2010

The influence of two estrogens on the sex and ovarian development of eastern oysters (Crassostrea virginica) maintained in a closed recirculating system

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THE INFLUENCE OF TWO ESTROGENS ON THE SEX AND OVARIAN DEVELOPMENT OF EASTERN OYSTERS (*Crassostrea virginica*) MAINTAINED IN A CLOSED RECIRCULATING SYSTEM

A Thesis

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

in

The School of Renewable Natural Resources

By
Wesley M. Burnside
B.S., Louisiana State University and Agricultural and Mechanical College, 2004
May 2010

To my family and friends with love and appreciation:

When life passes us by,
When old dreams become our second priorities,
When love not only touches your heart, but sweeps it away;
When all your friends are still your friends.

We have all been growing.
We are all on our way.
Life is passing us by,
Wishing love for all today.

Whether in this country, or far away,
Across long bridges or large rivers,
Many known cities and states away,
Small town, big city, or a flat in the U.K.

I wish you love and laughter!
Live your life: don't fight its changes
Or your life will live you …
As it passes you away.

I feel the Earth shifting beneath me,
The winds strong around;
Life is forever changing,
Forever shifting from place to place.

Grow.
Grow!
It's our turn to bloom.

Jeremy D. Hains | May 30, 2009

ACKNOWLEDGEMENTS

I thank Dr. Terrence Tiersch for serving as my graduate advisor; he not only encouraged me to improve my technical and critical thinking skills, but taught me leadership; he also helped me think "outside of the box." I thank Dr. John Lynn for always walking me through my biological quandaries; he was an immense help during experiment planning and execution. I thank Dr. John Supan for his wealth of oyster knowledge and his passion for sharing it; he was instrumental in experiment planning and providing the oysters for my research.

I thank everyone at the LSU Agricultural Center (LSUAC) Aquaculture Research Station (ARS) for help and support. Dr. Huiping Yang, with her extensive oyster experience, assisted with experimental design and oyster sampling, and was a great friend and supporter during the problems encountered during this project. Amy Guitreau was a major contributor during my time with this laboratory, is now one of my best friends. I thank the undergraduate students, especially Kasey Couch; he is a hard worker and friend who helped analyze a large portion of the oyster histology. Dr. Jill Jenkins also provided support and advice. I thank the ARS staff, especially Millie Williams, Sandy Malone, and Marla Jones. Kevin Melody and Vanessa Maxwell helped maintain and collect the oysters used in my experiments from the Sea Grant Grand Isle Bivalve Hatchery. Cheryl Crowder at the LSU School of Veterinary Medicine processed histological samples. I appreciate the LSUAC and National and Louisiana Sea Grant College Programs for providing financial support for this project.

Finally, I thank Paul Link and Elisa Ledet for their support, humor and statistical genius, and my other family and friends for their support during my cumbersome trek through this graduate program; they kept me grounded and on track. We have all been through a lot in the past few years and I am lucky to have such great people in my life.

PREFACE

As a multi-million dollar industry, oyster production is significant to the economy of the nation's coastal regions. In 2007, the U.S. oyster fishery was valued at $126.9 million (Prichard, 2008). Nationally, from 2001 to 2006, the annual worth of oyster meats increased by 27% and the price per pound increased by 44% (NMFS, 2008), a trend corresponding to the 83% (EIA, 2008) increase in consumer gasoline prices and 17% (NASS, 2008) increase in agricultural farm labor (Figure P.1). While national oyster fishery landings have remained consistent (34.80 ± 3.78 million lbs), the estimated production by commercial aquaculture decreased by 48% after 2004 (NMFS, 2008; Figure P.2). One explanation for this decline is the annual increase in energy and labor costs that affect aquaculture production more than fisheries due to the intensive management and costs required to culture oysters effectively. The advantages of commercial aquaculture are discussed in Chapter 1.

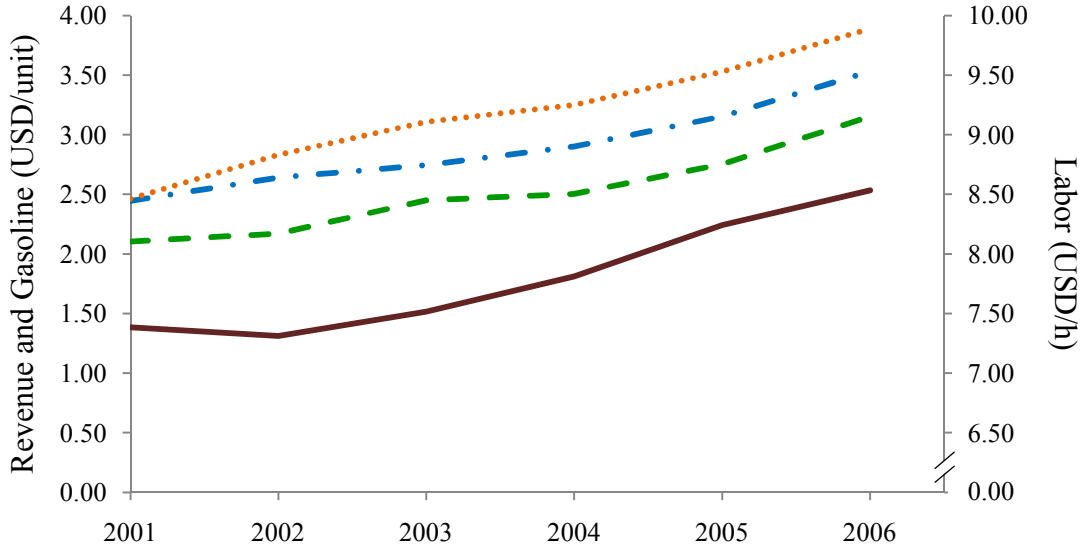

Figure P.1. Annual revenue (USD/lb) from oyster meats in the U.S. (— · —) and Louisiana (— —), as well as the consumer price of gasoline (USD/gallon, ——) and agricultural labor workers (USD/h, ·····) from 2001 to 2006.

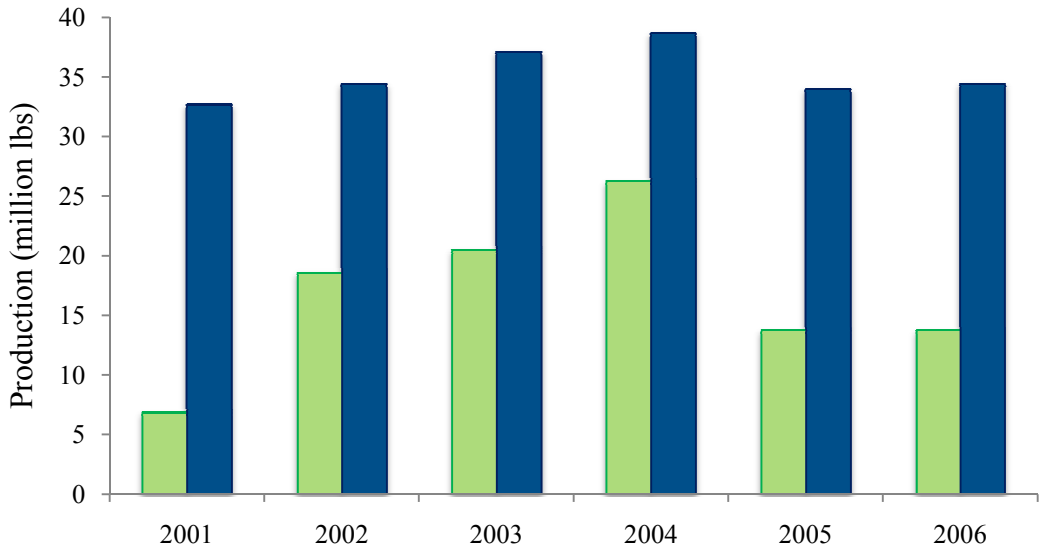

Figure P.2. Annual U.S. oyster production represented by the total meat yield (million lbs) from commercial aquaculture (■) and fisheries (■) from 2001 to 2006.

The eastern oyster *Crassostrea virginica* is the major oyster species harvested from the Gulf of Mexico. Valued at $80 million, the Gulf Coast oyster fishery accounted for 63% of the total U.S. oyster landings in 1997, yielding 22 million pounds of oyster meats (Prichard, 2008). Louisiana is the leader in the U.S. oyster industry, contributing 35% of the total landings between 2000 and 2006 (NMFS, 2008). Although the trend of oyster revenues from oyster landings is parallel to the national trend, the Louisiana revenue was 15% lower on a per-pound basis (Figure P.1).

After Hurricanes Katrina and Rita devastated the Gulf South in 2005, annual Louisiana eastern oyster landings decreased by 20%. The storms not only destroyed oyster fishing boats, processing plants, and storage facilities, but the Louisiana Department of Wildlife and Fisheries initially estimated 99% oyster mortality in some areas due to contamination by excess nutrients and toxins released from mobilized sediment. Excess nutrients increase the dissolved oxygen demand and results in anoxic water conditions, while the toxins interfere with normal physiological processes. These mobilized sediments also buried oyster reefs, destroying habitat

and smothering populations of oysters which died from asphyxiation (Sheikh, 2005). A new assessment in 2007 reported a range of 20 to 74% loss of Louisiana oyster reefs depending on the region, with no significant chemical or biological contamination. Because Louisiana oysters require as long as 2 years to reach market size, it could take years for these reefs to make a full recovery. However, intensive management of oyster leases by some oystermen could accelerate the rejuvenation process (Pflieger and Lumsden, 2007).

Even before the havoc caused by the hurricanes of 2005, the profitability of the oyster industry fluctuated with variations in seasonal meat yields which decrease by two-thirds during the summer spawning season (Allen and Downing, 1986). By culturing oysters with no or reduced spawning potential, these summer losses could be virtually eliminated. Currently, the production of triploid oysters is one way to achieve reproductive sterility. Gametogenesis is disrupted in triploids due to an additional set of chromosomes that interferes with normal reproduction; therefore, triploid oysters are associated with an increased growth rate, higher meat yields, increased glycogen stores, and improved meat quality, especially during the summer spawning months.

Unfortunately, triploid oysters are not easily produced. The most straightforward method, yielding all-triploid offspring, is by artificially spawning a tetraploid female (with four sets of chromosomes) with a normal diploid male (Guo et al., 1996). Thus, the biggest hurdle becomes producing the tetraploid female broodstock. Tetraploids are generally produced by genetic manipulation (polar body I inhibition) of triploid oocytes (Guo and Allen, 1994; Guo et al., 2002). Because triploids have low fecundity, methods for increasing oocyte availability are essential. The use and production of triploid and tetraploid individuals is discussed in greater detail in Chapter 1.

The goal of this project was to determine if the use of an estrogen, estradiol-17β or estradiol-3-benzoate, at a consistent environmental temperature would increase the percentage of females in populations, as well as increase ovarian development of eastern oysters maintained in an indoor recirculating system. Ultimately, it is hoped that findings can be applied to triploid oysters to increase the overall number of viable oocytes produced for use in tetraploid induction.

Structurally, this thesis is organized into three chapters and two appendices. The first chapter describes the basic and reproductive biology of the eastern oyster, as well as its commercial significance. Chapter 2 evaluates the effect of two estrogens, estradiol-17β and estradiol-3-benzoate, on ovarian development. The third chapter summarizes the overall findings of this project and suggests their applicability to the oyster industry. The appendices contain standard operating procedures (Appendix A) and the raw data collected in these experiments (Appendix B).

The chapters of this thesis are formatted according to the *Journal of Shellfish Research*. A modified version containing the data in Chapter 2 will be submitted for publication to this journal.

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ABSTRACT

As a multi-million dollar industry, production of the eastern oyster *Crassostrea virginica* is significant to the economy of the nation's coastal regions. The cost of commercial oyster aquaculture has higher inputs than the oyster fishery, but larger, better quality oysters can be produced for culture by the stable availability of triploid individuals.

The goal of this project was to determine if the use of an estrogen, estradiol-17β or estradiol-3-benzoate, at a consistent environmental temperature would increase the percentage of females in a population, as well as ovarian development of eastern oysters. Ultimately, this could be applied to triploids to increase the number of viable oocytes produced for use in tetraploid induction. Tetraploids are used in direct triploid production to produce 100% triploid offspring when crossed with diploids.

When estradiol-17β was administered to oysters obtained late in the spawning season (August), there was no significant change in sex distribution. The gonad-to-body ratios on day 10 were significantly greater than on day 14 ($P < 0.0001$) and the stages of ovarian development in oysters treated with 150 ng differed from the other treatments ($P = 0.002$); significant differences were also seen between days 10 and 14 in this treatment (150 ng; $P = 0.004$).

When estradiol-3-benzoate was administered to out-of-season oysters obtained in February prior to the spawning season, there was a significant difference in sex distribution from days 10 to 14 between the non-injected control ($P = 0.030$) and 37.5 ng treatment ($P = 0.010$). There was not enough gonadal tissue to calculate the gonad-to-body ratio, but there were no significant differences in the stages of ovarian development.

Overall, the decrease in ovarian size over time is indicative of exogenous factors negatively affecting gonadal development. By increased management of factors such as high

nutrient availability and decreased stress during transport and hormone administration could improve the response to estrogen treatment. Due to the short half-life of estrogens *in vivo*, a sustained-release system could also increase the treatment efficacy. Under the present conditions, there was not a clear, predictable effect of either estrogen on increasing gonadal development, maturity, or sex.

CHAPTER 1. INTRODUCTION

The eastern oyster (formerly known as the American oyster) *Crassostrea virginica* (formerly classified as *Ostrea virginaica*) (Gmelin, 1791) is bottom-dwelling, bivalve mollusc naturally distributed in estuaries from the Gulf of St. Lawrence, Canada, along the eastern North American coast to the Gulf of Mexico, Panama and the northern South American coast, as well as on the coasts of the Caribbean Islands (Figure 1.1; Carriker and Gaffney, 1996). Its natural spawning season varies by the geographic location of each population and annual climatic conditions. There are several exogenous factors that affect oyster physiology; three chief considerations include temperature, salinity, and nutrient availability. Temperature and salinity influence feeding, respiration and metabolism, growth and development, gonadal maturation, and immunological function (Shumway, 1996).

Figure 1.1. Distribution of the eastern oyster *Crassostrea virginica* (▬).

The eastern oyster is of high economic significance to the Gulf Coast, especially Louisiana. It is harvested through commercial fisheries, as well as cultured through various intensities of broodstock management. By understanding natural oyster physiology, gonadogenesis and spawning, factors such as endocrine signals, ploidy and temperature can be manipulated to increase the productivity of more intensive oyster operations. Because the oyster is an environmentally tolerant species, they are ideal for aquacultural production.

FACTORS AFFECTING PHYSIOLOGY

Oysters are ectothermic organisms, and as such, temperature a dominant critical factor affecting their physiology, especially gonadal sex and maturation (Prytherch, 1928). There is a direct correlation between increased ambient water temperatures and gametogenesis. The body composition of the oyster and condition of the gonad varies with seasonal temperature fluctuations. Because growth and metabolism are also promoted by high temperatures, rapid growth can occur at temperatures higher than the average growing season. During this periods of growth, the sex ratio is also affected; although oysters are considered to be a protandric species (initially male during the first annual breeding season, then changing to female), a greater proportion of female offspring develop under these conditions. In populations of one-year-old oysters, increases in mean size are associated with an increased occurrence of females (Coe, 1934; Coe, 1936). When indifferent oysters from the Long Island Sound were maintained from 10 to 30°C, oysters at 10°C underwent no gametic changes, while some oysters were able to develop and spawn at 15°C (Loosanoff and Davis, 1952). The ideal temperature for gametogenesis varies based on geographic location. In the Gulf States, changes in coastal water temperatures are not dramatic enough to greatly affect growth rate, but they do affect seasonal spawning activity.

Salinity limits the distribution of many aquatic species (Gunter, 1961; Wells, 1961), yet oysters are euryhaline and normally distributed throughout a wide range of salinities. The annual salinity can vary from 0 to 42.5 ppt in commercial oyster production (Ingle and Dawson, 1950a, 1950b, 1953). However, the range for optimal growth and development of eastern oysters is 14 to 28 ppt (Moore, 1900; Butler, 1949c, Chanley, 1958; Galtsoff, 1964). As with temperature, the most ideal salinity for a population of oysters can vary by geographic location, but consistent

concentrations are important. Among oyster reefs in the Gulf of Mexico, the average annual salinity varies and alters the physiology of different populations. At low salinity levels (< 7.5 to 10 ppt), gametogenesis is depressed or arrested (Loosanoff, 1953a, 1953b; Calabrese and Davis, 1970). At 10 ppt, oysters have higher mortality rates and decreased growth and development, while at 25 ppt, growth and reproduction rates peak. Although 15 ppt is not optimal for growth, the lower salinity can decrease predation by organisms limited to a more halinous environment (e.g. southern oyster drills *Stramonita haemastoma*, black drum *Pogonias cromis*) while the level of reproduction and larval survival remains high (Butler, 1954; Brown et al., 2004). Similarly to low salinities, high salinities (> 30 ppt) result in increased mortality and predation with decreased reproduction and larval survival (Bulter, 1954).

Although temperature and salinity are often examined independently, it is critical to weigh the interactions of these two factors. For example, during times of stress induced by low salinity levels (0 to 3 ppt), lower temperatures (8 to 12°C) can decrease mortality by lowering the metabolic rate of the oyster (Loosanoff, 1948; Andrews et al., 1959). On the contrary, at high temperatures (23 to 27°C), morbidity increases with changes in salinity (Loosanoff, 1948).

The nutrients available for oyster consumption are primarily affected by the particle size and seasonality of phytoplankton and other live seston. Although oysters are omnivorous, they do not gain much sustenance from detritus and bacteria (Langdon and Newell, 1990). There are numerous species of potentially consumable organisms, yet various attributes select some during filter-feeding over others. The particle size of algal material limits consumption by oysters of a certain size. For example, larvae (71 to 136 μm) preferentially consume algal cells less than 10 μm in diameter (Fritz et al., 1984); by 180 μm, larvae consume cells of 20 to 30 μm which largely consist of heterotrophic protozoans and dinoflagellates (Baldwin and Newell, 1991).

Poor digestibility can also be associated with the cell walls of some algal species (e.g. *Chlorella*, *Chlorococcum*, and *Platymonas* spp., *Phaeodactylum tricornutum*) (Langdon and Newell, 1996).

Like most diets, varying the composition of live particulates can greatly affect growth and development. Varying seasonally, the predominance of certain plankton species over others can affect the overall nutrient availability. The season affects photoperiod, selecting for species that require more or less light; it also affects temperature, selecting species that reproduce well in warmer or colder temperatures, and tide, which affects water depth allowing more or less light to penetrate the water column to select for species more or less tolerant of shading.

Ultimately, proper nutrition prior to reproductive activity is vital. During gonadal development and spawning, individual energy reserves, stored in the form of lipids and glycogen, are mobilized. Feeding at this time assists in this process, but it provides more to metabolic maintenance; it is less essential for gametogenesis. The level of initial glycogen stores prior to gonadal development can determine the quality of offspring produced (Loonsanoff and Davis, 1952). During seasonally high temperatures coinciding with the spawning season, glycogen stores are depleted by the energy demands required for reproduction (Mitchell, 1917; Medcof and Needler, 1941; Galtsoff et al., 1947). This depletion can account for losses of more than two-thirds of the meat weights (Allen and Downing, 1986).

REPRODUCTION

As stated above, eastern oysters are protandric, beginning life as a male and changing to female as their size and age increase, presumably because a greater reproductive capacity is required for egg production. Oocytes are approximately 50 μm in diameter (Thompson et al., 1996), while spermatozoa are composed of the head and midpiece with a combined length of approximately 40 μm (2 μm head and 38 μm tail) (Galtsoff, 1964). Oocytes are larger because

they contain vitellogenin, an energy source for early larval development before feeding occurs; larger oysters can store more glycogen, essential for vitellogenesis. The size of an oyster (determined by measuring its shell height) can be correlated to its age. Accordingly, oysters less than 35 mm are more likely to be male than female (Andrews, 1979).

In the event of physiological stress (Tranter, 1958) such as extreme temperatures or salinities, poor nutrient intake, or disease, oysters are more likely to be male because spermatogenesis requires considerably less energy than does vitellogenesis (Russell-Hunter, 1979), allowing more energy for physiological maintenance. Histologically, a mature male gonad will contain dense clusters of spermatozoa within its follicular lumen and genital canals, while the follicular lumen and genital canals of a mature female gonad contain the substantially larger, vitellogenin-rich primary oocytes (Figure 1.2). This project focused on ovarian development, so the detailed discussion of reproduction was limited to females.

Eastern oysters in Louisiana and other Gulf States are seasonally reproductively active. Gametogenesis occurs from late March to early April, followed by spawning from May until late August. In some cases, *recycling* oysters (discussed later) can spawn every four weeks until as late as October (Supan and Wilson, 2001). After spawning, residual gametes are destroyed by phagocytic hemocytes and resorbed (Kennedy and Krantz, 1982). This undifferentiated state of the gonad is referred to as *indifferent*. Because eastern oysters lack heteromorphic sex chromosomes and predetermined gametic progenitors such as oogonia and spermatogonia, this stage is characterized by a lack of gametes (Eble and Scro, 1996). Histologically, the gonad consists primarily of indifferent primordial germ cells (PCGs), connective tissue, and interstitial cells (Figure 1.2). Interstitial cells primarily support the connective tissue matrix, but also serve a variety of roles such as glycogen storage for gametogenesis (Eble and Scro, 1996).

