15)	Control	603.46
B11	August2004 (day15)	Low	621.06
B15	August2004 (day15)	Low	613.89
B19	August2004 (day15)	Low	746.35
B21	August2004 (day15)	Low	676.97
B23	August2004 (day15)	Low	623.14
B25	August2004 (day15)	Low	649.58
B28	August2004 (day15)	Low	587.68
B14	August2004 (day15)	Low	732.88
B18	August2004 (day15)	Low	663.31
B20	August2004 (day15)	Low	656.50
B22	August2004 (day15)	Low	646.95
B24	August2004 (day15)	Low	626.61
B27	August2004 (day15)	Low	717.39
C9	August2004 (day15)	Low	708.27
D12	August2004 (day15)	High	632.00
D15	August2004 (day15)	High	616.33
D20	August2004 (day15)	High	673.62
D22	August2004 (day15)	High	720.72
D26	August2004 (day15)	High	618.34
D30	August2004 (day15)	High	681.90
D14	August2004 (day15)	High	613.28
D18	August2004 (day15)	High	758.95
D21	August2004 (day15)	High	663.03
D25	August2004 (day15)	High	626.79

VITA

Roberto Quintana-Rodriguez was born in Tijuana, Baja California, México, on April 25, 1974. He attended the Faculty of Marine Sciences (FMC) at the Universidad Autonoma de Baja California, where he received his bachelor of science degree in oceanography with a specialization in aquaculture. During that time, he worked for one summer at a commercial abalone farm in Ensenada, México, and later, during his thesis research, at the laboratory of mollusks at the FMC, under the supervision of Prof. Alfredo Salas. In 1998 he began working for the company Abulones Cultivados S.A. de C.V. as a hatchery supervisor and two years later as an assistant manager for two more years. In 2003 he joined the School of Renewable Natural Resources were he worked toward a master's degree under the supervision of Dr. Tiersch, Dr. Supan, and Dr. Lynn. He is currently a candidate for the degree of Master of Science in fisheries (aquaculture) in summer 2005.