EFFECTS OF ANOXIA ON HISTOLOGY, BACTERIOLOGY, CONDITION INDEX,

GLYCOGEN LEVELS, AND FECUNDITY IN THE EASTERN OYSTER,

Crassostrea virginica.

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Susan B. Fogelson

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VITA

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THESIS ABSTRACT

EFFECTS OF ANOXIA ON HISTOLOGY, BACTERIOLOGY, CONDITION INDEX, GLYCOGEN LEVELS, AND FECUNDITY IN THE EASTERN OYSTER, *Crassostrea virginica*.

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The eastern oyster, *Crassostrea virginica*, is well known for its commercial and ecological roles in Mobile Bay, AL. During summer months oysters may be exposed to hypoxic and occasionally anoxic conditions which in turn can cause mortality. This study analyzed the effects of anoxia on oyster condition under summer temperatures.

Five hundred hatchery reared oysters ($\overline{X}$ = 48.05 ± 5.56 mm), were examined for the effects of anoxia on oyster condition. Oysters were exposed in the laboratory to anoxic (< 0.10 mg/L O_2) conditions for intervals of 24 h, 48 h and 60 h at 25ppt salinity and 28ºC ± 1ºC. Condition index was evaluated prior to experimentation to establish a baseline for comparison to control and test oysters which were sampled at the 60 h interval and 4 weeks post experiment. Histology, glycogen levels, and bacteriology

levels were evaluated for test oysters prior to the experiment to establish a baseline for comparison to samples taken during the experiment, at the 24 h, 48 h, 60 h intervals, and 4 weeks post experiment. Bacterial enumeration was done under aerobic and anaerobic conditions. Fecundity estimates were performed 18 weeks post-experiment on control and experimental oysters.

Histological examination indicated that anoxia causes stress in oyster tissue. Digestive tubule lumen ratios increased significantly from the baseline to the 60 h interval.

Condition indices were not significantly different ($P<0.05$) between baseline oysters ($\overline{X}$ =59.0±2.6) and test oysters at the 60 h interval ($\overline{X}$ =57.5±1.4). Similarly, there was no difference between the baseline oysters and the control oysters at the 60 h interval ($\overline{X}$ =55.9±2.16). Glycogen levels for 10 test oysters from all intervals did not differ from the baseline ($\overline{X}$ =72.5± 18.2) μmoles glucosyl units/g dry weight.

The bacterial count for test oysters ($\overline{X}$ =1.11 x 10^6 CFU/ml) from the 60h interval was significantly higher than counts for the baseline oysters and the 24 h and 48 h intervals. The most common bacteria isolated were *Clostridium sp* which confirms previous studies on the natural anaerobic flora of oysters.

No difference in fecundity could be established due to the lack of mature females in both the control and the experimental groups.

These findings support previous reports that mortality in oysters during anoxic events is due to bacterial infection and not lack of endogenous fuels.

ACKNOWLEDGEMENTS

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I. INTRODUCTION

The Eastern Oyster, *Crassostrea virginica* (Gmelin), is an ecologically and commercially important organism with a wide distribution from New Brunswick, Canada to the Gulf of Mexico. Oysters occur in dense intertidal and subtidal beds (Galstoff 1964, Burrell 1986, Carriker and Gaffney 1996). The eastern oyster is found primarily in subtidal areas in and around Mobile Bay. According to a 1995 survey by Alabama Department of Conservation and Natural Resources, Marine Resources Division, Mobile bay has approximately 1407.0 hectares of major public oyster reef (Tatum et al 1995). As the only oyster species harvested in Mobile Bay, the eastern oyster is an essential part of the local economy. The National Marine Fisheries Service reported that 16,804 metric tons of oysters valued at approximately $103 million were produced by the United States in 2003 with local oyster landings in Alabama producing 411.0 metric tons of oysters estimated at $2,120,392 (NMFS 2005).