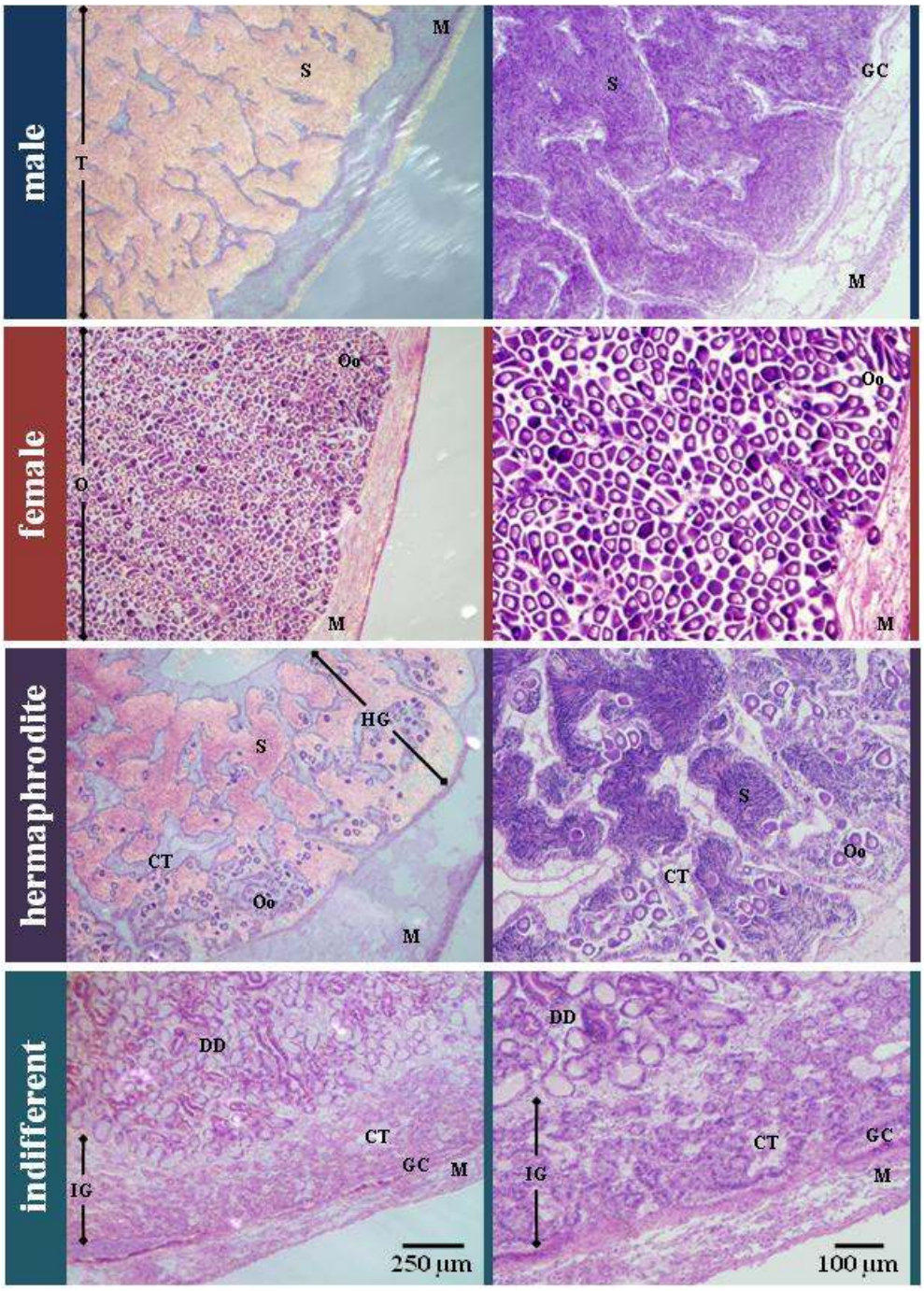

Figure 1.2. Gametic sex classification in the eastern oyster by evaluating histological sections from oysters collected in this study stained with hematoxylin and eosin. Identifiable features include loops of digestive diverticula (DD), genital canals (GC), hermaphroditic (HG) and indifferent gonad (IG), connective tissue (CT), mantle (M), oocytes (Oo), ovary (O), spermatozoa (S), and testis (T). Terminology adapted from a review by Eble and Scro (1996).

During *early development* in gonadogenesis, the primordial germ cells (PCGs) undergo rapid mitosis and differentiate into oogonia or spermatogonia depending on exogenous and endogenous cues (Eble and Scro, 1996). *Hermaphrodites*, individuals possessing oogonia and spermatogonia simultaneously, account for less than 0.5% of a population (Burkenroad, 1931; Needler, 1932; Coe, 1934; Berg, 1969; Kennedy, 1983). Histologically, a mature hermaphrodite has a mixture of full-grown primary oocytes and spermatozoa (Figure 1.2). In females, oogonia contain a large ovoid nucleus within granular basophilic cytoplasm. These ovarian follicles increase in size and oogenesis begins (Figure 1.3). The histological images in Figure 1.4 present the stages of ovarian development and recycling discussed in this section.

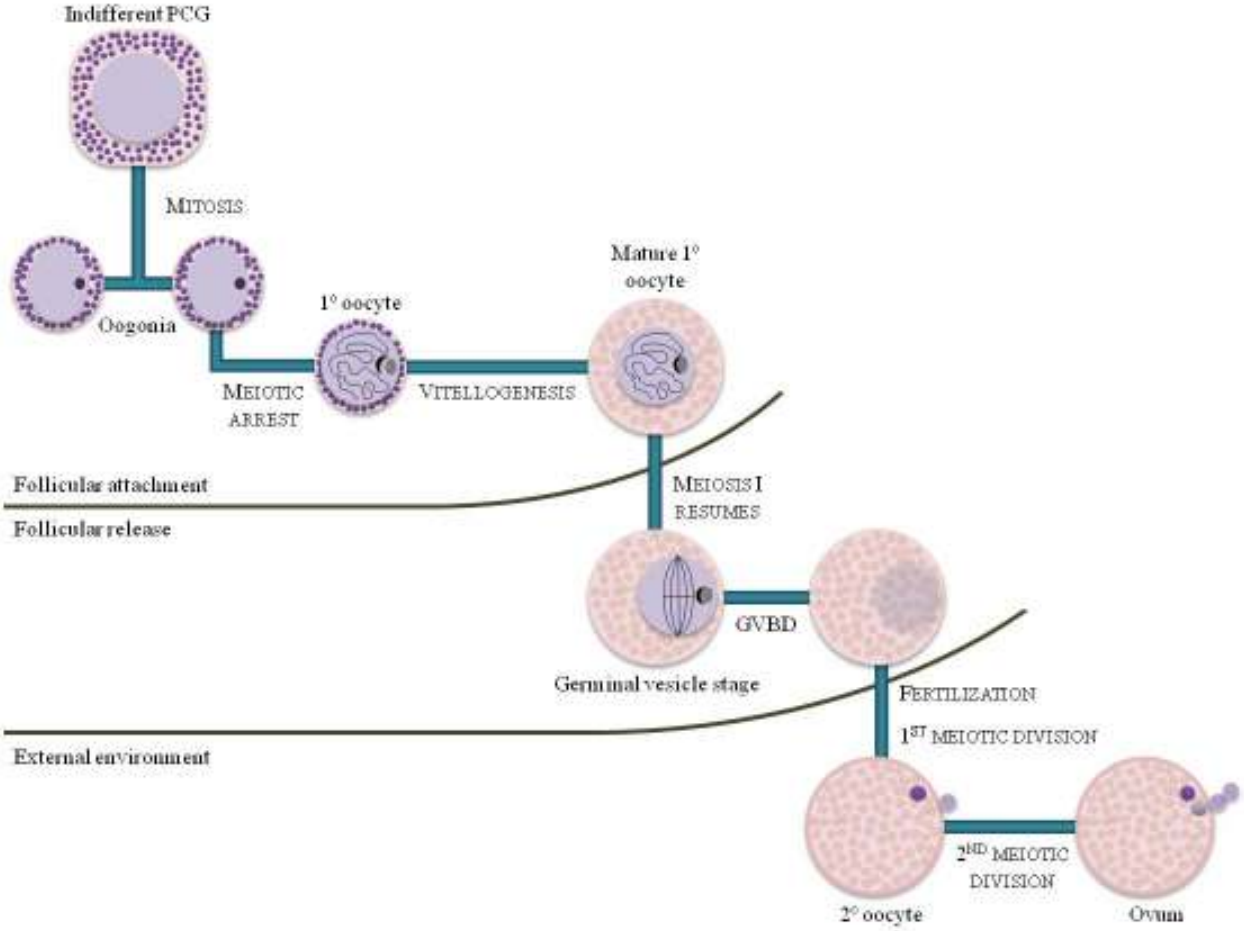

Figure 1.3. Characteristic schematic of landmarks in oyster oogenesis. Adapted from a review by Eble and Scro (1996).

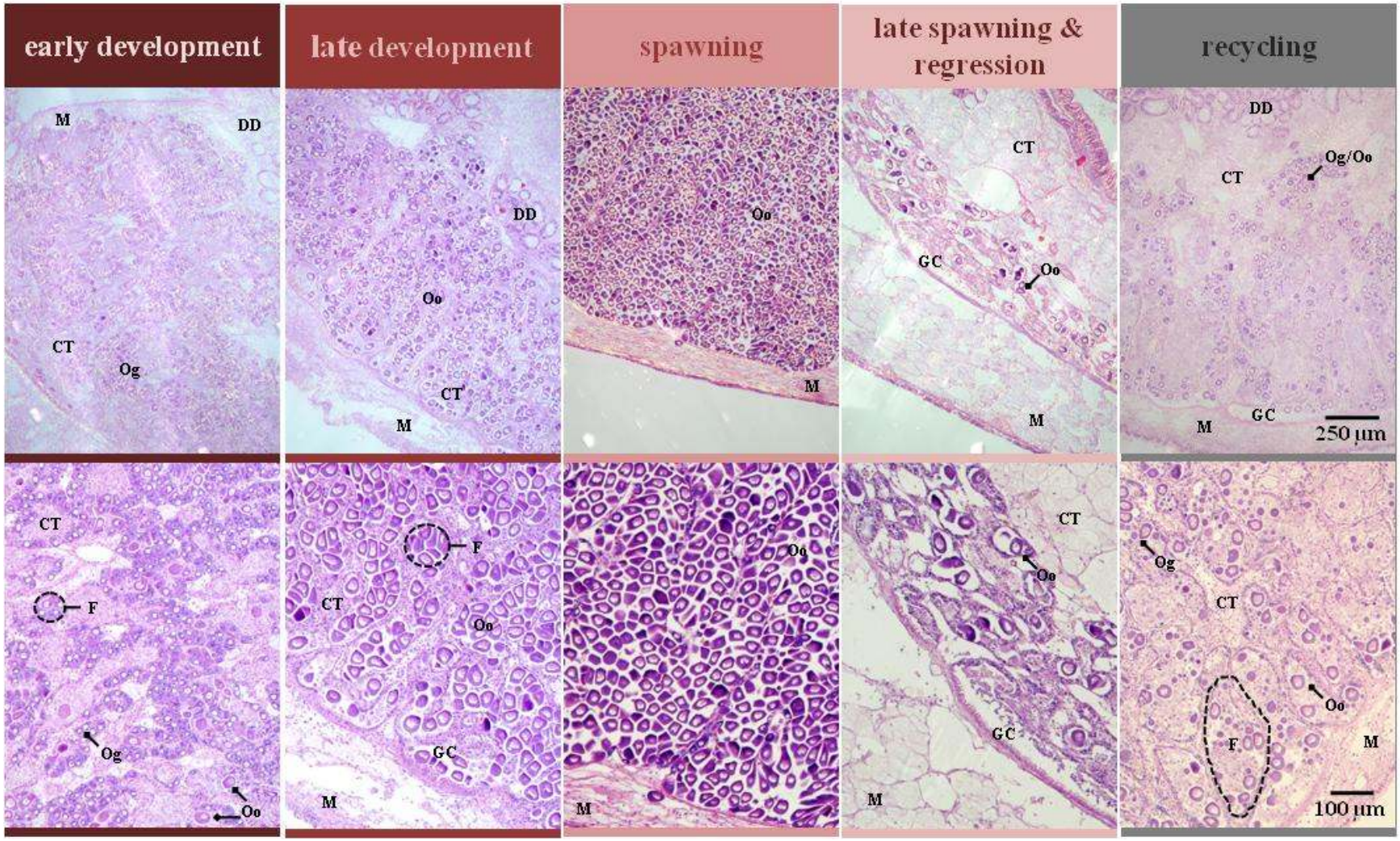

Figure 1.4. Classification of the four stages of ovarian development and recycling in the eastern oyster by evaluating histological sections from oysters collected in this study stained with hematoxylin and eosin. Identifiable features include loops of digestive diverticula (DD), follicles (F), genital canals (GC), connective tissue (CT), mantle (M), oogonia (Og) and oocytes (Oo). Terminology adapted from Kennedy and Krantz (1982), Eble and Scro (1996), and Supan and Wilson (2001).

During oogenesis (Figure 1.3), the oogonia divide and differentiate into primary oocytes. The nucleus increases in size, becoming more spherical; at this stage, the nucleus becomes known as the *germinal vesicle*. Meiosis I is arrested at the diplotene stage of early prophase to prepare for vitellogenesis and the ovarian follicles increase in size, anastamose, and invade the surrounding vascular tissue. During growth, vitellogenin accumulates in the swelling cytoplasmic granules which become slightly acidophilic (Eble and Scro, 1996). There are two parts of the eccentric nucleolus found in the nucleus of growing primary oocytes: the crescent-shaped karyosome (type K) and spherical plasmasome (type P); although initially separate, the parts become closely apposed as the primary oocyte matures (Kobayashi, 1954; Kennedy and Battle, 1964).

The follicles continue to develop and expand, while the amount of connective tissue is reduced to accommodate them. After vitellogenesis, the mature primary oocytes enter *late development* as they begin to detach from the germinal epithelia and migrate to the lumen (Kennedy and Krantz, 1982). This release stimulates the oocytes to resume meiosis I, and spindles form in the cytoplasm to prepare for the first nuclear division. The final stage of oocyte maturation is germinal vesicle breakdown (GVBD) at which time the nuclear envelope dissolves, and the oocyte is ready for fertilization (Kennedy and Battle, 1964).

The oocytes are pushed from their follicular lumen into genital canals by follicular contraction. S*pawning* is characterized by a gonad full of mature, irregularly arranged oocytes with no clear follicular divisions (Kennedy and Krantz, 1982). The spawning event is often triggered by a sharp change in temperature (Medcof, 1939) or the spawning of neighboring oysters (Galtsoff, 1938), resulting in synchronized spawning. Oocytes are forcefully flushed from the genital canals into the surrounding water by contraction of the adductor muscle, forcing

water through the mantle cavity which can be selectively opened or sealed by the pallial curtain (a ciliated fold in the mantle) (Eble and Scro, 1996).

In *advanced spawning and regression*, few unspawned oocytes remain in the lumen and follicles appear disorganized (Eble and Scro, 1996). The gonad may recycle to spawn again, or return to an indifferent state by the resorption of the remaining oocytes and gonadal tissue. During *recycling*, the ovary displays early and late developmental features in some areas of the gonad, while other regions appear atretic (Kennedy and Krantz, 1982; Supan and Wilson, 2001). Resorption is facilitated by phagocytic hemocytes from the hemolymphatic sinuses that supply the follicles as part of the combined open circulation system of the oyster. Interstitial cells proliferate and predominate to reinforce associated follicular connective tissue and store glycogen for the next spawning (Eble and Scro, 1996). Histological images in Figure 1.4 represent the four classifications of ovarian development and recycling described above.

Oogenesis continues outside of the ovary after the oocytes have been expelled from the follicles (Figure 1.3). The first meiotic division occurs after fertilization resulting in the secondary oocyte and release of the first polar body (PBI). At the second division, the second polar body (PBII) is released, signifying the end of oogenesis. A fertilized ovum has been produced.

ADVANTAGES OF COMMERCIAL AQUACULTURE

Although oysters are tolerant to extreme temperature and salinity, aquacultural production can lead to a more consistent product, despite higher initial input costs for equipment and labor. The ability to control site selection, gonadal maturation, artificial spawning, broodstock genetics, and fouling results in a superior product. However, the success of a commercial operation depends on the intensity of the culture technique implemented. Site

selection is the most important factor affecting production. Annual changes in temperature, salinity, and natural food availability all affect the attainable level of production. Although these factors are difficult and expensive to control, others (mentioned above) can be manipulated to increase production above the threshold of a fishery operation. Natural foods are considered best; algal cultures provide supplemental nutrition to oysters at various stages of hatchery production with a combination of naked flagellates (e.g. *Isochrysis galbana*) and diatoms (e.g. *Chaetoceros muelleri*, *Thalassiosira pseudonana*) that provide lipids, proteins and energy required for broodstock conditioning and larval culture (Castanga et al., 1996).

Oyster broodstock fed daily with cultured algae can be held in heated tanks for gonadal maturation at 24°C for 6 to 10 weeks, or to maintain a mature gonadal condition from 16 to 21°C (Castanga et al. 1996). The producer can decide when to artificially spawn individuals and prepare accordingly. Additionally, there is potential that selective breeding can improve the genetic quality and performance of stocks based on enhanced growth rates, increased food conversion, shell quality, or disease resistance, although there has not been much work in this area (Newkirk, 1996).

The lease can be managed by culling and separating to yield single-oyster culture to produce oysters of a more consistent size and shell morphology and increase their marketability to high-end restaurants for sale on the half shell. The removal of dead or dying oysters and debris decreases oyster fouling and loss; predators and disease can be minimized by growing oysters in mesh bags. Off-bottom systems decrease the time to market size when compared to on-bottom culture systems (Parsons, 1974; Castanga et al., 1996); the incorporation of a weekly drying regime can further decrease fouling, predation and disease (Maxwell and Supan, 2006). Another method of increasing profitability of oysters is the advent of polyploidy technology.

POLYPLOID TECHNOLOGY

Polyploid individuals contain more than the normal two sets of chromosomes. Although there are exceptions in a number of species, most progeny receive one set of chromosomes from each parent at fertilization when the male and female pronuclei fuse to form a diploid (2N) zygote. Animals with an odd number of chromosome sets, such as triploids (3N) or pentaploid (5N), face difficulties in gametogenesis. During meiosis in diploid individuals, the sets of chromosomes are divided equally to produce haploid (N) gametes; in triploids, the additional chromosome set does not divide evenly among the gametes and normal gametogenesis cannot occur (Thorgaard, 1983). Because triploid oysters expend less energy on reproduction, they have an increased growth rate, higher meat yields and glycogen stores, and improved meat quality, especially during the summer months when oysters generally consume as much as two-thirds of their body weight during normal gametogenesis (Allen and Downing, 1986). Unfortunately, triploid oysters are not an easy commodity to produce.

The successful development of tetraploid (4N) broodstock, which produce 100% triploids when mated with diploids (Figure 1.5; Guo et al., 1996) is becoming increasing

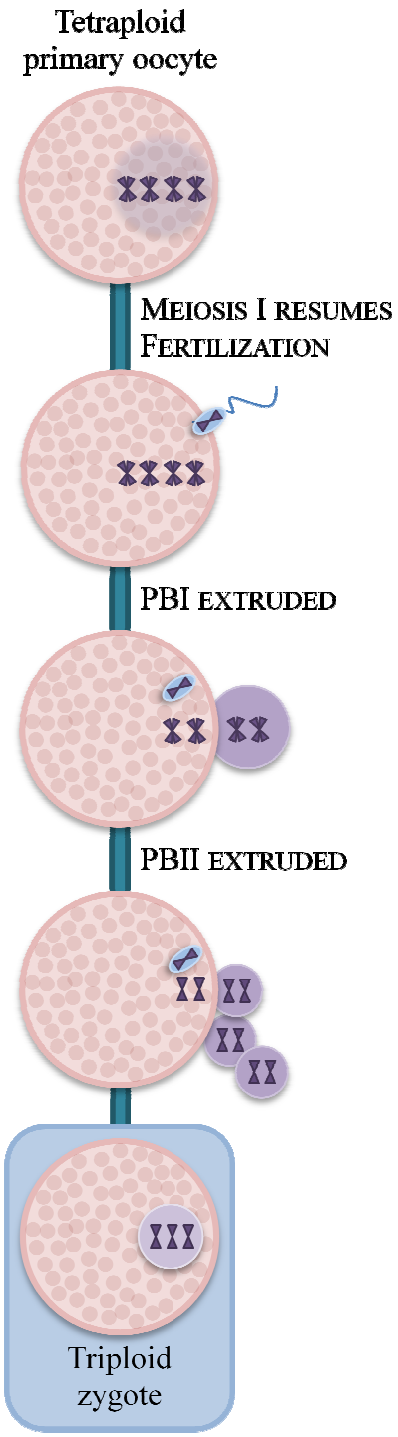

Figure 1.5. Chromosomal events and polar body (PB) extrusion that occurs during triploid production by mating tetraploid females with diploid males.

important to the commercial oyster industry to improve the profitability of intensive production. Because the alternative approach, the direct inhibition of PBI or PBII in fertilized eggs, commonly results in low larval survival and low or variable triploid induction, tetraploid technology is viewed as the key to triploid oyster production. Despite strong interest and repeated attempts, the induction of tetraploids remains a challenge in most molluscan species.

Tetraploid induction has been reported by use of two different approaches. The *direct method* (Figure 1.6) interferes with meiosis or mitosis in fertilized eggs of normal diploids; the other, the *indirect method* (Figure 1.7), inhibits PBI in eggs from triploid females fertilized with sperm from diploid males (Guo and Allen, 1994).

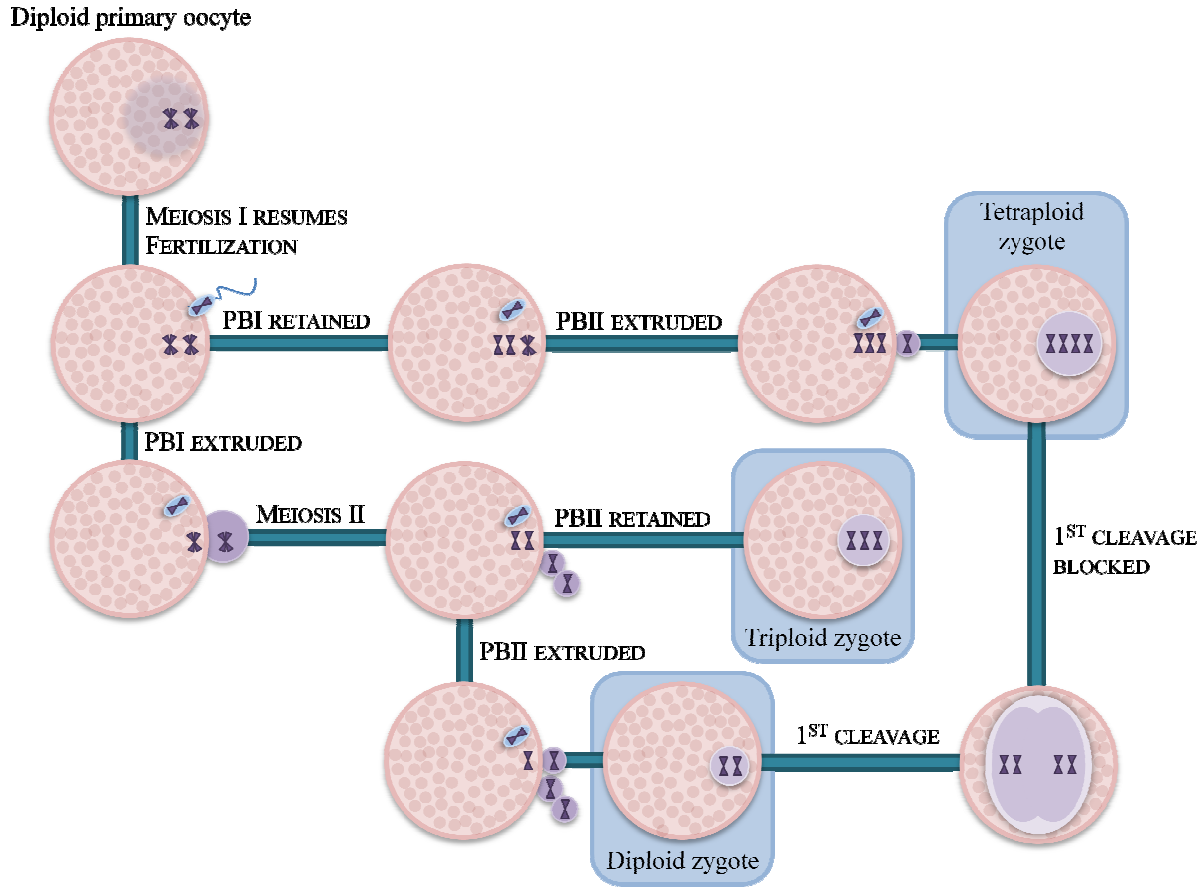

Figure 1.6. Direct methods of ploidy induction produced from diploid broodstock by polar body (PB) retention or blockage of the first cleavage of a diploid zygote.

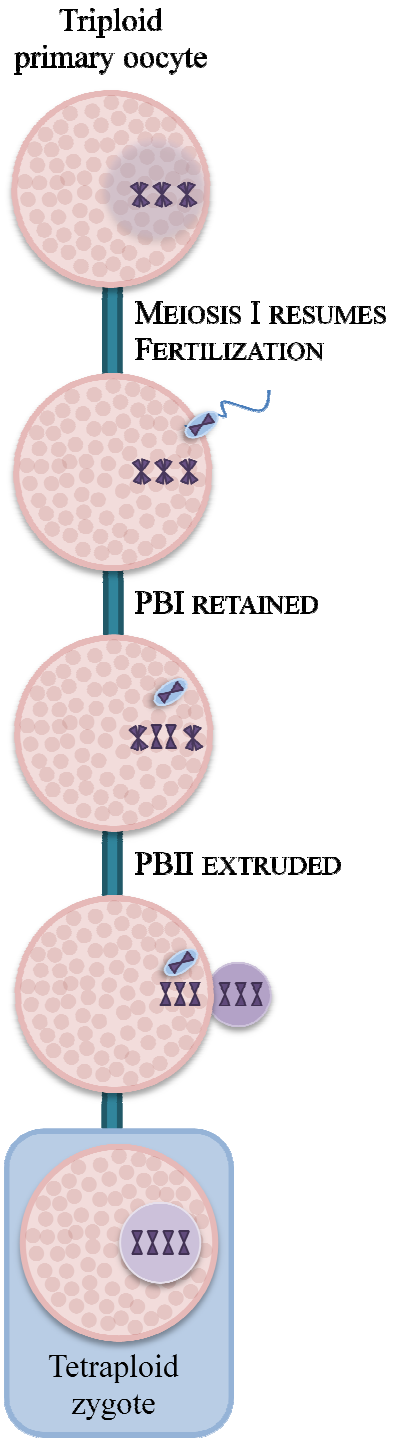

Figure 1.7. Indirect method of tetraploid induction produced from triploid female and diploid male broodstock by polar body (PB) I retention.

Using the direct method, tetraploids have survived metamorphosis in species including the blue mussel *Mytilus galloprovincialis* (Scarpa et al., 1993), Manila clam *Tapes philippinarum* (Allen et al., 1994), zhikong scallop *Chlamys farreri* (Yang et al., 2000), and dwarf surfclam *Mulinia lateralis* (Peruzzi and Guo, 2002; Yang and Guo, 2004). However, each of these studies yielded less than 10 tetraploid individuals, and tetraploid breeding populations were not established. Tetraploid broodstock have been established in the Pacific oyster and are used to produce triploids on the western coast of the United States by the indirect method (Guo and Allen, 1994). Viable tetraploids in other bivalve species have been produced by this method including the pearl oyster *Pinctada martensii* Dunker (He et al., 2000), eastern oyster (Guo et al., 2002), Suminoe oyster *Crassostrea ariakensis* (Allen et al., 2003), and Catarina scallop *Argopecten ventricosus* (Maldonado et al., 2003).

Unfortunately, application of the indirect method is limited by the low availability of eggs from triploid females due to their highly dysfunctional gametogenesis; this makes it difficult to produce tetraploids by the indirect method due to the rarity of triploid eggs. Therefore, approaches to increase triploid gametogenesis are required for routine

tetraploid induction. The administration of hormones is one method that has been reported to encourage ovarian maturation and spawning in bivalve species and was selected as the topic of this thesis to address this problem.

REPRODUCTIVE HORMONES IN BIVALVES

Until recently, little research was conducted on the presence or action of endogenous endocrine hormones in molluscs. Some scientists hypothesize that hormone synthesis in vertebrates evolved from filter feeders, such as bivalves, that can process steroids produced by microalgae or bacteria (Pollio et al., 1994; Panter et al., 1999). Accordingly, when an oyster is exposed to aqueous estradiol in the surrounding water, the hormone is absorbed by the filtration (e.g. gills, labial palps) and digestive systems, or diffused passively across membranes (e.g. mantle, gonad tissues) (Le Curieux-Belfond et al., 2005). Basic biological and agricultural research has investigated classic vertebrate hormones commonly associated with reproduction in invertebrates and determined that testosterone, estradiol-17β, and progesterone are present in the main classes of molluscs, including bivalves (Lafont and Mathieu, 2007).

More specifically, a number of studies have addressed the effect of various hormones on ovarian maturation and spawning of bivalves including the eastern oyster, Pacific oyster, Yesso (Japanese) scallop *Patinopecten yessoensis*, sea scallop *Placopecten magellanicus*, soft-shell clam *Mya arenaria*, and zebra mussel *Dreissena polymorpha*. These hormones include prostaglandins, serotonin, testosterone and two estrogens (Table 1.1). The physiological mechanisms of action of these hormones are not well understood in these species. Although studies of hormone-receptor interactions in marine invertebrates have been reported, researchers have yet to reach consensus in this area. The next section reviews the roles of estrogen in bivalves.

Table 1.1. Review of the actions of endogenous hormones involved in ovarian maturation and spawning in commonly studied bivalves.

Hormone action	Common name	Species	Reference
Estradiol-17β			
Increases seasonally with ovarian development	Pacific oyster Yesso scallop	*Crassostrea gigas* *Patinopecten yessoensis*	Matsumoto et al., 1997
Male-to-female sex change	Pacific oyster	*Crassostrea gigas*	Mori, 1969 Mori et al., 1969
Induces synchronous oocyte maturation	Eastern oyster	*Crassostrea virginica*	Quintana, 2005 Lynn, 2006
	Soft-shell clam	*Mya arenaria*	Gauthier-Clerc et al., 2006
Promotes ovarian development	Sea scallop	*Placopecten magellanicus*	Wang and Croll, 2004
Promotes vitellogenesis	Pacific oyster Yesso scallop	*Crassostrea gigas* *Patinopecten yessoensis*	Li et al., 1998 Osada et al., 2003
Depresses gonadal catecholamines	Yesso scallop	*Patinopecten yessoensis*	Osada and Nomura, 1989
Stimulates serotonin receptors to form on the oocyte surface	Pacific oyster Yesso scallop	*Crassostrea gigas* *Patinopecten yessoensis*	Osada et al., 1998
Estradiol-3-benzoate			
Increases ovarian development	Pacific oyster	*Crassostrea gigas*	Mori, 1969
Male-to-female sex change	Pacific oyster	*Crassostrea gigas*	Mori et al., 1969
Prostaglandins			
Increases during ovarian development (PGE_2 & $PGF_{2\alpha}$)	Yesso scallop	*Patinopecten yessoensis*	Osada et al., 1990
Enhances the effect of serotonin on spawning (PGE_2)	Yesso scallop	*Patinopecten yessoensis*	Matsutani and Nomura, 1987
Inhibits the effect of serotonin on spawning ($PGF_{2\alpha}$)	Yesso scallop	*Patinopecten yessoensis*	Matsutani and Nomura, 1987
Serotonin			
Induces oocyte maturation and spawning	Zebra mussels	*Dreissena polymorpha*	Fong, 1998
Induces spawning	Yesso scallop Eastern oyster	*Patinopecten yessoensis* *Crassostrea virginica*	Matsutani and Nomura, 1982 Lynn, 2006
Testosterone			
Increases during ovarian development	Soft-shell clam	*Mya arenaria*	Gauthier-Clerc et al., 2006
High doses promote ovarian degeneration	Sea scallop	*Placopecten magellanicus*	Wang and Croll, 2004

Estrogens are the most widely studied hormones in bivalves. Generally, estrogens are examined for influences within the gonad, primarily the ovary. Estradiol-17β is the circulating form of estrogen produced in molluscs (Matsumoto et al., 1997; Gauthier-Clerc et al., 2006). It has been found to promote vitellogenesis (Li et al., 1998, Osada et al., 2003) and to synchronize and mature oocytes within the ovary (Quintana, 2005; Lynn, 2006; Gauthier-Clerc et al., 2006). The formation of vitellogenin is crucial in the maturation of primary oocytes during oogenesis, as is synchronization of development to ensure that most or all oocytes are available for simultaneous spawning.

Additionally, estradiol-17β affects physiological neurotransmitter dynamics in bivalves. It depresses gonadal catecholamine levels (Osada and Nomura, 1989), stimulates the formation of serotonin receptors on the surface of oocytes during oogenesis (Osada et al., 1998), and promotes serotonin-induced spawning (Osada et al., 1992). As in many species, catecholamines (e.g. noradrenalin, dopamine) are significantly increased in response to stress in the circulating hemolymph of oysters (Lacoste, 2001). Such hormones have an inhibitory affect on reproduction during times of stress that may be caused by physical disturbance or adverse temperature or salinity. By decreasing the activity of catecholamines within the ovary, more energy could be available for gonadal maturation, rather than in response to stress.

In marine invertebrates, serotonin (5-hydroxytryptamine; 5-HT) has been linked to ovarian maturation and spawning (Matsutani and Nomura, 1982; Fong, 1998; Vaca and Alfaro, 1999; Lynn, 2006). It has a role in reinitiating meiosis I, and induction of GVBD in mature primary oocytes (Toraya et al., 1987; Kyozuka et al., 1997) and egg release (Osada et al., 1998). By increasing serotonin surface-receptor expression on the maturing primary oocyte during

oogenesis, estradiol-17β boosts sensitivity to the presence of serotonin to promote GVBD and spawning (Osada et al., 1998).

Exogenous estradiol-17β not only stimulates ovarian maturation and spawning; it can also induce male-to-female sex change (Mori, 1969). Other synthetic forms of estrogen have been designed for human and animal use. The most commonly used drug, estradiol-3-benzoate, is an estrogen analog that is reported to have a less potent, but longer lasting effect after administration (Chien, 1992). It is also reported to increase ovarian development (Mori, 1969) and induce male-to-female sex change in Pacific oysters (Mori et al., 1969). Estradiol-3-benzoate is further discussed in Chapter 2.

Additional research has linked estradiol-17β to physiological processes in addition to gonadal development and maturation; however, molluscs undergo dynamic changes in biochemical composition to meet the energy demands of gametogenesis. Much of the research in this area was performed in the Mediterranean mussel *Mytilus galloprovincialis* (Canesi et al., 2007). Estradiol-17β was found to modulate lipid and glucose metabolism in the digestive system. In addition, it was also reported that estradiol-17β can play an immunostimulatory role by boosting antioxidant defenses (Canesi et al., 2007) and increasing the lytic and phagocytic responses by hemocytes (Canesi et al., 2004, 2006). However, over-stimulation by high hormone levels has an immunosuppressive effect (Canesi et al., 2006). A similar immune response to estradiol-17β was found in the Yesso scallop *Patinopecten yessoensis* (Matsutani and Nomura, 1986). Because reproductively active individuals have great metabolic energy demands, mobilization of energy stores is vital. The immunostimulatory response is important to protect oysters from pathogens during ovarian maturation, as well as to provide a mechanism to resorb residual oocytes within the ovary after spawning.

The goal of this project was to determine if the use of an estrogen, estradiol-17β or estradiol-3-benzoate, at a consistent environmental temperature would increase the percentage of females in a population, as well as stimulate ovarian development of eastern oysters maintained in an indoor recirculating system. Ultimately, it is hoped that findings can be applied to triploid oysters to increase the overall number of viable oocytes produced for use in tetraploid induction.

The next chapter evaluates the effect of two estrogens, estradiol-17β and estradiol-3-benzoate, on ovarian development. The hormone structure and primary functions, as well as previous research involving estrogens are discussed as they relate to ovarian maturation in a number of species. The third chapter summarizes the overall findings of this project and suggests their applicability to the oyster industry.

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CHAPTER 2. SEX DISTRIBUTION AND OVARIAN DEVELOPMENT OF EASTERN OYSTERS INFLUENCED BY ESTRADIOL-17β OR ESTRADIOL-3-BENZOATE

In response to declining eastern oyster landings attributed to disease, predation, and pollution, approaches to boost annual yields are warranted (Supan, 2000). The use of hormones to improve ovarian fecundity has been used in a number of agricultural species to increase gamete production, synchronize reproduction and oocyte maturation among groups of individuals, and extend periods of reproductive productivity.

Estradiol-17β (Figure 2.1) is a cholesterol-derived steroid hormone, resulting from the aromatization of testosterone, primarily produced in the granulosa cells in the follicles of the ovary; it is the most potent natural form of estrogen. In mammals, the mechanism of estradiol-17β activity is controlled by two estrogen receptors (ER), α and β. To date, the exact synthetic pathway and mechanism of action of estradiol-17β in bivalves is unknown, but its concentration increases *in vivo* at the time of reproductive activity in a variety of molluscan species (Matsumoto et al., 1997; Gauthier-Clerc et al., 2006).

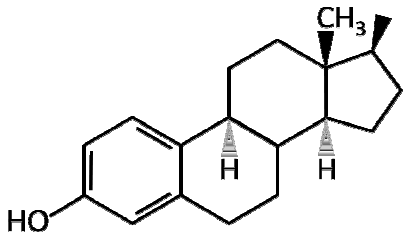

Figure 2.1. Chemical structure of estradiol-17β.

The role of estradiol-17β in bivalve reproductive physiology has been investigated since the late 1960s (Mori, 1969, Mori et al., 1969). Research has determined that it promotes vitellogenesis (Li et al., 1998, Osada et al., 2003), as well as synchronizes and matures oocytes within the ovary (Quintana, 2005; Lynn, 2006; Gauthier-Clerc et al., 2006). Additionally, estradiol-17β upregulates serotonin receptors on the surface of oocytes (Osada et al., 1998). Serotonin has been found to induce spawning in bivalves (Matsutani and Nomura, 1982; Fong, 1998; Lynn, 2006) and has been used in conjunction with estradiol-17β in the eastern oyster to

synchronize ovarian maturation and spawning (Lynn, 2006). When delivered exogenously, estradiol-17β can induce male-to-female sex change (Mori, 1969).

Estradiol-17β is by far the most widely administered form of estrogen in primates and agricultural species (Chien, 1992). This is primarily because it is the biologically active form produced naturally *in vivo*, and subsequently the most studied. Although injection with estradiol-17β is fast-acting, its half-life is only about an hour in mammals; to increase exposure time, esterified forms of the hormone have been used (Chien, 1992; Becker et al., 2005).

Estradiol-3-benzoate (Figure 2.2) is a stable synthetic estrogen analog with a benzoic acid esterified to the third carbon of estradiol-17β; when hydrolyzed *in vivo*, the physiologically active form, estradiol-17β, is released. One intramuscular injection can provide steady concentrations of estradiol-17β for several days. Although its potency is half that of estradiol-17β, the prolonged availability may increase its efficacy (Chien, 1992; Becker et al., 2005).

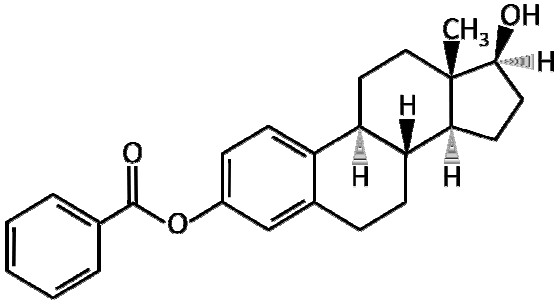

Figure 2.2. Chemical structure of estradiol-3-benzoate.

Estradiol-3-benzoate activity is comparable to the activity of estradiol-17β in humans (Blackmore, 1999). After cattle were injected with estradiol-17β, a sharp spike of activity resulted shortly after administration; alternatively, estradiol-3-benzoate resulted in a lower peak activity post-injection, but the total activity was greater due to sustained plasma levels over time (Souza et al., 2005). Studies in rats have shown that two doses (3 to 36 h apart) of estradiol-17β maybe be more effective than one injection of estradiol-3-benzoate (Sodersten et al., 1981; Clark and Roy, 1987). There is limited research in the use of estradiol-3-benzoate in invertebrates.

In the Pacific oyster *Crassostrea gigas*, the administration of estradiol-3-benzoate has been reported to increase the percentage of females in a population by 15% (Mori et al., 1969) and promote ovarian development (Mori, 1969). Based on this success, the use estradiol-3-benzoate has the potential to improve oyster production as an alternative to estradiol-17β. However, there have been no studies in the eastern oyster. Ultimately, it is necessary to determine the optimal concentration for ovarian maturation and the relationship between estradiol-3-benzoate concentration and the sex ratio of a population.

Based on previous studies (discussed above), the use estradiol-17β or estradiol-3-benzoate has the potential to promote ovarian development and maturation. However, more research is needed in the eastern oyster to determine the optimal conditions for ovarian maturation and the relationship between estrogen dose and activity, as well as the effect on the sex ratio of a population. Increasing the number of females in a population by sex change and stimulating ovarian maturation would provide a greater number of meiotically synchronized oocytes from broodstock for use in artificial spawning in the hatchery.

The goal of this study was to determine if exogenous estradiol-17β or estradiol-3-benzoate administered at a consistent temperature in a closed indoor recirculating system would increase the percentage of females and stimulate ovarian development of eastern oysters. The objectives of this study were to determine the: 1) gametic sex distribution; 2) gonad-to-body ratio, and 3) ovarian development of year-old eastern oysters injected with estradiol-17β in early August (H_0: $\mu_{\text{non-injected}} = \mu_{0\ \text{ng}} = \mu_{75\ \text{ng}} = \mu_{150\ \text{ng}}$; H_1: $\mu_{\text{non-injected}} \neq \mu_{0\ \text{ng}} \neq \mu_{75\ \text{ng}} \neq \mu_{150\ \text{ng}}$), or estradiol-3-benzoate in early February during the indifferent state of the gonad (H_0: $\mu_{\text{non-injected}} = \mu_{37.5\ \text{ng}} = \mu_{75\ \text{ng}} = \mu_{150\ \text{ng}}$; H_1: $\mu_{\text{non-injected}} \neq \mu_{37.5\ \text{ng}} \neq \mu_{75\ \text{ng}} \neq \mu_{150\ \text{ng}}$).