Oysters are an integral part of the Mobile Bay benthic community providing biogenic habitat that supports many infaunal and epifaunal species (Burrell 1986, Lenihan and Peterson 1998, Heck et al 2005). Oyster reefs have a positive relationship with organism density, biomass and species richness (Lenihan and Peterson 1998). In a study by Wells (1961), 303 different species were associated with subtidal and intertidaloyster reefs in Beaufort, North Carolina. Coen and Luckenbach (2000)

collected 42 species of recreationally and commercially important fish, shrimp, and crab species at experimental and natural reefs in South Carolina. Oyster reefs are also home to various types of algae and invertebrates. Lenihan and Peterson (1998) cited that mud crabs (*Panopeus herbstii* and *Eurypanopeus depressus*) and blennies (*Chasmodes bosquianus* and *Hypsoblennius hentzi*) use the unique crevices that oyster reefs create as shelter. Dame and Libes (1993) provided evidence that oysters are responsible for nutrient retention in terms of total nitrogen, total phosphorus and ammonium in tidal creeks thus regulating availability of nutrients to local phytoplankton. Increasing oyster abundance in an ecosystem will decrease phytoplankton productivity and increase benthic fauna as described in the "top down" theory proposed by Ulanowicz and Tuttle (1992).

Oysters are of great importance as an environmental filter (Dame et al 1980, Newell 1988, Ulanowicz and Tuttle 1992). Adult oysters can filter water at a rate of 15 L/h in optimal conditions (Galtsoff 1964). Oysters primarily feed on phytoplankton but are also known to ingest detrital particles and bacterial associated with the particles (Galtsoff 1964). Dame (1980) calculated that roughly 1.5×10^7 m^3/tide (approximately 6 hours) of water can be pumped by dense oyster beds measuring 450g dry body weight/m^2 in North Inlet estuary, South Carolina. This calculation provided evidence that oysters in North Inlet estuary are capable of influencing suspended particulate matter of the surrounding ecosystem. Oysters filter suspended particulate from the water column which is then deposited as feces and pseudofeces. Oysters only use approximately 70% of ingested organic material and the remaining 30% is excreted as dense mucous-bound biodeposits (Newell 1988). These biodeposits in turn provide a nutritional source for

microbiota, meiofauna, and macrofauna which in turn makes them a link between pelagic and benthic food webs (Newell 1988).

Oyster reefs on the eastern side of Mobile Bay, have been chronically affected by low oxygen events dating back to 1876 (May 1973). A phenomenon known as a "Jubilee" occurs most often along the eastern shore of the bay in the warm summer months when pockets of low oxygen bottom water upwell along the shore. Stressed fish and invertebrates will move shoreward in an attempt to avoid the low oxygen waters. But immobile benthic animal such as oysters may be exposed to extended periods of low oxygen (Lenihan and Peterson 1998). "Jubilees" are a product of salinity stratification coupled with the lack of water circulation which then induces low dissolved oxygen in the stratified bottom waters (May 1973, Turner et al 1987). "Jubilees" occur during the summer months where warmer water encourages lower oxygen saturation and a net increase in oxygen consumption rates by microbial decomposition of organic matter settling to the estuarine floor (May 1973, Turner et al 1987, Rabalais 2002). According to May (1973), "Jubilee" events are set in motion by easterly wind induced currents that cause a northeasterly deflection of low oxygen bottom waters by incoming tides.

The geology of Mobile Bay contributes to the conditions favorable for hypoxic waters to develop. Mobile Bay has an average depth of 3 m except for the dredged shipping channel that is 120 m wide with an average depth of 12 m deep which runs the entire length of the bay (Schroeder and Wiseman 1986). Mobile Bay receives 95% of its freshwater input by the Mobile River system (Schroeder 1979). Mobile Bay is considered a positive estuary in which surface salinities are less than salinities in the open ocean (Pritchard 1952) and exhibits characteristics of both drowned river valley and bar

built estuaries (Schroeder and Wiseman 1986). These conditions are favorable for density stratification where increased freshwater input from the river can lead to density stratification where denser high salinity water from the Gulf of Mexico will remain at the bottom while the fresh water stratifies on the top. Turner et al (1987) has suggested that shallow estuaries like Mobile Bay have a high incidence of density stratification and are a productive source of organic matter, which in turn may lead to hypoxic bottom waters. Changes in benthic structure, such as the introduction of the shipping channel (May 1973) and spoil banks (Hoese 1987), has contributed to the formation of hypoxic conditions. Natural mixing of water by tidal exchange is prevented by the shipping channel. Dammed off pockets of high salinity water are trapped in the channel whereas before the introduction of the shipping channel the bathymetry of Mobile Bay was such that water circulation was not hindered by these man made spoil banks and shoals (May 1973).