MATERIALS AND METHODS

Animals

Two cohorts of eastern oysters were obtained from the Sea Grant Grand Isle Bivalve Hatchery (29°15'12"N, 90°03'26"W, Caminada Bay, LA). The first cohort, used to study the effects of estradiol-17β, was 12-month-old and collected in August 2006 when the ambient water was ~30°C at roughly 31 ppt salinity. The second cohort, used to study the effects of estradiol-3-benzoate, was 7-month-old and obtained February 2007 when the ambient water was ~16°C at roughly 28 ppt salinity. They were transported 4 to 6 h in burlap sacks dampened with seawater in an ice chest to minimize changes in temperature and decrease drying.

Oysters were placed into three 300-L tanks of a closed recirculating system at the LSU Agricultural Center Aquaculture Research Station (30°22'04"N, 91°10'91"W, Baton Rouge, LA) at ~26°C and 26 ppt salinity (Instant Ocean® Sea Salt, United Pet Group, Inc., Mentor, OH) and fed a commercial shellfish diet (Shellfish Diet 1800®, Reed Mariculture, Campbell, CA) composed of *Isochrysis* (30%), *Pavlova* (20%), *Tetraselmis* (20%), and *Thalassiosira weissfloffi* (30%) at 9% dry matter and ~2 billion cells/mL as directed for 18 days.

Each tank was divided into 4 treatments. In the estradiol-17β study, the treatments included a non-injected control, and three doses of estradiol-17β (0, 75, or 150 ng/oyster), while the treatments for the estradiol-3-benzoate study included a non-injected control, and three doses of estradiol-3-benzoate (37.5, 75, or 150 ng/oyster).

Sample collection and histological analysis

For each experiment, an initial sampling was performed for baseline analysis (n = 40 oysters) on day 0. The remaining oysters (n = 90 oysters/treatment) were acclimated in the system for 2 d. Each oyster was measured (hinge to bill) and notched at the bill of the shell to

allow injection into the adductor muscle (Appendix A, SOP 1). The body weight was estimated for each size class (SOP 2). Following a 2-d recovery after notching, the estradiol-17β or estradiol-3-benzoate working solution was prepared for each size class based on their estimated weight (SOP 3) and the appropriate dose was injected into the adductor muscle; a second dose was injected 3 d later (SOP 1). Oysters were sampled at 10 and 14 d after the initial injection (SOP 4); the tissue sections were stored in Davidson's fixative (SOP 5; Shaw and Battle, 1957).

The histological samples were processed and prepared with hematoxylin and eosin staining by the Histology Preparation Facility in the Department of Comparative Biomedical Sciences at the LSU School of Veterinary Medicine (Baton Rouge, LA). The gametic sex (Figure 1.2) (Eble and Scro, 1996) and ovarian development (Figure 1.4) (Kennedy and Krantz, 1982; Eble and Scro, 1996; Supan and Wilson, 2001) were determined by histological examination using light microscopy (200-X). The gonad-to-body ratio was determined by computer-based image analysis (SOP 6; Quintana, 2005) in the estradiol-17β study, but could not be performed in the estradiol-3-benzoate study due to patchy, incomplete gonadal development.

Statistical analysis

All analyses were performed using SAS® statistical software (SAS 9.1.3, SAS Institute Inc., Cary, NC). The sex distribution and stages of ovarian development were each compared using a Chi-squared analysis (PROC FREQ) with Fishers Exact Test. The gonad-to-body ratios were compared using a four-way analysis of variance (PROC MIXED). For both procedures, the insignificant variables were removed individually, beginning with the highest order (treatment, then day, then sex or stage), from the model stepwise until only values of significance remained ($P < 0.05$). Because recycling was not seen in the estradiol-17β study, it was not included in the analysis of ovarian development.

RESULTS

Estradiol-17β

Gametic sex distribution. Among all treatments and days, the percentage of females was lower than males. No indifferent individuals were present in the baseline analysis (day 0). There were two hermaphrodites on day 0 and the 0 ng dose on day 10. The greatest percentage of females (47%) was in the 75 ng dose on day 10; the smallest (27%) was in the non-injected control on day 10. The highest percentage of males (62%) was observed in the 75 ng dose on day 14; the lowest (40%) was in the 0 ng dose on day 14. There were no significant differences in the percentages among treatments by day ($P > 0.14$) or on day 10 or 14 ($P = 0.81$) (Figure 2.3).

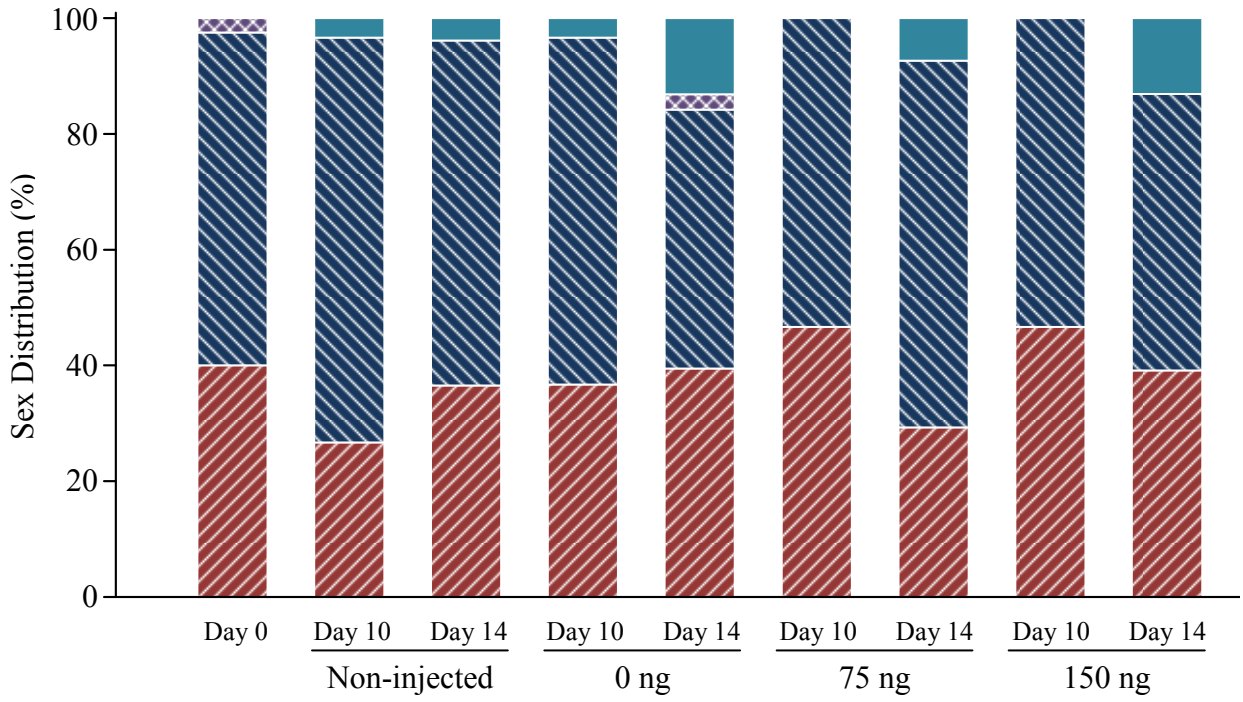

Figure 2.3. Eastern oysters were sampled in August 2006 at 0, 10 and 14 days after estradiol-17β injection. Gametic sex was indentified and individuals were classified as female (▨), male (▨), hermaphroditic (▨), or indifferent (■). There was no significant difference in the percentages among treatments ($P > 0.14$) on days 10 or 14 ($P = 0.81$).

Gonad-to-Body Ratio. Among males, the smallest gonad-to-body ratio was (0.21 ± 0.01, $\overline{x}$ ± SEM) from males on day 14 treated with 150 ng of estradiol-17β. Among the females, only the non-injected control on day 10 exceeded the gonad-to-body ratio from day 0. The highest gonad-to-body ratio (0.38 ± 0.02) was from non-injected females on day 10. The ratios observed on day 10 were significantly higher than those of day 14 ($P < 0.0001$); however, there was no significant difference among the treatments ($P = 0.49$) (Figure 2.4).

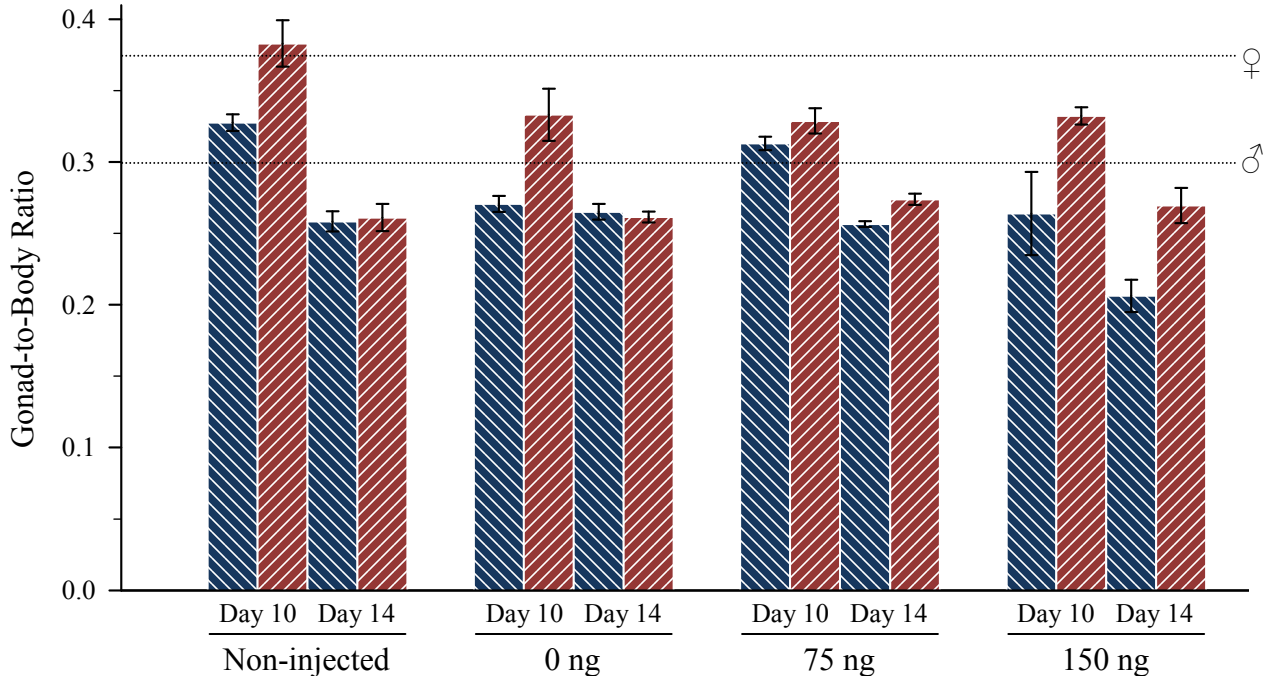

Figure 2.4. In August 2006, the gonad-to-body ratio of male (▚) and female (▨) eastern oysters sampled 10 and 14 days after treatment with estradiol-17β was determined. The baseline values of male (♂) and female (♀) oysters on day 0 is also shown. The ratios observed on day 10 were significantly greater than those of day 14 ($P < 0.0001$), but there was no significant difference among treatments ($P = 0.49$).

Ovarian development. Upon baseline analysis on day 0, all female individuals sampled were in late ovarian development and no indifferent or recycling individuals were detected. By day 10, the percentage of indifferent individuals in the non-injected control and 0 ng dose was approximately 10%, while no indifferent individuals were detected in the 75 and 150 ng doses.

The majority of the females on day 10 were in the late stage of development for all treatments with the exception of the 150 ng dose in which half of the females were in the late development stage and the other half were spawning. In the other treatments, spawning accounted for less than 36% of the sampled population. There were no females in advanced spawning and regression on day 10 (Figure 2.5).

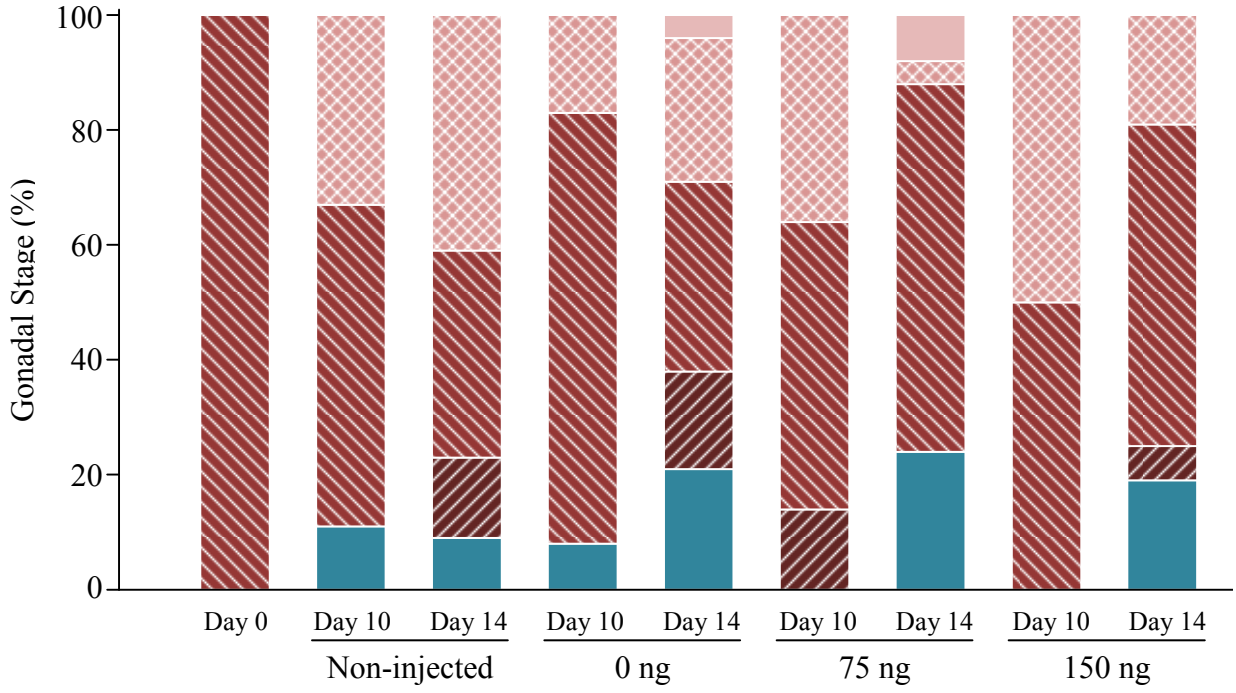

Figure 2.5. Five gonadal states were used to classify eastern oysters collected in August 2006 and treated with estradiol-17β: indifferent ( ), early development ( ), late development ( ), spawning ( ), and advanced spawning and gonadal regression ( ). The stages of oysters treated with 150 ng differed from the other treatments ($P = 0.002$); this treatment also differed by day ($P = 0.004$). No other treatments had an interation of stage by day ($P > 0.10$).

By day 14, the percentage of indifferent individuals ranged from 9 to 24% among all treatments. There were no females in early development for the 75 ng dose. The percentage of spawning females slightly increased in the control group and 0 ng dose from day 10 to 14, while this percentage decreased in the 75 and 150 ng doses. Advanced spawning and regression was

only detected in the 0 and 75 ng doses accounted for less than 8% of individuals in these treatments.

Overall, the stages of oysters treated with 150 ng of estradiol-17β was significantly different from the other treatments ($P = 0.002$); this treatment was also significantly different between day 10 and 14 ($P = 0.004$). No other treatment interactions of stage by day were observed ($P > 0.10$) (Figure 2.5).

Estradiol-3-benzoate

Gametic sex distribution. Among all treatments and days, the percentage of indifferent animals accounted for the highest percentage in all treatments by day except for the 75 ng dose on day 10; there were an equal percentage of indifferent, male and female oysters. There were a number of hermaphrodites; the highest frequency was in the control on day 14.

There was a higher percentage of males except in the non-injected control and 37.5 ng dose on day 14. The highest percentage of males (43%) was in the 75 ng dose on day 14; the lowest (10%) was in the 37.5 ng dose on day 14. The greatest percentage of females (23%) was in the 37.5 ng dose on day 14; the smallest (10%) was in the 75 and 150 ng doses on day 14. The differences in sex distribution between days 10 and 14 for the non-injected control ($P = 0.03$) and 37.5 ng dose ($P = 0.01$) were significant, but there were no significant differences for the 75 or 150 ng dose from day 10 to 14 ($P > 0.20$).

When comparing the significant change in sex distribution between the non-injected control and 37.5 ng dose, it appears that the number of females increased over time; the higher frequency of hermaphrodites in day 14, but total absence in day 10 suggests further similarities between these two treatments. The major difference between the two treatments appears to be in the indifferent and male animals. In the non-injected control, the number of indifferent oysters

decreased by 30%, which appeared to account for the approximate 14% increase in females, 13% in hermaphrodites and 3% in males. Conversely, the increased number of females and hermaphrodites in the 37.5 ng treatment appear to be accounted for by 30% reduction in the number of males (Figure 2.6).

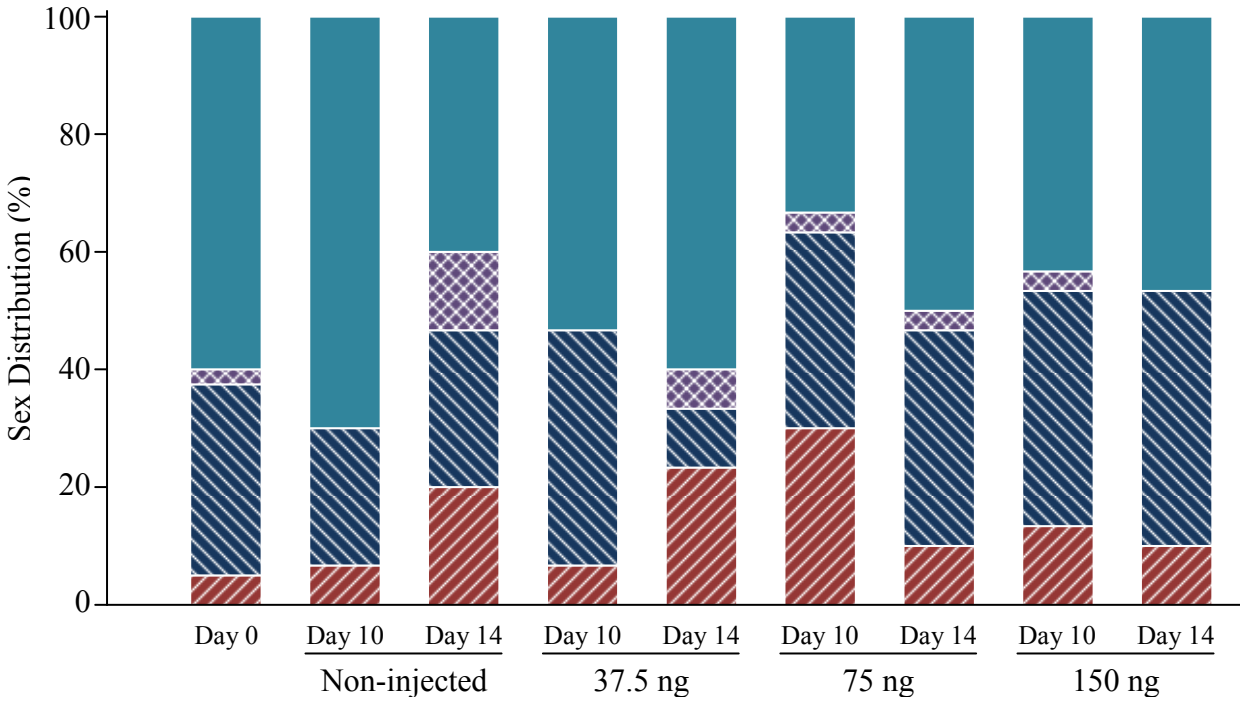

Figure 2.6. Eastern oysters were sampled in February 2007 at 0, 10 and 14 d after estradiol-3-benzoate injection. Gametic sex was identified and individuals as were classified as female ( ), male ( ), hermaphroditic ( ), or indifferent ( ). The difference in sex distribution was significant on day 10 and 14 for the non-injected control ($P = 0.03$) and 37.5 ng dose ($P = 0.01$).

Ovarian development. Upon baseline analysis on day 0, indifferent individuals were the most frequent (92%) when the experiment began. This was also the case for all treatments on days 10 and 14, with the smallest percentage. Although there was not enough gonadal material present to determine the gonad-to-body ratio, patches of ovarian development occurred; the gonadal development overall was incomplete. There was some early development in all treatments on days 10 and 14. Late development occurred on day 14 in all three doses of estradiol-3-benzoate. None of the oysters were staged in spawning or in late spawning and

gonadal regression. Recycling oysters were observed in a total of 5 individuals in the non-injected control and 75 ng dose on day 10, as well as the non-injected control and 37.5 and 75 ng doses on day 14. Overall, there was no significant difference among treatments on day 10 or 14 ($P > 0.26$) (Figure 2.7).