Within the last 50 years there has been an increased occurrence of hypoxic (<2mg O_2/L) and sometimes anoxic (<0.1mg O_2/L) events in the Gulf of Mexico (May 1973, Diaz and Rosenberg 1995, Rabalais et al 2002). Increased occurrence of oyster mortality due to hypoxic events in Mobile bay may be credited to increased nutrients as shown by Osterman et al (2005) who attributed increased occurrence and intensity of hypoxic events on the Louisisana shelf to increased fluvial nutrients into Mississippi River discharge. Nutrient loads entering the estuary may enhance phytoplankton blooms which then lead to increased organic matter in the estuaries. Turner and Rabalais (2004) found that sediment cores taken from the Mississipi River plume support increased nitrogen loads into the estuary starting from the 1950's. Increases in the rate of production and

accumulation of excess nutrients that stimulate organism growth in an aquatic system also called eutrophication, lead to proliferation of detrital bacterial which can lead to hypoxic events (de Zwaan 2001a).

Hypoxia/anoxia has been proven to cause mortality (Lenihan and Peterson 1998, Saoud 2000) in oysters and can prevent damaged reefs from recovery (Lenihan and Peterson 1998; Rabalais 2001). A reef restoration study by Saoud et al (2000) in Bon Secour Bay, Alabama, provided evidence that anoxia/hypoxia causes mortality in juvenile oysters over a 5 day period of anoxia (<1mg/L O_2) at 28ºC. Baker and Mann (1992) also found that settlement of juvenile oysters was greatly reduced in hypoxic conditions and was almost non-existent in anoxic conditions. A follow-up study by Baker and Mann (1994) supported previous conclusions that hypoxia and anoxia have detrimental effects on the development, growth and survival on post-settlement oysters. Juvenile oysters exposed to microxic (<0.07 mg/L O_2) conditions for 24 h had stunted growth and eventually had total mortality at 120 h whereas normoxic oysters had only 8% mortality at 120 h.

Other factors that have attributed to oyster mortality include decline of reef height by harvesting and dredging (Rothschild 1994). Decreased oyster reef height through habitat degradation coupled with hypoxic/anoxic events increased oyster mortality in a study by Lenihan and Peterson (1998). Reduced water flow at reefs with lowered height can cause reduced water mixing and siltation which resulted in mortality in the Neuse River estuary, North Carolina. Oysters at a water depth of 6 m had 92 ±10% mortality

whereas oysters at a water depth of 3 m had 28 ± 9% mortality when exposed to periods of severe hypoxia/anoxia in the Neuse River estuary in 1993 (Lenihan and Peterson 1998).

Hypoxic/anoxic events put stress on the benthic community and in turn cause mass mortality of benthic invertebrates such as *C. virginica*. Because of the commercial importance of oysters as well as the significant role in the environment, it is vital to understand the effects of hypoxia/anoxia on the condition of these animals.

Hypoxia/Anoxia Tolerance in Oysters

Oysters are known to withstand a wide range of salinity (5ppt-39ppt) and temperature fluctuations (10ºC-32ºC) (Burrell 1986, Shumway 1996) and as a facultative anaerobe oysters can survive without the presence of oxygen for extended periods (Hochachka et al 1973). Stickle et al (1989) showed that oysters can slow metabolism to 75% of the normal metabolic rate to survive hypoxic events. Under anoxic conditions bivalves will revert to anaerobic glycolysis to maintain basic metabolic requirements (Hochachka et al 1973). Matthew and Damodoran (1997) demonstrated a decrease in glycogen under hypoxic conditions for the clam and, *Sunsetta scripta* and the green mussel, *Perna viridis*, showing that anaerobic glycolysis pathways are being used. Two products of anaerobic glycolysis in bivalves are L-alanine and D-lactate and have been proven to increase during periods of anoxia in the cockle, *Cerastoderma edule* (Babarro and de Zwaan 2001).

Temperature, salinity and pH play a major role in the hypoxia/anoxia tolerance in bivalves. Increased temperature has been shown to decrease survival times during a hypoxic/anoxic event in bivalve species. According to Shumway and Koehn (1982)

oysters acclimated at higher salinity coupled with low temperature can regulate oxygen consumption under decreasing oxygen tensions better than oysters acclimated at lower salinity with high temperatures. Stickle et al (1989) provided evidence that hypoxia tolerance at 20ºC is greater than at 30ºC for *C. virginica*. Theede et al (1969) also showed that an increase in temperature had an increased effect on cellular resistance to oxygen deficiency. Survival times of oysters decreased with an increase in temperature during a burial study (Dunnington 1968). De Zwaan and Barbarro (2001a) found that there is a decrease in pH during static incubation versus a stable pH in flow-through incubation in near anoxic trials with the Baltic clam, *Macoma balthica*. This decrease in pH was positively correlated to bacterial proliferation during the near anoxic experiment and can be positively correlated with shorter mortality times.