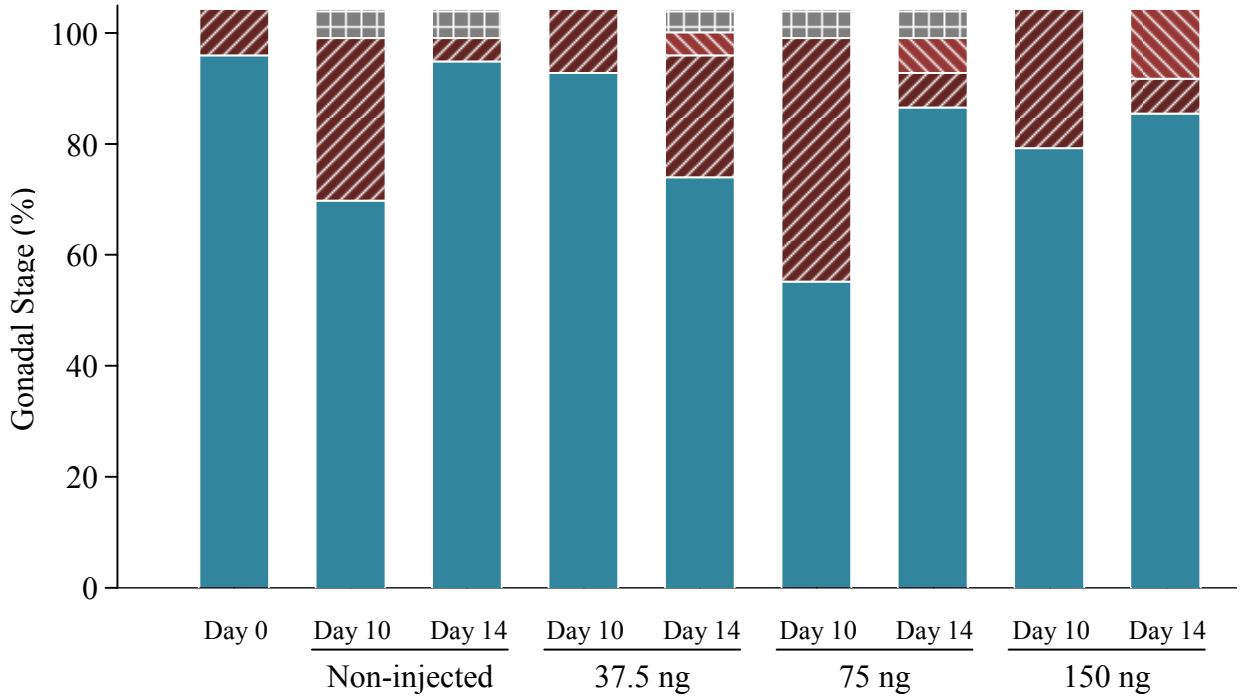

Figure 2.7. Six gonadal states were used to classify eastern oysters collected in February 2007 and treated with estradiol-3-benzoate: indifferent ( ), early development ( ), late development ( ), spawning ( ), advanced spawning and gonadal regression ( ), and recycling ( ). There was no significant different among treatments on day 10 or 14 ($P > 0.26$).

DISCUSSION

In these experiments, the effect of two exogenous estrogens on sex distribution, gonad-to-body ratio, and distribution of the stages of ovarian development was examined in eastern oysters maintained at a static temperature in a recirculating system. Because of the different oyster ages and times of collection, the two studies were not compared directly; instead, each drug was examined independently to determine how it affected the gonadal state a point in the reproductive cycle when it was expected to be indifferent. During each experiment, the effect of

either drug on sex differentiation and ovarian development was not apparent and there was no dose-dependent response.

When estradiol-17β was administered intramuscularly to 12-month-old oysters in August 2006, the percentage of female individuals was not significantly increased and did not overwhelm the percentage of male individuals. After the 7-month-old oysters collected in February 2007 were treated with estradiol-3-benzoate, there was a significant difference between the sex distribution from days 10 to 14 in the non-injected control and the 37.5 ng treatment; it is unclear why no change was observed in the higher doses.

The percentage of hermaphrodites in estradiol-17β-treated oysters (<1% of the total sample population) is consistent with previous observations in which hermaphrodites account for less than 0.5% of the examined populations, while the percentage seen after estradiol-3-benzoate treatment (3.6%) was over 7 times greater (Burkenroad, 1931; Needler, 1932; Coe, 1934; Berg, 1969; Kennedy, 1983). This high percentage does not appear to be linked to estradiol-3-benzoate administration because the highest percentage observed (13%) was in the non-injected control.

With a mean shell height of 58.1 ± 9.5 mm ($\overline{x} \pm s$; median = 57.49; n = 342) for estradiol-17β-treated oysters, the baseline of females (40%) was ten times less than previously reported for oysters greater than 40 mm (Burkenroad, 1931). Similarly, there was a mean shell height of 53.96 ± 7.42 mm ($\overline{x} \pm s$; median = 54.11; n = 280) in estradiol-3-benzoate-treated oysters with a female baseline ratio (5%) was less than the 75% previously reported. However, the same study also reported that the sex ratio of cultured oysters with valve margins of less than 4 cm apart was 1:1 for males and females (Burkenroad, 1931). This could be accounted for by the high density culture methods of the hatchery where these animals were obtained. The lack of indifferent

individuals in the baseline analysis of the oysters in the present study obtained in early August suggests that the population was still reproductively active. Conversely, the oysters collected in February before the breeding season began were primarily indifferent (60%). The higher incidence (33%) of males at this time could be accounted for by the nutrient availability and younger age.

Nutrient availability can affect gametic sex because oogenesis requires more energy than spermatogenesis due to the high vitellogenin content of oocytes (the highest lipid deposits in sperm are located in the plasma membrane) (Russell-Hunter, 1979). Wild foods, as opposed to commercially cultivated algal suspensions, contain a wide variety of natural fauna differing in nutrient content and particle size for selective feeding. In an indoor system, the use of commercial broodstock diets may be nutritionally inadequate for female gonadal differentiation in indifferent individuals. The availability of wild food also varies by season, and gonadal maturation generally coincides with increased nutrient supply in the spring and summer months.

In terms of gonadal development, the gonad-to-body ratios in estradiol-17β-treated oysters suggested that overall development was declining over time. Between days 10 and 14, the drop in the gonad-to-body ratio indicated a significant decrease in gonadal tissue. This decrease in gonad percentage could be attributed to stress-inducing exogenous factors. In the oyster, the presence of stressors increases circulating catecholamine levels; in nature, these stressors include acute changes in the temperature and salinity of the surrounding waters or physical disruption, as well as continuous exposure to an inappropriate temperature, salinity, nutrient availability or parasite load (Lacoste, 2001).

Prolonged stress can result in energy shunts to physiological maintenance to maintain homeostasis, rather than reproductive development (Lacaste, 2001). This can lead to the

metabolism of glycogen stores and gonadal resorption, especially if the stress decreases normal feeding behavior. As previously discussed, nutrient availability is a common limiting factor in gonadal development and may have decreased the reproductive efficiency in this study.

The oysters used in this study were transported for 4 to 6 h from natural waters to an indoor recirculation system and acclimated 2 d before treatment. Acute stress from physical movement and temperature change could have resulted from the partial cooling and drying that occurred during relocation. This stress could have induced spawning once the oysters were placed into warm tanks, reducing the gonad size and affecting development. In August, when the oysters were transported to the experimental tanks, there was a 4°C decrease from the coastal water temperature where the oysters were collected. Similarly, the oysters collected in February were exposed to an increase of 10°C. Because temperature is the most important factor affecting physiology, this acute change could have affected reproductive performance (Shumway, 1996).

Additionally, if one oyster in a tank spawned, it could have induced the spawning of the other individuals in that tank (Medcof, 1939). Because the gonad of the oyster is examined at a given point in time, it would not be possible to determine if spawning occurred unless recycling was seen histologically. This was not the case for any individuals examined in the estradiol-17β experiment. Even if recycling did occur in this experiment, as in the estradiol-3-benzoate experiment, it would be difficult to determine if spawning occurred just prior to sampling, or after indoor relocation. There were no signs of spawning (gametes released) in the tank after relocation.

When comparing ovarian development among treatments in oysters treated with estradiol-17β, the significant difference seen in the 150 ng treatment could indicate that this dose was best for maturing the ovary to spawning; the significant difference between days 10 and 14

in the 150 ng dose could be indicative of recycling. Additionally, the doubling in the ratio of indifferent oysters supports this possibility. Although it has been reported that 4 weeks are required for an oyster to recycle after spawning under natural conditions (Supan and Wilson, 2001), there was no determination of how much time elapses from one stage of gonadal development to the next; therefore, it would be difficult to extrapolate if some of these oysters spawned and recycled. There are indications for recycling that can be determined histologically, but it requires sampling the oyster at the particular point in time during ovarian development when late spawning and regression overlaps with early development, before the gonad resynchronizes. Because the baseline sample for the oysters collected in August consisted exclusively of animals in late development, it is likely that some oysters spawned and recycled.

Acute and long-term exogenous factors could have affected this study performed in an indoor recirculating system. Increases in the occurrence of females and advances in the stage of gonadal development are possible by stimulating gametogenesis prior to or late in the spawning season by using exogenous estrogen. Evaluation of gamete quality would be advantageous because although ovarian size decreased, the gamete quality may have increased in oysters treated with estradiol-17β or estradiol-3-benzoate due to increased vitellogenin deposition and upregulation of serotonin receptors of the surface of the oocyte; therefore, future research should incorporate fertilization and larval survival trials to evaluate this possibility.

Ultimately, the use of estrogens to promote an increase in the occurrence of females, as well as stimulate ovarian development and maturation, has been demonstrated by studies in bivalves, although all previous work was performed in broodstock maintained in natural seawater. The ability to synchronize and mature female broodstock by estrogen administration

and to control temperature in an indoor recirculating system has implications for decreasing predation and disease, and increasing control of the environmental factors affecting reproduction.

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CHAPTER 3. SUMMARY AND CONCLUSIONS

The eastern oyster has cultural and commercial relevance and value in Louisiana. Although Hurricanes Katrina and Rita in 2005 damaged this industry by losses estimated between 20 and 74%, intensive management of oyster leases could accelerate recovery and allow Louisiana to remain the leader in the U.S. oyster industry (Pflieger and Lumsden, 2007). Additionally, the profitability of the oyster industry fluctuates with variations in seasonal meat yields which decrease by as much as two-thirds during the summer spawning season (Allen and Downing, 1986). The application of triploid technology can achieve higher meat quality and yields year-round.

The most consistent triploid production method is by crossing of tetraploid females with diploid males, resulting in 100% triploids (Guo et al., 1996). The hurdle to overcome is the poor reproductive efficiency that makes triploid individuals so marketable; this complicates the production of tetraploid broodstock by the direct method because it requires triploid oocytes (Guo and Allen, 1994; Guo et al., 2002). Additional advances in promoting triploid oogenesis are essential. The administration of estrogen may be the key to successful ovarian maturation in triploids, but the dose and route of administration must be determined.

In this study, two estrogens were evaluated: the naturally occurring estradiol-17β and estradiol-3-benzoate, a synthetic analog. Because they were administered at two different times of the year to two different oyster populations, the results were not intended to be compared directly. Estradiol-17β was administered to 12-month-old oysters collected in August, near the end of spawning season when the oysters were expected to have completed at least one spawning and should be indifferent or in early development. Estradiol-3-benzoate was administered to 7-month-old oysters collected in February prior to the spawning season when they are expected to

be reproductively inactive and indifferent. It was hypothesized that administering hormones to indifferent oysters and holding at a constant temperature (26°C) would promote ovarian differentiation, development and maturation from an indifferent gonad.

Although the aim of both treatments was to target indifferent oysters, the water temperature and season of the animals affected the sex ratios of the two populations, as well as their gonadal states. Because October is the last month in which oysters are found to recycle and the natural spawning season does not occur until May, the February oysters were far from being reproductively active. This appeared to be the case in the baseline sampling (day 0) of the experimental populations in which 60% of the oysters collected in February were indifferent, compared to a total lack of indifferent animals from those collected in August. The August oysters may have already spawned and recycled at least once.

Although previous research has demonstrated the endogenous presence of estradiol-17β and other reproductive hormones in molluscs, the synthesis and action of these hormones are still being studied. The presence of aromatase, which converts androstenedione into estrone in the synthesis of estrogen from testosterone (Matthiessen and Gibbs, 1998; Oberdörster and McClellan-Green, 2002), as well as 17β-hydroxysterol dehydrogenase which metabolizes estradiol-17β into estrone (Le Curieux-Belfond et al., 2001) in molluscs further demonstrates a vertebrate-like hormone synthesis pathway of sex hormones. Laboratories have attempted to isolate the functional estrogen receptor (ER) in bivalves, but have not yet been successful.

Estrogen receptor-like immunoreactivity has been demonstrated in the nuclei of oocytes and follicular cells of bivalves (Dorange and Le Pennec, 1989; Osada et al., 2003). Western blots of hemocyte protein revealed the presence of immunoreactive ER α- and ER β-like proteins (Canesi et al., 2004). Additionally, a 266-bp fragment was isolated in *Mytilus* and found to

match the human ER β gene exactly (Stefano et al., 2003). Although 86 to 89% DNA sequence agreement was discovered between the ERs of the Pacific oyster *Crassostrea gigas* and human in the DNA-binding domain and 45% in the ligand-binding domain, the receptor was deemed unresponsive to estrogen after a reporter gene assay. The ERs were primarily localized in oocytes at the site of vitellogenesis, and in the nuclei of follicular cells (Matumoto et al. 2007). These studies determined the presence of the ER in molluscs, but were unable to demonstrate their ability to function in response to estrogen.

Although ER sequences in bivalves and other species have shown homology with those in mammals, it is important to note that sequences are not identical among mammalian species (Köhler et al., 2007). Recently, there have been claims of the complete absence of estrogen receptors in molluscs based on the inability to demonstrate estrogen-specific activation of the receptors that have been identified (Keay and Thornton, 2009); however, no alternative mechanisms of action have been suggested.

The efficacy of a drug is often determined by its mode of delivery. As previously discussed, estrogens are lipid soluble and readily absorbed. Due to rapid metabolism, the action of estradiol-17β is limited to about an hour in humans, while esterified forms, such as estradiol-3-benzoate have been used to increase the exposure time to several days due to the hydrolysis required to release the active estradiol-17β from the analog (Chien, 1992; Becker et al., 2005). In one study, intramuscular (IM) injection was used to deliver the hormones. Because oysters have an open circulatory system, the proposed route of estradiol-17β perfusion though tissues is from the adductor muscle, though the gills, and to the heart where the hormone is circulated to the peripheral tissues (gonad, mantle, labial palps and digestive gland). In the same study, high performance liquid chromatography was used to examine estradiol-17β metabolism after IM

injection of radioactive estradiol-17β (4 μM/oyster). Only half of the drug was detectable in oysters after 10 min, while the other 50% was excreted into the environment; the remaining estradiol-17β was metabolized to estrone in all tissues in less than 2 h (Le Curieux-Belfond, 2005).

Although oysters in the present experiments were kept dry 30 min after injection before returning to the water, release of the hormone into the tank could have occurred. Because estrogen uptake readily occurs through filtration (Le Curieux-Belfond, 2005), levels of exogenous estrogen could affect reproduction; however, it is difficult to determine if the resulting estrogen levels would be high enough to have an effect. The injection method is more controlled than placing the animals in water containing high levels of estrogen because the immersion method makes it difficult to measure the dose of estrogen received by individual oysters.

Because of the short half-life of estrogens, a controlled-release drug delivery system could be more effective. Normally, estrogen is released during gonadogenesis and ovarian maturation. By providing a consistent, elevated level of estrogen, it may be possible to increase the number of reproductive mature females in a population. One example of a slow-release delivery system that has been used in mammals employs crystalline hormone implants in silicone tubing; lipophilic steroid hormones are dissolved within the silicone and released at a constant rate. The rate of delivery depends on the surface area and thickness of the silicone tubing (Dziuk and Cook, 1966; Johnston and Davidson, 1979). Circulating hormone concentrations remain relatively constant due to the consistent rate of release by the silicone compared to spikes seen in the circulation as a result of injections (Legan et al., 1975).

As in many animal production systems, the administration of estrogens to increase reproductive fecundity may be the key to ovarian maturation in triploid oysters for the production of tetraploid broodstock by the indirect method. Currently, work by various groups aims to determine the mechanisms of action and activity of estrogens in molluscs. The appropriate concentration and delivery method of the drug would maximize efficacy. Future research should employ controlled-release delivery systems and compare estradiol-17β activity with that of estradiol-3-benzoate in the eastern oyster. As the leading oyster producer in the United States, technologies such as triploidy would keep Louisiana at the forefront the industry. After losing many of the oyster landings after the hurricanes of 2005, producers need methods to increase production and are open to new technology more than ever.

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APPENDIX A: STANDARD OPERATING PROCEDURES

Six standard operating procedures (SOPs) have been written to supplement the Materials and Methods section of Chapter 2. Materials and step-by-step directions were provided. When appropriate, the references from which these techniques were adapted are also given.

SOP 1. OYSTER PREPARATION AND ESTRADIOL DELIVERY

Materials

Heavy-duty 4-1/2" (115 mm) small angle grinder (D28114, DEWALT, Hampstead, MD)
Estradiol working solution (SOP.4)

Procedure

1. Acclimate oysters in the closed recirculating system for 2 d.

2. Use the grinder to notch each oyster at the bill of the shell to allow injection into the adductor muscle.

3. Measure and classify the oysters by size (e.g. 40-50, 50-60, 60-70, and 70-80 mm).

4. Allow oysters to recover from notching for 2 d.

5. Inject the appropriate dose and volume of estradiol working solution into the adductor muscle.

6. Keep the oyster dry 30 min prior to returning it to appropriate tank.

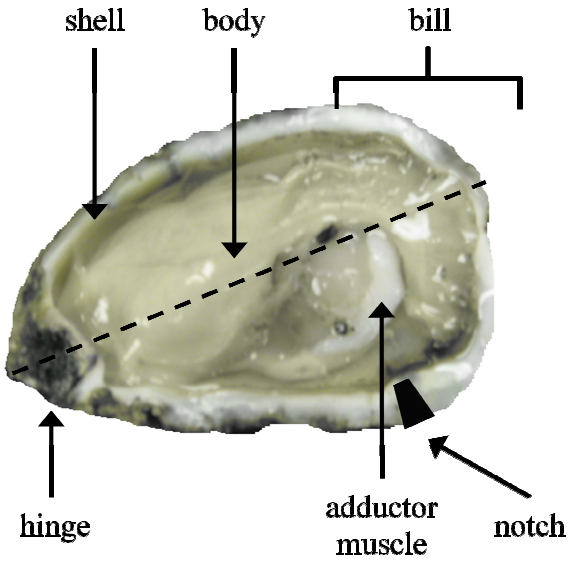

Figure A.1. Photograph of an opened oyster identifying the location of the adductor muscle in relation to the bill. The oyster shell is notched to provide access to this muscle. The dotted line represents the shell height measured from the hinge to the bill.

7. After 3 days, inject a second dose of estradiol and return the oysters to the appropriate tank 30 min after injection.

SOP 2. CORRELATION OF SHELL HEIGHT TO BODY WEIGHT

Materials

Oyster sample population	Digital balance
Digital calipers	Microsoft Excel (Microsoft Corp., Redmond, WA)
Commercial oyster shucking knife	Personal computer

Procedure

1. Using the digital calipers, determine the shell height (mm) by measuring the distance from hinge to bill for each oyster in the subpopulation (Figure A.1).

2. Shuck each oyster and determine respective body weights (g) using the digital balance.

3. Enter the data into a Microsoft Excel spreadsheet and create a scatter plot [x = shell height (mm); y = body weight (g)] with a power fit trendline to determine an R^2 value.

4. If $R^2 \geq 0.7$, use the equation to predict the average body weight (y) in each shell-height size class (x).

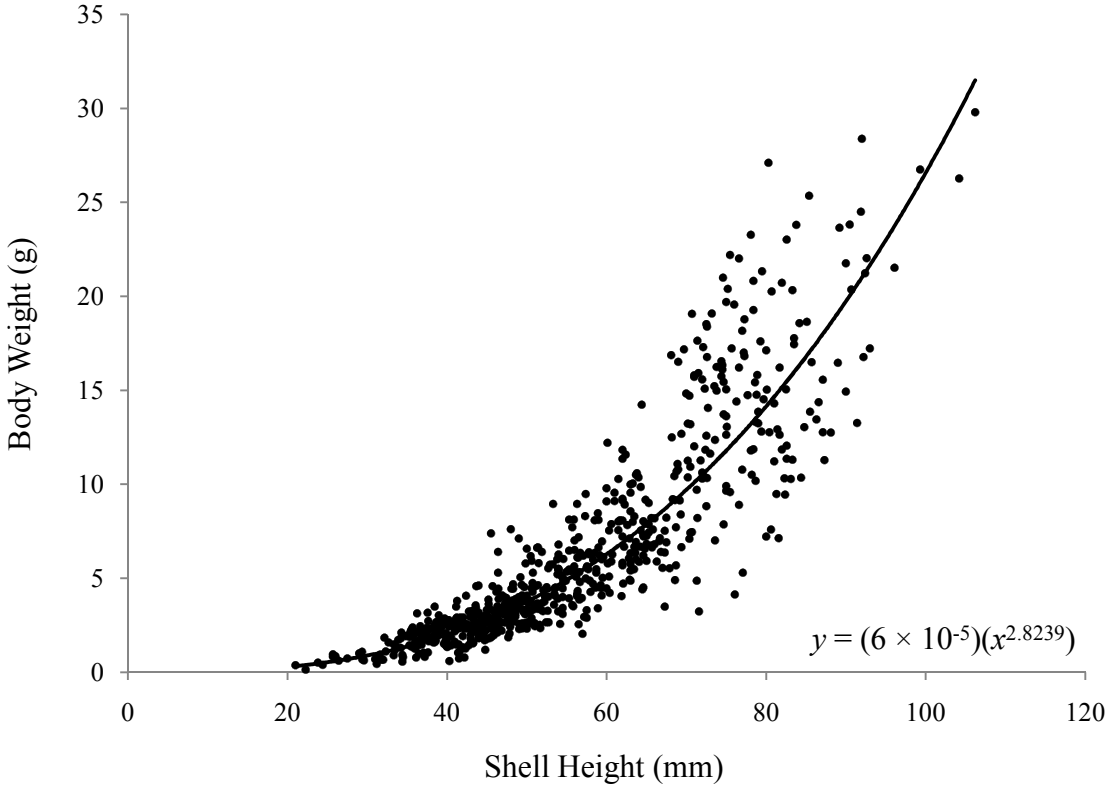

Figure A.2. Power-fit trendline estimating the correlation between shell height (mm) and body weight (g) from previously collected data ($R^2 = 0.85$).

SOP 3. ESTRADIOL SOLUTIONS

Materials

β-Estadiol [FW = 272.39, min 98%] (E8875, Sigma-Aldrich, Inc., St. Louis, MO)
β-Estadiol 3-benoate [FW = 376.5, approx. 98%] (E8515, Sigma-Aldrich, Inc., St. Louis, MO)
Dimethyl sulfoxide (DMSO) [FW = 78.13] (Fisher Scientific, Fair Lawn, NJ)
Filtered artificial seawater (0.45 μm, 20 ppt, Instant Ocean® Sea Salt, United Pet Group, Inc., Mentor, OH)
10-mL glass scintillation vials
Digital balance

Procedure

1. Create shell-height size classes in 10 mm increments (e.g. 30 to 40, 40 to 50, and 50 to 60 mm).

2. Using the equation from the model developed in SOP 3, determine the estimated body weight (g) for each size class using an average shell height (e.g. 35, 45, and 55 mm).

3. Multiply the estimated body weight by the estradiol dose (e.g. 37.5, 75, or 150 ng), and multiply by 1000.

4. Next, divide the result from Step 3 by the final target volume of solution (e.g. 50, 100, 150 μL). Volumes should be chosen based on the size class to insure the solution does not exceed the volumetric capacity of the adductor muscle of the oyster.

5. Using a digital balance, measure the weight (mg) of the estradiol.

6. To determine the volume (mL) of DMSO to add, divide the weight (mg) from Step 5 by the concentration (μg/μL).

7. In a 10 mL glass scintillation vial, dissolve the estradiol in the DMSO and shake vigorously. This is the **stock solution** and should be prepared fresh to ensure efficacy.

8. To prepare the **working solution**, dilute the stock solution 1:1000 in filtered seawater.

SOP 4. SAMPLE COLLECTION

Materials

Digital balance
Commercial oyster shucking knife
Razor blade
Histological tissue cassette (Omnisette, Fisher Scientific, Pittsburg, PA)
1-L glass jar with screw lid
750 mL Davidson's fixative (working solution, SOP 5)
Parafilm® (Pechiney Plastic Packaging Company, Inc., Chicago, IL)

Procedure

1. Record the whole weight (g), body weight (g), and shell height (mm) of each oyster.

2. Open the oyster with a shucking knife, separating adductor muscle from both valves.

3. Section oysters using the method described by Morales-Alamo and Mann (1989) by placing the oyster ventral side up and using the razor blade to make the first cut at the labial palp-gill junction and the second 4 mm posterior to the first (to remove a section containing gill tissue).

4. Remove the transverse section and place it into a tissue cassette facing side of the labial palp-gill junction down.

5. Place the closed cassette into a 1-L glass jar containing 750 mL of Davidson's fixative (SOP 5) working solution.

6. Seal the glass jar with Parafilm® prior to storage.

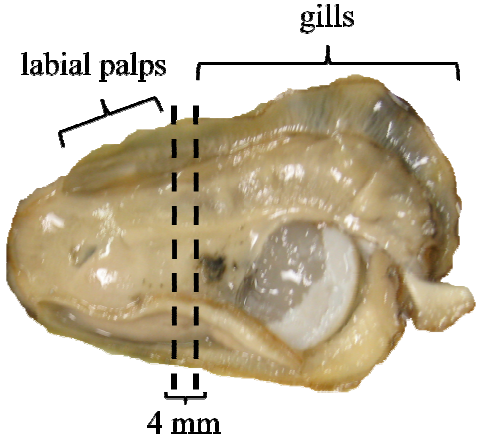

Figure A.3. Photograph of an oyster body identifying the regions of the labial palps and gills. The dotted lines mark the location of the two cuts performed for histological sectioning.

7. Protect the fixed sections from light until histological processing and staining with hematoxylin and eosin by the Histology Preparation Facility in the Department of Comparative Biomedical Sciences at the LSU School of Veterinary Medicine.

REFERENCE

Morales-Alamo, R., and R. Mann. 1989. Anatomical features in histological sections of *Crassostrea virginica* (Gmelin, 1971) as an aid in measurements of gonad area for reproductive assessment Journal of Shellfish Research 8:71-82.

SOP 5. PREPARATION OF DAVIDSON'S FIXATIVE

Materials

Fume hood
Protective eyewear
Latex gloves
Stir plate with magnetic rod
750 mL ethanol 95% (AAPER Alcohol, Shelbyville, KY)
500 mL formalin 37% [FW = 30.03] (Fisher Scientific, Fair Lawn, NJ)
250 mL glacial acetic acid [FW = 60.05] (Mallinckrodt Baker Inc., Phillipsburg, NJ)
750 mL filtered seawater (0.45 μm) from oyster tank

Procedure

1. Wear appropriate gloves and eyewear.

2. Within a fume hood, combine the three reagents stepwise to allow each to thoroughly mix on the stir plate before adding the next (**stock solution**).

3. Store the **stock solution** for as long as 6 months in a dark place.

4. To prepare the **working solution**, slowly add 750 mL of filtered seawater to the stock solution while using a stir plate to continuously mix the solution.

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SOP 6. GONAD-TO-BODY RATIO DETERMINATION

Materials

Histological slides of a stained transverse oyster section
Flatbed scanner (HP Scanjet 3670 Scanner, Hewlett Packard, Palo Alto, CA)
Metaview 6.1 (Universal Imaging Corporation, Downingtown, PA)
Personal computer (Dell Precision workstation 360, Dell Inc., Austin, TX)

Procedure

1. Arrange the slides in two rows of five on a flatbed scanner.

2. Acquire an image at 300 dpi and save it in tagged image file format (TIFF).

3. Open the image in Metaview 6.1 and create an individual image file for each oyster section.

4. Using the area tool, trace the gonadal area of each section and record the data, followed by the perimeter of the entire body. These measurements require approximately 3 min/oyster.

5. To determine the gonad-to-body ratio, divide the total area of the gonad by the total area of the body.

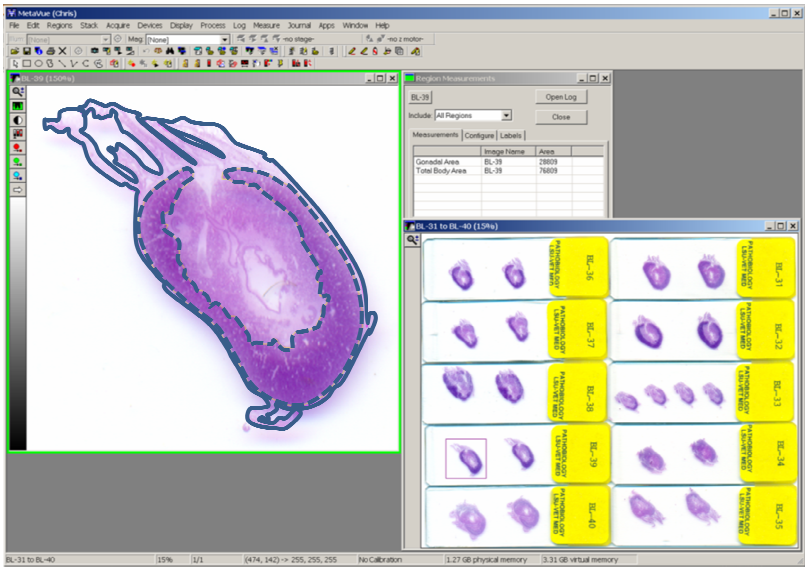

Figure A.4. Screen image capture of tracing and recording the area of the gonad (dotted outline) and area of the body contour (solid outline) using Metaview 6.1 image analysis software. The software calculates the area and lists the data in a spreadsheet next to the image.

REFERENCE

Quintana, R. 2005. Effect of estradiol-17β on the gonadal development of diploid and triploid female eastern oysters. Electronic Theses and Dissertaions, Louisiana State University, Baton Rouge, LA. 123 pp.

APPENDIX B: INDIVIDUAL OYSTER DATA

The two subsequent unanalyzed (raw) data tables include all data collected (in addition to a digital copy located in the back cover of this thesis) on individual oysters from the experiments using estradiol-17β (Table B.1) and estradiol-3-benzoate (Table B.2). These include sampling date, day post-injection, tank number (1, 2, or 3), treatment and individual oyster number (untreated baseline sample on day 0 = BL, non-injected control = C, or estrogen dose = E_0, $E_{37.5}$, E_{75} or E_{150}), shell height (mm), whole and body weights (g), gonad-to-body ratio (G:B), gametic sex (male = M; female = F; hermaphrodite = H, and indifferent = I) and stage of ovarian development (indifferent = 0; early development = 1; late development = 2; spawning = 3; late spawning and ovarian regression = 4, and recycling = R).

Table B.1. Raw data collected to determine the influence of estradiol-17β on the gametic sex distribution and ovarian development of 12-month-old eastern oysters collected from the Sea Grant Grand Isle Bivalve Hatchery (29°15'12"N, 90°03'26"W, Caminada Bay, LA) in August 2006 when the ambient water was ~30°C and 31 ppt salinity.

Sampling date	Day	Tank	Treatment		Shell height (mm)	Weight (g)		G:B	Gametic sex	Stage
						whole	body			
07/28/06	0	–	BL	1	39.04	32.13	4.28	0.32	M	–
07/28/06	0	–	BL	2	27.58	31.78	4.07	0.37	M	–
07/28/06	0	–	BL	3	34.40	65.05	6.17	0.35	F	2
07/28/06	0	–	BL	4	22.30	24.03	4.38	0.36	M	–
07/28/06	0	–	BL	5	30.50	40.40	5.02	0.35	M	–
07/28/06	0	–	BL	6	39.06	50.19	5.70	0.32	M	–
07/28/06	0	–	BL	7	44.43	41.48	4.89	0.31	M	–
07/28/06	0	–	BL	8	65.14	35.56	3.98	0.19	M	–
07/28/06	0	–	BL	9	64.87	34.38	5.13	0.29	M	–
07/28/06	0	–	BL	10	62.32	48.60	4.53	0.38	F	2
07/28/06	0	–	BL	11	48.14	29.36	2.94	0.14	M	–
07/28/06	0	–	BL	12	57.18	39.36	4.10	0.28	M	–
07/28/06	0	–	BL	13	81.05	47.12	5.04	0.29	F	2
07/28/06	0	–	BL	14	55.30	38.41	3.73	0.06	H	–
07/28/06	0	–	BL	15	85.05	51.68	5.50	0.41	F	2
07/28/06	0	–	BL	16	49.73	23.87	2.14	0.38	F	2
07/28/06	0	–	BL	17	68.50	54.91	4.33	0.27	F	2
07/28/06	0	–	BL	18	48.78	23.64	2.43	0.42	M	–
07/28/06	0	–	BL	19	78.80	40.84	4.25	0.31	M	–
07/28/06	0	–	BL	20	24.23	66.63	7.45	0.39	M	–
07/28/06	0	–	BL	21	56.54	33.49	3.70	0.31	F	2
07/28/06	0	–	BL	22	63.13	40.29	4.95	0.42	F	2
07/28/06	0	–	BL	23	58.51	34.83	5.88	0.38	F	2
07/28/06	0	–	BL	24	51.08	20.17	2.33	0.42	M	–
07/28/06	0	–	BL	25	55.69	32.52	4.91	0.08	M	–
07/28/06	0	–	BL	26	67.16	39.48	6.83	0.29	M	–
07/28/06	0	–	BL	27	40.92	11.87	1.87	0.31	F	2
07/28/06	0	–	BL	28	55.30	31.94	6.39	0.32	M	–

Table B.1 continued

07/28/06	0	–	BL	29	56.21	27.54	3.55	0.35	F	2
07/28/06	0	–	BL	30	49.26	19.66	3.27	0.35	F	2
07/28/06	0	–	BL	31	47.68	26.82	3.90	0.43	F	2
07/28/06	0	–	BL	32	53.64	32.28	4.44	0.39	M	–
07/28/06	0	–	BL	33	54.14	19.11	1.98	0.16	M	–
07/28/06	0	–	BL	34	45.17	20.79	2.74	0.59	F	2
07/28/06	0	–	BL	35	67.55	45.70	5.92	0.15	F	2
07/28/06	0	–	BL	36	47.25	23.84	3.07	0.32	M	–
07/28/06	0	–	BL	37	61.80	30.66	4.72	0.31	M	–
07/28/06	0	–	BL	38	63.11	67.87	6.80	0.36	M	–
07/28/06	0	–	BL	39	54.79	30.22	4.12	0.37	M	–
07/28/06	0	–	BL	40	63.00	42.27	5.51	0.38	F	2
08/09/06	10	1	C	1	60.36	46.03	4.05	0.48	M	–
08/09/06	10	1	C	2	60.15	33.62	3.96	0.33	M	–
08/09/06	10	1	C	3	59.63	38.96	3.97	0.36	F	2
08/09/06	10	1	C	4	51.32	17.72	2.03	0.33	M	–
08/09/06	10	1	C	5	64.21	35.20	4.68	0.40	M	–
08/09/06	10	1	C	6	60.32	25.07	2.90	0.48	F	3
08/09/06	10	1	C	7	45.10	24.38	2.56	0.30	M	–
08/09/06	10	1	C	8	43.89	19.37	2.87	0.52	F	2
08/09/06	10	1	C	9	47.34	23.13	2.99	0.31	M	–
08/09/06	10	1	E_0	10	66.31	45.73	4.68	0.36	F	3
08/09/06	10	1	E_0	1	68.31	46.11	4.59	0.49	F	3
08/09/06	10	1	E_0	2	66.20	57.87	6.20	0.39	F	2
08/09/06	10	1	E_0	3	61.06	37.49	4.54	0.25	M	–
08/09/06	10	1	E_0	4	62.12	46.83	5.25	0.13	M	–
08/09/06	10	1	E_0	5	60.02	40.48	5.38	0.25	M	–
08/09/06	10	1	E_0	6	58.13	28.21	2.64	0.19	M	–
08/09/06	10	1	E_0	7	58.93	26.51	2.90	0.40	M	–
08/09/06	10	1	E_0	8	65.89	41.78	5.31	0.39	F	3
08/09/06	10	1	E_0	9	62.13	40.41	4.20	0.43	F	2
08/09/06	10	1	E_0	10	61.99	49.17	5.20	0.32	F	2
08/09/06	10	1	E_{75}	1	60.67	42.88	3.97	0.31	M	–
08/09/06	10	1	E_{75}	2	55.70	30.14	2.35	0.39	M	–
08/09/06	10	1	E_{75}	3	59.68	28.44	2.56	0.40	F	3
08/09/06	10	1	E_{75}	4	60.00	35.09	3.90	0.11	M	–
08/09/06	10	1	E_{75}	5	61.77	28.46	2.48	0.34	M	–
08/09/06	10	1	E_{75}	6	51.70	39.23	3.07	0.33	F	2
08/09/06	10	1	E_{75}	7	61.41	30.41	5.06	0.46	F	3
08/09/06	10	1	E_{75}	8	40.19	15.56	2.66	0.25	F	2
08/09/06	10	1	E_{75}	9	47.99	22.00	4.02	0.31	M	–
08/09/06	10	1	E_{75}	10	62.29	45.48	6.29	0.35	M	–
08/09/06	10	1	E_{150}	1	47.68	32.21	4.67	0.15	M	–
08/09/06	10	1	E_{150}	2	70.53	54.58	5.57	0.14	M	–
08/09/06	10	1	E_{150}	3	64.54	35.72	5.88	0.06	M	–
08/09/06	10	1	E_{150}	4	66.16	55.70	6.76	0.49	F	3

Table B.1 continued

08/09/06	10	1	E_{150}	5	61.05	40.77	5.50	0.06	F	1
08/09/06	10	1	E_{150}	6	55.35	33.34	4.78	0.33	F	2
08/09/06	10	1	E_{150}	7	60.11	37.28	4.86	0.29	F	3
08/09/06	10	1	E_{150}	8	51.93	23.79	2.56	0.44	F	2
08/09/06	10	1	E_{150}	9	50.27	15.40	2.75	0.38	F	3
08/09/06	10	1	E_{150}	10	49.39	26.26	3.39	0.36	F	3
08/09/06	10	2	C	1	54.23	23.50	2.79	0.31	M	–
08/09/06	10	2	C	2	56.57	33.17	4.46	0.42	M	–
08/09/06	10	2	C	3	52.75	31.66	2.06	0.16	M	–
08/09/06	10	2	C	4	52.64	20.51	2.52	0.00	I	0
08/09/06	10	2	C	5	56.88	31.17	3.43	0.33	F	2
08/09/06	10	2	C	6	54.77	25.70	2.32	0.25	M	–
08/09/06	10	2	C	7	51.08	33.35	3.14	0.18	M	–
08/09/06	10	2	C	8	55.01	22.60	3.21	0.42	M	–
08/09/06	10	2	C	9	53.96	30.30	3.92	0.39	M	–
08/09/06	10	2	C	10	52.76	30.45	5.15	0.35	F	2
08/09/06	10	2	E_0	1	55.07	27.85	2.50	0.10	M	–
08/09/06	10	2	E_0	2	59.76	37.89	3.01	0.38	M	–
08/09/06	10	2	E_0	3	60.42	37.75	3.23	0.14	M	–
08/09/06	10	2	E_0	4	73.94	67.93	7.28	0.35	M	–
08/09/06	10	2	E_0	5	52.50	33.41	3.67	0.40	F	2
08/09/06	10	2	E_0	6	57.93	24.60	2.90	0.10	I	0
08/09/06	10	2	E_0	7	53.53	21.37	2.14	0.11	F	2
08/09/06	10	2	E_0	8	59.60	40.06	5.50	0.37	F	2
08/09/06	10	2	E_0	9	54.82	26.02	3.85	0.49	M	–
08/09/06	10	2	E_0	10	52.95	31.38	3.62	0.30	M	–
08/09/06	10	2	E_{75}	1	68.14	43.59	4.63	0.30	M	–
08/09/06	10	2	E_{75}	2	57.39	34.60	3.71	0.40	M	–
08/09/06	10	2	E_{75}	3	75.73	67.77	9.06	0.29	F	2
08/09/06	10	2	E_{75}	4	46.77	21.34	2.27	0.27	F	2
08/09/06	10	2	E_{75}	5	62.63	34.20	5.94	0.32	M	–
08/09/06	10	2	E_{75}	6	45.33	24.34	3.01	0.23	F	2
08/09/06	10	2	E_{75}	7	61.76	38.37	6.33	0.52	F	3
08/09/06	10	2	E_{75}	8	56.15	38.24	5.68	0.37	F	3
08/09/06	10	2	E_{75}	9	44.68	28.15	4.13	0.38	M	–
08/09/06	10	2	E_{75}	10	64.09	50.35	6.99	0.28	M	–
08/09/06	10	2	E_{150}	1	64.63	44.40	6.61	0.26	M	–
08/09/06	10	2	E_{150}	2	50.59	15.90	2.69	0.36	M	–
08/09/06	10	2	E_{150}	3	61.49	50.31	6.77	0.10	F	1
08/09/06	10	2	E_{150}	4	52.81	20.82	2.29	0.39	F	2
08/09/06	10	2	E_{150}	5	61.57	40.36	6.54	0.31	M	–
08/09/06	10	2	E_{150}	6	59.89	31.07	4.14	0.35	M	–
08/09/06	10	2	E_{150}	7	40.34	13.32	2.05	0.52	M	–
08/09/06	10	2	E_{150}	8	44.66	17.25	2.47	0.44	F	3
08/09/06	10	2	E_{150}	9	67.92	39.61	6.30	0.30	M	–
08/09/06	10	2	E_{150}	10	61.49	33.22	43.80	0.48	M	–

Table B.1 continued

08/09/06	10	3	C	1	52.96	19.74	3.33	0.29	F	3
08/09/06	10	3	C	2	57.02	45.74	6.36	0.42	M	–
08/09/06	10	3	C	3	54.82	26.16	3.28	0.40	M	–
08/09/06	10	3	C	4	61.90	38.39	4.32	0.27	M	–
08/09/06	10	3	C	5	49.68	26.70	2.65	0.28	M	–
08/09/06	10	3	C	6	54.50	36.07	3.38	0.31	M	–
08/09/06	10	3	C	7	60.03	29.30	3.49	0.47	F	3
08/09/06	10	3	C	8	66.54	36.64	3.58	0.42	M	–
08/09/06	10	3	C	9	59.74	52.19	4.96	0.25	M	–
08/09/06	10	3	C	10	45.55	20.60	1.62	0.23	M	–
08/09/06	10	3	E_0	1	49.36	25.57	5.88	0.03	M	–
08/09/06	10	3	E_0	2	66.24	43.99	4.16	0.32	F	2
08/09/06	10	3	E_0	3	56.34	37.72	3.38	0.27	M	–
08/09/06	10	3	E_0	4	49.66	32.99	4.19	0.43	M	–
08/09/06	10	3	E_0	5	64.84	32.14	7.14	0.35	F	2
08/09/06	10	3	E_0	6	67.17	65.84	1.94	0.25	F	2
08/09/06	10	3	E_0	7	51.26	21.77	4.88	0.47	M	–
08/09/06	10	3	E_0	8	55.02	42.01	4.57	0.04	M	–
08/09/06	10	3	E_0	9	66.60	44.18	4.75	0.34	M	–
08/09/06	10	3	E_0	10	46.78	27.10	2.23	0.34	M	–
08/09/06	10	3	E_{75}	1	68.60	49.96	5.56	0.34	M	–
08/09/06	10	3	E_{75}	2	53.58	31.31	4.44	0.29	M	–
08/09/06	10	3	E_{75}	3	77.71	51.35	6.11	0.31	M	–
08/09/06	10	3	E_{75}	4	56.64	60.84	4.24	0.33	M	–
08/09/06	10	3	E_{75}	5	57.25	38.71	4.90	0.40	F	3
08/09/06	10	3	E_{75}	6	66.97	51.37	6.56	0.32	F	2
08/09/06	10	3	E_{75}	7	51.50	27.42	3.17	0.24	M	–
08/09/06	10	3	E_{75}	8	57.00	22.50	2.74	0.29	F	3
08/09/06	10	3	E_{75}	9	74.93	59.00	6.97	0.26	F	3
08/09/06	10	3	E_{75}	10	66.45	47.02	7.29	0.19	F	2
08/09/06	10	3	E_{150}	1	46.65	15.62	2.04	0.41	F	2
08/09/06	10	3	E_{150}	2	53.12	29.22	4.40	0.40	M	–
08/09/06	10	3	E_{150}	3	51.56	26.09	2.95	0.42	M	–
08/09/06	10	3	E_{150}	4	57.90	37.28	4.77	0.33	M	–
08/09/06	10	3	E_{150}	5	59.98	36.15	5.27	0.35	F	2
08/09/06	10	3	E_{150}	6	52.57	43.25	8.25	0.35	F	2
08/09/06	10	3	E_{150}	7	66.39	42.69	6.63	0.38	M	–
08/09/06	10	3	E_{150}	8	64.17	30.80	5.29	0.20	M	–
08/09/06	10	3	E_{150}	9	68.19	48.62	8.18	0.29	F	2
08/09/06	10	3	E_{150}	10	64.57	39.27	5.34	0.13	M	–
08/13/06	14	1	C	1	49.95	24.34	2.70	0.24	F	1
08/13/06	14	1	C	2	55.72	26.20	3.73	0.30	F	2
08/13/06	14	1	C	3	56.37	40.28	3.06	0.16	M	–
08/13/06	14	1	C	4	59.11	36.73	3.97	0.41	M	–
08/13/06	14	1	C	5	72.88	89.19	13.56	0.35	F	3
08/13/06	14	1	C	6	58.22	24.98	4.64	0.17	F	2

Table B.1 continued

08/13/06	14	1	C	7	56.19	39.37	6.09	0.22	M	–
08/13/06	14	1	C	8	48.78	20.10	3.72	0.44	M	–
08/13/06	14	1	C	9	57.34	45.71	8.38	0.35	M	–
08/13/06	14	1	C	10	74.07	34.80	4.18	0.34	F	3
08/13/06	14	1	C	11	51.25	27.18	3.55	0.13	I	0
08/13/06	14	1	C	12	65.95	31.70	4.20	0.07	F	2
08/13/06	14	1	C	13	41.92	18.01	2.38	0.25	M	–
08/13/06	14	1	C	14	43.36	20.50	3.01	0.38	M	–
08/13/06	14	1	C	15	52.27	21.88	2.38	0.24	M	–
08/13/06	14	1	C	16	49.33	27.34	3.41	0.20	M	–
08/13/06	14	1	C	17	44.51	19.54	2.38	0.11	M	–
08/13/06	14	1	E_0	1	50.39	35.63	5.61	0.34	F	2
08/13/06	14	1	E_0	2	69.15	47.40	8.20	0.26	M	–
08/13/06	14	1	E_0	3	68.40	42.95	6.52	0.31	F	3
08/13/06	14	1	E_0	4	49.46	22.40	3.21	0.17	M	–
08/13/06	14	1	E_0	5	63.51	27.52	3.07	0.29	M	–
08/13/06	14	1	E_0	6	64.90	40.60	6.28	0.28	F	2
08/13/06	14	1	E_0	7	69.46	38.31	7.98	0.20	M	–
08/13/06	14	1	E_0	8	50.80	33.64	4.09	0.54	M	–
08/13/06	14	1	E_0	9	58.88	32.84	4.24	0.25	F	1
08/13/06	14	1	E_0	10	55.68	31.28	3.43	0.23	F	4
08/13/06	14	1	E_0	11	59.71	32.41	4.77	0.26	M	–
08/13/06	14	1	E_0	12	69.34	31.40	4.32	0.00	I	0
08/13/06	14	1	E_0	13	50.62	21.06	2.58	0.32	M	–
08/13/06	14	1	E_0	14	56.09	27.98	5.07	0.27	F	2
08/13/06	14	1	E_{75}	1	–	–	3.38	0.36	M	–
08/13/06	14	1	E_{75}	2	–	–	3.14	0.29	M	–
08/13/06	14	1	E_{75}	3	54.53	–	5.28	0.25	M	–
08/13/06	14	1	E_{75}	4	47.16	17.56	1.52	0.19	M	–
08/13/06	14	1	E_{75}	5	54.38	22.73	3.89	0.14	M	–
08/13/06	14	1	E_{75}	6	56.81	33.18	3.83	0.13	I	0
08/13/06	14	1	E_{75}	7	70.22	51.54	5.40	0.28	M	–
08/13/06	14	1	E_{75}	8	67.75	38.02	5.51	0.37	F	2
08/13/06	14	1	E_{75}	9	62.92	29.92	4.75	0.09	F	1
08/13/06	14	1	E_{75}	10	69.94	43.48	4.87	0.00	I	0
08/13/06	14	1	E_{75}	11	63.35	38.73	5.65	0.35	F	3
08/13/06	14	1	E_{75}	12	52.13	22.09	3.03	0.30	M	–
08/13/06	14	1	E_{75}	13	59.62	31.26	4.21	0.39	M	–
08/13/06	14	1	E_{75}	14	57.08	28.58	3.52	0.18	M	–
08/13/06	14	1	E_{75}	15	65.49	54.07	7.47	0.00	I	0
08/13/06	14	1	E_{150}	1	53.13	40.46	5.87	0.19	M	–
08/13/06	14	1	E_{150}	2	57.70	28.94	3.55	0.40	F	3
08/13/06	14	1	E_{150}	3	53.60	19.42	3.01	0.13	M	–
08/13/06	14	1	E_{150}	4	66.33	35.84	4.92	0.30	M	–
08/13/06	14	1	E_{150}	5	46.38	28.79	3.97	0.38	F	2
08/13/06	14	1	E_{150}	6	53.88	19.30	2.69	0.18	F	4

Table B.1 continued

08/13/06	14	1	E_{150}	7	59.05	45.18	4.09	0.49	F	2
08/13/06	14	1	E_{150}	8	55.10	38.76	5.95	0.36	M	–
08/13/06	14	1	E_{150}	9	51.61	25.38	2.49	0.00	I	0
08/13/06	14	1	E_{150}	10	56.92	39.01	4.28	0.11	M	–
08/13/06	14	1	E_{150}	11	59.75	39.42	5.24	0.30	M	–
08/13/06	14	1	E_{150}	12	67.00	57.18	9.20	0.36	M	–
08/13/06	14	1	E_{150}	13	49.49	25.25	3.57	0.29	F	2
08/13/06	14	1	E_{150}	14	80.41	82.36	11.73	0.23	F	2
08/13/06	14	1	E_{150}	15	66.11	33.31	3.28	0.05	I	0
08/13/06	14	2	C	1	76.05	34.93	6.75	0.31	M	–
08/13/06	14	2	C	2	62.39	43.65	3.63	0.18	F	3
08/13/06	14	2	C	3	59.78	38.70	4.33	0.29	M	–
08/13/06	14	2	C	4	67.44	41.84	5.88	0.12	F	3
08/13/06	14	2	C	5	55.57	24.25	3.60	0.22	M	–
08/13/06	14	2	C	6	47.74	20.97	2.95	0.35	M	–
08/13/06	14	2	C	7	54.84	27.04	3.48	0.32	M	–
08/13/06	14	2	C	8	66.44	49.46	7.06	0.44	F	3
08/13/06	14	2	C	9	47.07	36.33	5.17	0.32	F	2
08/13/06	14	2	C	10	60.22	35.23	3.61	0.21	F	2
08/13/06	14	2	C	11	47.36	29.65	4.03	0.04	M	–
08/13/06	14	2	C	12	60.79	37.16	7.20	0.29	F	2
08/13/06	14	2	C	13	79.99	72.00	7.70	0.37	M	–
08/13/06	14	2	C	14	47.95	16.65	2.20	0.38	M	–
08/13/06	14	2	C	15	49.48	20.12	2.95	0.38	M	–
08/13/06	14	2	C	16	60.95	29.09	4.10	0.21	M	–
08/13/06	14	2	C	17	43.88	22.02	3.45	0.16	F	1
08/13/06	14	2	C	18	51.56	34.39	6.03	0.10	F	1
08/13/06	14	2	E_0	1	76.61	65.88	7.82	0.34	F	2
08/13/06	14	2	E_0	2	51.61	33.55	3.90	0.26	M	–
08/13/06	14	2	E_0	3	60.70	41.34	4.51	0.13	H	–
08/13/06	14	2	E_0	4	55.83	31.29	3.10	0.24	M	–
08/13/06	14	2	E_0	5	60.85	39.11	4.49	0.09	M	–
08/13/06	14	2	E_0	6	55.08	20.80	2.55	0.18	M	–
08/13/06	14	2	E_0	7	50.51	25.91	2.68	0.28	F	2
08/13/06	14	2	E_0	8	72.13	53.57	7.00	0.00	I	0
08/13/06	14	2	E_0	9	53.29	24.08	4.02	0.27	F	2
08/13/06	14	2	E_0	10	70.91	53.62	7.87	0.04	F	2
08/13/06	14	2	E_0	11	53.70	22.99	3.39	0.37	M	–
08/13/06	14	2	E_0	12	61.39	37.72	5.20	0.34	F	3
08/13/06	14	2	E_0	13	56.58	25.98	3.09	0.39	M	–
08/13/06	14	2	E_0	14	40.85	18.76	2.16	0.00	I	0
08/13/06	14	2	E_{75}	1	45.95	19.10	3.33	0.19	M	–
08/13/06	14	2	E_{75}	2	77.77	59.51	9.89	0.33	F	2
08/13/06	14	2	E_{75}	3	56.36	32.78	5.33	0.31	F	2
08/13/06	14	2	E_{75}	4	64.47	31.80	4.15	0.21	M	–
08/13/06	14	2	E_{75}	5	59.72	40.51	6.59	0.41	F	2

Table B.1 continued

08/13/06	14	2	E_{75}	6	50.70	22.14	3.19	0.25	M	–
08/13/06	14	2	E_{75}	7	55.76	60.49	5.23	0.29	M	–
08/13/06	14	2	E_{75}	8	66.10	36.00	5.15	0.42	M	–
08/13/06	14	2	E_{75}	9	86.42	45.05	4.41	0.18	F	2
08/13/06	14	2	E_{75}	10	46.03	37.04	4.31	0.21	F	2
08/13/06	14	2	E_{75}	11	45.13	21.08	1.89	0.05	M	–
08/13/06	14	2	E_{75}	12	51.01	23.02	3.77	0.40	M	–
08/13/06	14	2	E_{75}	13	66.43	46.67	7.79	0.13	F	2
08/13/06	14	2	E_{75}	14	51.15	21.76	3.13	0.25	M	–
08/13/06	14	2	E_{150}	1	51.10	32.46	5.17	0.18	M	–
08/13/06	14	2	E_{150}	2	61.16	47.10	5.93	0.26	F	2
08/13/06	14	2	E_{150}	3	57.24	28.26	3.92	0.08	M	–
08/13/06	14	2	E_{150}	4	63.28	39.23	5.02	0.25	F	2
08/13/06	14	2	E_{150}	5	48.58	24.91	3.27	0.13	F	2
08/13/06	14	2	E_{150}	6	73.66	52.57	7.04	0.10	I	0
08/13/06	14	2	E_{150}	7	56.29	31.37	4.92	0.40	M	–
08/13/06	14	2	E_{150}	8	66.40	39.69	6.53	0.39	F	2
08/13/06	14	2	E_{150}	9	56.69	50.40	7.77	0.28	F	2
08/13/06	14	2	E_{150}	10	56.41	23.48	3.45	0.35	M	–
08/13/06	14	2	E_{150}	11	51.83	30.57	5.48	0.26	M	–
08/13/06	14	2	E_{150}	12	54.23	29.52	4.02	0.10	M	–
08/13/06	14	2	E_{150}	13	57.49	35.72	5.20	0.24	M	–
08/13/06	14	2	E_{150}	14	72.85	65.64	9.40	0.38	M	–
08/13/06	14	2	E_{150}	15	62.75	41.68	5.13	0.20	M	–
08/13/06	14	2	E_{150}	16	71.82	51.76	10.45	0.11	F	4
08/13/06	14	2	E_{150}	17	80.13	71.33	9.10	0.05	M	–
08/13/06	14	2	E_{150}	18	65.31	44.38	6.26	0.19	F	2
08/13/06	14	3	C	1	61.81	66.76	6.46	0.00	I	0
08/13/06	14	3	C	2	58.89	30.80	3.62	0.12	M	–
08/13/06	14	3	C	3	56.44	42.48	4.26	0.41	F	3
08/13/06	14	3	C	4	51.08	20.19	2.51	0.22	M	–
08/13/06	14	3	C	5	43.78	20.86	3.35	0.48	F	3
08/13/06	14	3	C	6	53.47	21.92	3.53	0.25	M	–
08/13/06	14	3	C	7	65.83	32.68	3.28	0.14	F	2
08/13/06	14	3	C	8	59.39	37.31	4.46	0.34	M	–
08/13/06	14	3	C	9	51.24	31.69	3.59	0.34	F	3
08/13/06	14	3	C	10	55.58	28.52	3.77	0.30	M	–
08/13/06	14	3	C	11	66.04	26.40	3.39	0.23	F	2
08/13/06	14	3	C	12	54.66	27.75	3.01	0.04	M	–
08/13/06	14	3	C	13	51.81	22.43	2.52	0.08	M	–
08/13/06	14	3	C	14	61.27	43.46	5.11	0.33	M	–
08/13/06	14	3	C	15	58.98	33.89	6.24	0.29	M	–
08/13/06	14	3	C	16	52.53	38.92	5.44	0.10	M	–
08/13/06	14	3	C	17	53.79	18.79	2.64	0.27	M	–
08/13/06	14	3	C	18	52.75	24.52	3.19	0.24	F	3
08/13/06	14	3	E_0	1	72.38	52.40	8.40	0.12	F	1

Table B.1 continued

08/13/06	14	3	E_0	2	61.94	37.44	6.49	0.22	F	2
08/13/06	14	3	E_0	3	72.13	58.88	6.50	0.09	M	–
08/13/06	14	3	E_0	4	60.61	35.62	4.94	0.34	M	–
08/13/06	14	3	E_0	5	63.91	43.77	5.59	0.21	M	–
08/13/06	14	3	E_0	6	68.05	48.02	7.94	0.25	F	3
08/13/06	14	3	E_0	7	63.69	62.27	8.87	0.18	F	1
08/13/06	14	3	E_0	8	69.48	43.17	5.16	0.00	I	0
08/13/06	14	3	E_0	9	69.40	44.71	6.33	0.22	F	1
08/13/06	14	3	E_0	10	71.26	40.09	4.88	0.35	F	3
08/13/06	14	3	E_0	11	58.31	35.40	2.95	0.00	I	0
08/13/06	14	3	E_0	12	63.03	44.58	5.52	0.34	M	–
08/13/06	14	3	E_0	13	55.68	28.97	4.80	0.33	F	3
08/13/06	14	3	E_0	14	66.53	35.76	4.78	0.34	F	3
08/13/06	14	3	E_{75}	1	50.05	29.63	2.81	0.28	M	–
08/13/06	14	3	E_{75}	2	58.95	28.95	4.07	0.28	M	–
08/13/06	14	3	E_{75}	3	55.25	20.72	2.49	0.25	F	2
08/13/06	14	3	E_{75}	4	59.43	22.57	1.98	0.14	M	–
08/13/06	14	3	E_{75}	5	66.25	39.35	4.95	0.25	M	–
08/13/06	14	3	E_{75}	6	57.76	23.60	4.37	0.30	F	3
08/13/06	14	3	E_{75}	7	50.82	23.03	3.86	0.38	F	3
08/13/06	14	3	E_{75}	8	60.65	42.03	5.94	0.26	M	–
08/13/06	14	3	E_{75}	9	55.46	28.03	4.09	0.38	M	–
08/13/06	14	3	E_{75}	10	63.12	24.82	3.50	0.12	M	–
08/13/06	14	3	E_{75}	11	70.81	63.53	5.34	0.19	M	–
08/13/06	14	3	E_{75}	12	83.77	45.63	7.07	0.24	F	2
08/13/06	14	3	E_{75}	13	70.81	56.62	8.84	0.31	M	–
08/13/06	14	3	E_{150}	1	61.15	32.62	4.32	0.00	I	0
08/13/06	14	3	E_{150}	2	53.96	18.25	1.80	0.00	I	0
08/13/06	14	3	E_{150}	3	54.22	26.19	2.89	0.14	M	–
08/13/06	14	3	E_{150}	4	46.03	20.55	2.45	0.28	F	2
08/13/06	14	3	E_{150}	5	46.70	16.59	2.81	0.26	M	–
08/13/06	14	3	E_{150}	6	53.09	39.96	5.52	0.28	F	2
08/13/06	14	3	E_{150}	7	62.52	55.53	5.34	0.29	M	–
08/13/06	14	3	E_{150}	8	60.91	38.71	4.47	0.03	M	–
08/13/06	14	3	E_{150}	9	63.85	37.25	3.15	0.18	F	2
08/13/06	14	3	E_{150}	10	68.30	43.74	4.00	0.27	F	2
08/13/06	14	3	E_{150}	11	72.40	59.71	5.37	0.37	F	2
08/13/06	14	3	E_{150}	12	79.68	82.40	6.63	0.13	F	2
08/13/06	14	3	E_{150}	13	71.51	47.12	6.00	0.00	I	0
08/13/06	14	3	E_{150}	14	54.06	28.18	2.54	0.01	M	–

Table B.2. Raw data collected to determine the influence of estradiol-3-benzoate on the gametic sex distribution and ovarian development of 7-month-old eastern oysters collected from the Sea Grant Grand Isle Bivalve Hatchery (29°15'12"N, 90°03'26"W, Caminada Bay, LA) in February 2007 when the ambient water was ~16°C and 28 ppt salinity.

Sampling date	Day	Tank	Treatment		Shell height (mm)	Weight (g)		Gametic sex	Stage
						whole	body		
02/01/07	0	–	BL	1	53.79	16.85	2.50	I	0
02/01/07	0	–	BL	2	59.50	32.83	3.57	F	1
02/01/07	0	–	BL	3	58.15	39.55	5.08	M	–
02/01/07	0	–	BL	4	47.48	15.51	1.88	I	0
02/01/07	0	–	BL	5	44.75	13.91	2.00	M	–
02/01/07	0	–	BL	6	66.30	43.81	4.84	I	0
02/01/07	0	–	BL	7	64.01	37.45	4.65	I	0
02/01/07	0	–	BL	8	66.25	33.68	4.51	I	0
02/01/07	0	–	BL	9	49.97	18.22	3.41	I	0
02/01/07	0	–	BL	10	48.36	18.93	3.11	I	0
02/01/07	0	–	BL	11	53.65	25.18	4.06	M	–
02/01/07	0	–	BL	12	63.77	40.10	5.71	I	0
02/01/07	0	–	BL	13	64.21	32.05	4.30	M	–
02/01/07	0	–	BL	14	58.43	31.13	3.59	M	–
02/01/07	0	–	BL	15	65.23	30.65	5.06	I	0
02/01/07	0	–	BL	16	62.66	43.95	6.91	M	–
02/01/07	0	–	BL	17	48.95	25.98	3.41	I	0
02/01/07	0	–	BL	18	49.97	24.85	3.83	F	1
02/01/07	0	–	BL	19	48.63	19.89	3.39	I	0
02/01/07	0	–	BL	20	52.31	36.39	4.52	M	–
02/01/07	0	–	BL	21	57.03	21.80	3.54	I	0
02/01/07	0	–	BL	22	54.13	18.12	2.38	I	0
02/01/07	0	–	BL	23	59.50	25.04	5.54	M	–
02/01/07	0	–	BL	24	52.62	19.76	1.82	M	–
02/01/07	0	–	BL	25	42.89	21.23	3.92	M	–
02/01/07	0	–	BL	26	52.13	27.59	3.71	I	0
02/01/07	0	–	BL	27	42.18	14.59	2.31	I	0
02/01/07	0	–	BL	28	52.96	28.33	2.23	I	0
02/01/07	0	–	BL	29	66.44	40.16	4.74	I	0
02/01/07	0	–	BL	30	46.99	22.20	3.58	I	0
02/01/07	0	–	BL	31	55.17	29.19	4.10	M	–
02/01/07	0	–	BL	32	48.03	25.87	3.56	M	–
02/01/07	0	–	BL	33	58.38	22.67	2.55	I	0
02/01/07	0	–	BL	34	60.00	31.89	4.97	I	0
02/01/07	0	–	BL	35	58.22	24.81	4.38	H	–
02/01/07	0	–	BL	36	50.74	27.27	3.63	I	0
02/01/07	0	–	BL	37	48.13	20.90	4.31	I	0
02/01/07	0	–	BL	38	51.27	16.79	3.29	I	0

Table B.2 continued

02/01/07	0	–	BL	39	47.08	15.33	2.31	I	0
02/01/07	0	–	BL	40	44.71	13.99	1.76	M	–
02/13/07	10	1	C	1	57.60	39.50	5.11	I	0
02/13/07	10	1	C	2	61.50	25.60	4.14	I	0
02/13/07	10	1	C	3	45.93	19.19	2.47	I	0
02/13/07	10	1	C	4	45.74	22.03	2.82	M	–
02/13/07	10	1	C	5	57.81	30.90	3.55	I	0
02/13/07	10	1	C	6	42.96	28.02	4.24	I	0
02/13/07	10	1	C	7	41.51	24.09	3.45	M	–
02/13/07	10	1	C	8	47.80	21.19	3.45	F	1
02/13/07	10	1	C	9	63.95	51.49	7.82	I	0
02/13/07	10	1	C	10	20.78	41.97	6.47	F	R
02/13/07	10	1	$E_{37.5}$	1	58.28	25.06	3.14	I	0
02/13/07	10	1	$E_{37.5}$	2	42.14	15.25	1.59	I	0
02/13/07	10	1	$E_{37.5}$	3	61.65	34.89	4.06	M	–
02/13/07	10	1	$E_{37.5}$	4	66.71	55.00	5.21	I	0
02/13/07	10	1	$E_{37.5}$	5	57.92	47.88	7.58	I	0
02/13/07	10	1	$E_{37.5}$	6	55.32	37.41	3.19	I	0
02/13/07	10	1	$E_{37.5}$	7	57.22	29.90	3.29	M	–
02/13/07	10	1	$E_{37.5}$	8	55.93	28.45	3.25	M	–
02/13/07	10	1	$E_{37.5}$	9	56.01	28.39	3.56	I	0
02/13/07	10	1	$E_{37.5}$	10	44.37	14.05	0.95	I	0
02/13/07	10	1	E_{75}	1	57.42	29.67	3.88	I	0
02/13/07	10	1	E_{75}	2	56.99	56.49	7.88	M	–
02/13/07	10	1	E_{75}	3	58.44	38.11	4.54	H	–
02/13/07	10	1	E_{75}	4	54.87	18.87	1.88	M	–
02/13/07	10	1	E_{75}	5	57.16	39.39	4.68	M	–
02/13/07	10	1	E_{75}	6	51.90	22.79	3.77	M	–
02/13/07	10	1	E_{75}	7	44.05	21.76	2.93	M	–
02/13/07	10	1	E_{75}	8	60.27	19.24	2.63	M	–
02/13/07	10	1	E_{75}	9	55.05	27.04	3.32	I	0
02/13/07	10	1	E_{75}	10	49.97	17.70	2.40	F	1
02/13/07	10	1	E_{150}	1	62.64	39.18	5.02	I	0
02/13/07	10	1	E_{150}	2	61.36	39.13	5.07	M	–
02/13/07	10	1	E_{150}	3	65.85	45.58	6.00	I	0
02/13/07	10	1	E_{150}	4	64.38	43.82	5.36	H	–
02/13/07	10	1	E_{150}	5	61.91	23.63	3.73	I	0
02/13/07	10	1	E_{150}	6	56.24	30.55	4.79	M	–
02/13/07	10	1	E_{150}	7	57.04	35.18	5.61	F	1
02/13/07	10	1	E_{150}	8	51.29	26.12	3.87	M	–
02/13/07	10	1	E_{150}	9	71.33	32.68	5.76	M	–
02/13/07	10	1	E_{150}	10	52.51	22.97	3.19	I	0
02/13/07	10	2	C	1	64.12	35.73	5.01	I	0
02/13/07	10	2	C	2	46.84	30.78	4.45	I	0

Table B.2 continued

02/13/07	10	2	C	3	43.40	26.09	3.73	I	0
02/13/07	10	2	C	4	37.61	20.81	2.51	I	0
02/13/07	10	2	C	5	45.02	16.52	2.65	I	0
02/13/07	10	2	C	6	56.28	24.17	3.94	I	0
02/13/07	10	2	C	7	69.88	42.02	6.28	M	–
02/13/07	10	2	C	8	45.00	20.94	3.07	I	0
02/13/07	10	2	C	9	51.51	20.69	3.01	I	0
02/13/07	10	2	C	10	52.50	28.30	3.62	I	0
02/13/07	10	2	$E_{37.5}$	1	42.71	24.10	3.36	I	0
02/13/07	10	2	$E_{37.5}$	2	62.61	35.80	6.84	I	0
02/13/07	10	2	$E_{37.5}$	3	44.38	24.96	3.89	I	0
02/13/07	10	2	$E_{37.5}$	4	54.20	17.68	2.28	I	0
02/13/07	10	2	$E_{37.5}$	5	50.41	23.19	3.32	M	–
02/13/07	10	2	$E_{37.5}$	6	66.81	28.75	4.76	M	–
02/13/07	10	2	$E_{37.5}$	7	44.15	16.59	1.62	I	0
02/13/07	10	2	$E_{37.5}$	8	51.88	28.16	4.48	M	–
02/13/07	10	2	$E_{37.5}$	9	53.34	30.89	4.85	F	1
02/13/07	10	2	$E_{37.5}$	10	46.88	15.40	2.20	I	0
02/13/07	10	2	E_{75}	1	52.56	35.88	4.55	I	0
02/13/07	10	2	E_{75}	2	60.19	32.88	4.02	M	–
02/13/07	10	2	E_{75}	3	51.48	32.62	6.16	M	–
02/13/07	10	2	E_{75}	4	53.06	32.22	5.90	I	0
02/13/07	10	2	E_{75}	5	62.19	31.17	4.51	F	R
02/13/07	10	2	E_{75}	6	66.48	41.63	7.11	F	1
02/13/07	10	2	E_{75}	7	45.46	19.87	3.27	M	–
02/13/07	10	2	E_{75}	8	45.44	14.60	1.82	M	–
02/13/07	10	2	E_{75}	9	49.74	33.98	3.97	F	1
02/13/07	10	2	E_{75}	10	54.73	19.08	2.76	I	0
02/13/07	10	2	E_{150}	1	63.13	33.81	5.29	M	–
02/13/07	10	2	E_{150}	2	50.99	36.39	4.75	M	–
02/13/07	10	2	E_{150}	3	50.81	16.97	2.15	M	–
02/13/07	10	2	E_{150}	4	64.54	37.13	5.31	F	1
02/13/07	10	2	E_{150}	5	61.07	34.70	5.74	M	–
02/13/07	10	2	E_{150}	6	58.37	25.24	2.52	I	0
02/13/07	10	2	E_{150}	7	61.28	45.61	6.84	M	–
02/13/07	10	2	E_{150}	8	58.58	35.90	6.33	I	0
02/13/07	10	2	E_{150}	9	58.51	29.78	4.08	I	0
02/13/07	10	2	E_{150}	10	52.45	21.31	2.92	I	0
02/13/07	10	3	C	1	51.97	26.57	2.82	I	0
02/13/07	10	3	C	2	64.00	26.61	3.35	I	0
02/13/07	10	3	C	3	58.41	28.89	3.41	M	–
02/13/07	10	3	C	4	49.59	20.88	2.41	I	0
02/13/07	10	3	C	5	51.61	18.97	3.12	I	0
02/13/07	10	3	C	6	48.85	32.61	5.03	M	–

Table B.2 continued

02/13/07	10	3	C	7	47.57	20.07	3.27	M	–
02/13/07	10	3	C	8	44.89	19.19	2.61	I	0
02/13/07	10	3	C	9	48.42	25.73	3.89	I	0
02/13/07	10	3	C	10	56.80	23.31	3.36	M	–
02/13/07	10	3	$E_{37.5}$	1	63.30	28.61	4.11	M	–
02/13/07	10	3	$E_{37.5}$	2	59.69	43.39	6.62	M	–
02/13/07	10	3	$E_{37.5}$	3	50.65	17.24	2.57	I	0
02/13/07	10	3	$E_{37.5}$	4	49.08	18.93	2.73	I	0
02/13/07	10	3	$E_{37.5}$	5	62.77	31.40	5.61	M	–
02/13/07	10	3	$E_{37.5}$	6	57.04	30.91	4.11	F	1
02/13/07	10	3	$E_{37.5}$	7	62.49	34.14	4.89	I	0
02/13/07	10	3	$E_{37.5}$	8	56.82	27.96	4.21	M	–
02/13/07	10	3	$E_{37.5}$	9	64.04	36.62	4.80	M	–
02/13/07	10	3	$E_{37.5}$	10	39.04	12.68	2.61	M	–
02/13/07	10	3	E_{75}	1	51.87	32.74	6.09	I	0
02/13/07	10	3	E_{75}	2	63.48	30.95	4.94	I	0
02/13/07	10	3	E_{75}	3	58.87	46.61	6.40	F	1
02/13/07	10	3	E_{75}	4	66.79	41.40	6.02	I	0
02/13/07	10	3	E_{75}	5	58.68	13.41	1.51	I	0
02/13/07	10	3	E_{75}	6	64.54	38.26	8.03	F	1
02/13/07	10	3	E_{75}	7	54.08	19.97	3.25	I	0
02/13/07	10	3	E_{75}	8	48.79	16.85	1.53	F	1
02/13/07	10	3	E_{75}	9	37.69	17.14	1.65	F	1
02/13/07	10	3	E_{75}	10	58.88	17.34	1.58	F	1
02/13/07	10	3	E_{150}	1	51.99	32.34	5.10	I	0
02/13/07	10	3	E_{150}	2	64.58	32.10	3.88	M	–
02/13/07	10	3	E_{150}	3	54.61	33.92	4.01	M	–
02/13/07	10	3	E_{150}	4	58.08	31.26	3.92	I	0
02/13/07	10	3	E_{150}	5	52.95	30.40	3.86	F	1
02/13/07	10	3	E_{150}	6	57.96	37.86	–	F	1
02/13/07	10	3	E_{150}	7	44.55	20.20	2.36	M	–
02/13/07	10	3	E_{150}	8	59.13	37.39	5.38	I	0
02/13/07	10	3	E_{150}	9	50.35	22.64	2.95	I	0
02/13/07	10	3	E_{150}	10	53.16	23.00	3.59	I	0
02/17/07	14	1	C	1	61.00	39.94	6.22	M	–
02/17/07	14	1	C	2	55.15	33.73	4.07	M	–
02/17/07	14	1	C	3	65.23	45.18	6.88	F	1
02/17/07	14	1	C	4	55.59	40.44	5.29	I	0
02/17/07	14	1	C	5	57.41	38.91	5.50	I	0
02/17/07	14	1	C	6	54.12	32.76	4.74	F	1
02/17/07	14	1	C	7	47.30	27.70	3.51	H	–
02/17/07	14	1	C	8	57.00	44.78	4.82	F	1
02/17/07	14	1	C	9	44.44	23.71	4.32	F	1
02/17/07	14	1	C	10	48.60	25.70	4.16	H	–

Table B.2 continued

02/17/07	14	1	$E_{37.5}$	1	62.25	38.07	4.19	F	1
02/17/07	14	1	$E_{37.5}$	2	59.02	33.20	4.56	M	–
02/17/07	14	1	$E_{37.5}$	3	64.36	17.82	1.63	I	0
02/17/07	14	1	$E_{37.5}$	4	53.67	34.45	4.71	F	1
02/17/07	14	1	$E_{37.5}$	5	38.59	26.68	3.93	I	0
02/17/07	14	1	$E_{37.5}$	6	60.63	40.41	4.80	I	0
02/17/07	14	1	$E_{37.5}$	7	56.00	32.23	4.77	F	1
02/17/07	14	1	$E_{37.5}$	8	51.82	31.24	4.63	F	1
02/17/07	14	1	$E_{37.5}$	9	56.55	29.07	3.79	I	0
02/17/07	14	1	$E_{37.5}$	10	51.16	29.28	3.69	I	0
02/17/07	14	1	E_{75}	1	51.33	30.40	4.28	I	0
02/17/07	14	1	E_{75}	2	57.28	32.04	4.43	I	0
02/17/07	14	1	E_{75}	3	53.74	32.88	4.73	I	0
02/17/07	14	1	E_{75}	4	63.61	41.31	6.21	I	0
02/17/07	14	1	E_{75}	5	45.73	22.38	2.76	M	–
02/17/07	14	1	E_{75}	6	63.09	54.99	7.52	M	–
02/17/07	14	1	E_{75}	7	48.69	40.20	6.45	M	–
02/17/07	14	1	E_{75}	8	54.85	29.20	4.87	M	–
02/17/07	14	1	E_{75}	9	56.90	32.39	3.43	I	0
02/17/07	14	1	E_{75}	10	55.99	39.70	6.72	I	0
02/17/07	14	1	E_{150}	1	45.74	28.36	2.88	I	0
02/17/07	14	1	E_{150}	2	70.91	35.52	5.88	I	0
02/17/07	14	1	E_{150}	3	58.05	36.98	4.20	M	–
02/17/07	14	1	E_{150}	4	60.35	44.92	6.02	I	0
02/17/07	14	1	E_{150}	5	50.82	35.99	4.36	I	0
02/17/07	14	1	E_{150}	6	61.78	40.40	4.56	I	0
02/17/07	14	1	E_{150}	7	47.25	18.53	2.51	M	–
02/17/07	14	1	E_{150}	8	51.98	23.99	3.01	M	–
02/17/07	14	1	E_{150}	9	40.62	10.56	1.25	F	2
02/17/07	14	1	E_{150}	10	46.81	21.38	3.89	I	–
02/17/07	14	2	C	1	62.94	33.69	5.19	F	1
02/18/07	14	2	C	2	51.66	34.39	5.90	I	0
02/19/07	14	2	C	3	41.04	20.29	2.69	I	0
02/20/07	14	2	C	4	48.58	34.11	2.32	I	0
02/21/07	14	2	C	5	48.59	20.89	1.91	I	0
02/22/07	14	2	C	6	46.63	22.93	2.01	I	0
02/23/07	14	2	C	7	47.79	20.82	2.69	H	–
02/24/07	14	2	C	8	36.85	18.94	2.05	I	0
02/25/07	14	2	C	9	48.51	25.68	2.24	I	0
02/26/07	14	2	C	10	49.97	19.92	2.69	M	–
02/27/07	14	2	$E_{37.5}$	1	44.62	28.52	3.00	H	–
02/28/07	14	2	$E_{37.5}$	2	62.52	31.64	4.10	I	0
03/01/07	14	2	$E_{37.5}$	3	58.66	29.71	3.44	I	0
03/02/07	14	2	$E_{37.5}$	4	59.20	34.01	4.99	I	0

Table B.2 continued

03/03/07	14	2	$E_{37.5}$	5	60.69	33.36	5.15	F	2
03/04/07	14	2	$E_{37.5}$	6	42.02	23.98	4.63	M	–
03/05/07	14	2	$E_{37.5}$	7	51.04	17.36	2.28	I	0
03/06/07	14	2	$E_{37.5}$	8	39.17	19.45	3.61	F	R
03/07/07	14	2	$E_{37.5}$	9	49.27	28.04	4.53	I	0
03/08/07	14	2	$E_{37.5}$	10	45.07	14.57	3.10	I	0
03/09/07	14	2	E_{75}	1	62.40	51.80	6.07	F	2
03/10/07	14	2	E_{75}	2	62.90	39.54	4.72	M	–
03/11/07	14	2	E_{75}	3	59.41	34.48	4.27	M	–
03/12/07	14	2	E_{75}	4	53.24	19.45	2.40	I	0
03/13/07	14	2	E_{75}	5	59.63	28.21	5.09	F	1
03/14/07	14	2	E_{75}	6	56.64	31.95	3.87	M	–
03/15/07	14	2	E_{75}	7	61.60	28.66	4.60	I	0
03/16/07	14	2	E_{75}	8	45.06	20.38	3.01	I	0
03/17/07	14	2	E_{75}	9	53.81	30.53	4.35	M	–
03/18/07	14	2	E_{75}	10	52.72	29.87	4.17	M	–
03/19/07	14	2	E_{150}	1	48.13	25.81	4.75	I	0
03/20/07	14	2	E_{150}	2	48.38	29.54	4.45	M	–
03/21/07	14	2	E_{150}	3	57.11	35.76	5.24	M	–
03/22/07	14	2	E_{150}	4	53.86	33.67	4.66	I	0
03/23/07	14	2	E_{150}	5	50.20	21.53	3.26	I	0
03/24/07	14	2	E_{150}	6	47.63	19.05	2.43	M	–
03/25/07	14	2	E_{150}	7	52.80	30.41	4.61	F	2
03/26/07	14	2	E_{150}	8	54.37	22.58	2.76	M	–
03/27/07	14	2	E_{150}	9	48.19	24.86	4.33	I	0
03/28/07	14	2	E_{150}	10	64.62	27.23	4.01	I	0
03/28/07	14	3	C	1	45.13	35.55	5.01	M	–
03/28/07	14	3	C	2	54.98	34.26	5.34	F	R
03/28/07	14	3	C	3	54.94	41.40	4.29	I	0
03/28/07	14	3	C	4	59.54	34.47	3.69	H	–
03/28/07	14	3	C	5	57.77	39.50	5.30	M	–
03/28/07	14	3	C	6	46.22	23.27	2.43	I	0
03/28/07	14	3	C	7	54.25	35.44	4.34	M	–
03/28/07	14	3	C	8	56.24	43.58	5.69	I	0
03/28/07	14	3	C	9	59.71	19.64	3.10	M	–
03/28/07	14	3	C	10	51.14	28.14	3.96	M	–
03/28/07	14	3	$E_{37.5}$	1	56.57	37.43	4.33	I	0
03/28/07	14	3	$E_{37.5}$	2	58.42	29.61	2.96	I	0
03/28/07	14	3	$E_{37.5}$	3	54.10	29.62	3.06	I	0
03/28/07	14	3	$E_{37.5}$	4	55.52	12.66	1.67	I	0
03/28/07	14	3	$E_{37.5}$	5	65.46	44.78	5.76	H	–
03/28/07	14	3	$E_{37.5}$	6	62.13	21.53	3.45	I	0
03/28/07	14	3	$E_{37.5}$	7	45.99	25.70	3.34	I	0
03/28/07	14	3	$E_{37.5}$	8	60.09	44.33	6.09	I	–

Table B.2 continued

03/28/07	14	3	$E_{37.5}$	9	50.83	20.55	4.43	F	1
03/28/07	14	3	$E_{37.5}$	10	62.23	48.71	–	M	–
03/28/07	14	3	E_{75}	1	53.09	33.12	4.69	I	0
03/28/07	14	3	E_{75}	2	47.97	27.09	4.41	M	–
03/28/07	14	3	E_{75}	3	51.89	19.11	2.18	I	0
03/28/07	14	3	E_{75}	4	64.89	38.28	3.61	M	–
03/28/07	14	3	E_{75}	5	49.15	41.03	4.82	I	0
03/28/07	14	3	E_{75}	6	50.03	29.47	3.67	F	R
03/28/07	14	3	E_{75}	7	48.36	25.07	3.17	I	0
03/28/07	14	3	E_{75}	8	57.09	29.78	4.26	I	0
03/28/07	14	3	E_{75}	9	52.19	24.76	4.48	H	–
03/28/07	14	3	E_{75}	10	54.52	36.23	3.23	I	0
03/28/07	14	3	E_{150}	1	40.22	20.08	2.63	M	–
03/28/07	14	3	E_{150}	2	43.93	23.63	4.03	I	0
03/28/07	14	3	E_{150}	3	49.57	41.81	4.54	M	–
03/28/07	14	3	E_{150}	4	56.93	29.37	3.50	M	–
03/28/07	14	3	E_{150}	5	52.67	20.86	2.99	I	0
03/28/07	14	3	E_{150}	6	54.68	34.88	4.16	M	–
03/28/07	14	3	E_{150}	7	33.11	16.05	2.38	I	0
03/28/07	14	3	E_{150}	8	56.26	35.61	5.74	M	–
03/28/07	14	3	E_{150}	9	43.16	15.30	2.42	M	–
03/28/07	14	3	E_{150}	10	48.63	20.06	2.96	F	1

VITA

Wesley Michael Burnside was born in Metairie, Louisiana. He is an alumnus of Grace King High School. In the fall of 2001, he moved to Baton Rouge to attend Louisiana State University; he received his Bachelor of Science in animal, dairy and poultry sciences with a concentration in science and technology. During his undergraduate work, he was mentored by Dr. Robert Godke and others at the LSUAC Embryo Biotechnology Laboratory in St. Gabriel, Louisiana, as a student worker and undergraduate researcher; he studied assisted reproductive techniques and biotechnology in farm mammals. Wesley also worked as a veterinary assistant at Causeway Animal Hospital from February 2000 to January 2005. Seven months prior to graduation in December 2004, he began as an undergraduate student worker at the LSUAC Aquaculture Research station where he started to transfer his knowledge of reproductive physiology from mammals to fish and shellfish. In January 2005, he began a master's degree program in the School of Renewable Natural Resources with the guidance of Dr. Terrence Tiersch, Dr. John Lynn and Dr. John Supan as a Graduate Research Assistant until June 2007.

From June 2007 until September 2008, Wesley was a Research Associate II in the Gene Therapy Program at the LSU Health Sciences Center–New Orleans where he worked on an immunological approach to breast cancer treatment and the development of a novel mouse research model engrafted with human stem cells. In September of 2008, Wesley moved to Glasgow, Scotland, UK, to attend the University of Glasgow to pursue a degree in veterinary medicine and surgery. He is employed by the Weipers Centre for Equine Health at the university as a student technician part-time and on-call for surgical emergencies. Wesley is a candidate for the degree of Master of Science in wildlife in March 2010.