A negative relationship exists between survival rates of the ocean quahog, *Cyprina islandica,* peppery furrow shell, *Scrobicularia plana*, and soft clam, *Mya arenaria*) with the presence of hydrogen sulfide and pH values around 7 during hypoxic trials (Theede et al 1969). Looking at the dwarf surf clam, *Mulina lateralis*, Shumway and Scott (1983) also investigated effects of hydrogen sulfide during anoxia and found that mortality came much faster with the presence of hydrogen sulfide which indicates the presence of sulfate reducing anaerobic bacteria. During anoxic challenges, de Zwaan (2001b) concluded that the survival time of the cockle, *C. edule*, was directly correlated with the presence of anaerobic bacteria. A follow up study using the striped venus clam, *Chamelea gallina*, cockle, *C. edule*, and blood clam, *Scapharca inaequivalvis* showed a greater survival of anoxia challenged animals in tanks treated with the antibiotic Chloramphenicol. The conclusion of the second study suggests that mortality seems to

be caused by a proliferation of pathogenic anaerobic bacteria which causes tissue damage and not associated with degraded water quality. The positive effects of the antibiotic suggest that bacteria may be introduced into the system as a result of the bivalve natural flora (de Zwaan et al 2002).

Boyd and Burnett (1999) suggested that reactive oxygen intermediates (ROIs), an important defense mechanism produced by oyster hemocytes, may be affected by the stress of a hypoxic/anoxic event. The invasion of an oyster by a foreign particle is met by a phagocitic hemocyte which produces O_2^-, H_2O_2 (Anderson et al 1992) and HOCl (Austin and Paynter 1995). ROIs can cause extensive damage to cells by causing membrane damage, breakdown of DNA, enzyme inhibition and amino acid oxidation thus killing the invading cell (Anderson et al 1992). Using heat killed, washed yeast cells to induce ROI production, Boyd and Burnett (1999) showed that under hypoxic conditions oyster hemocytes produce only 33% of the normal ROIs. Decreased numbers of reactive oxygen intermediates may put an oyster at risk to succumb to infection.

Measures of Oyster Health

Histological examination of oyster tissues can be used to determine if the animal is stressed. Seasonal changes occur during tidal cycles in digestive-gland tubules of the Pacific oyster, *Crassostrea gigas*. Shaw and Battle (1957) described five main stages as: the holding stage where digestive tubule cells swell, the absorptive stage where food particles are taken into digestive tubule cells, the second absorptive stage where some cellular disintegration occurs, the disintegration phase and the reconstitution phase. Winstead (1995) showed that normal cuboidal epithelia of the digestive-gland tubules

transforms into simple, low cuboidal epithelia with a large circular lumen during salinity and starvation stress. Oysters can regenerate digestive cells after a period of stress to resume normal physiology (Eble and Scro 1996). Mix and Sparks (1971a) described the regenerative properties of digestive-gland tubules after various doses of ionizing radiation.

Evidence of irreversible tissue damage from ionizing radiation of oyster gonads was demonstrated by Mix and Sparks (1971b). Acute inflammatory responses have been documented by Pauley and Sparks (1965) who examined the pathological changes in tissues of the pacific oyster, due to direct injection of turpentine. At present, there have been no documented studies on the effects of anoxia/hypoxia on oyster tissues.

Leukocytes play a major role in the immune response of the oyster. Sparks (1976) describes the process by which leukocytes infiltrate into affected areas to begin repair when talc is injected into oyster tissue. Experimental bacterial infection with *Bacillus thuringiensis* and *Micobacterium smegmais* in the eastern oyster by Feng (1966) showed the ingestion of bacteria by leukocytes in the circulatory system through histological examination.

The natural bacterial flora of eastern oysters was determined by Murchelano and Brown (1968) as well as Hariharan et al (1995). Harriharan et al (1995) found the dominant anaerobic bacterium isolated from oysters was *Clostridium perfringens*. Certain bacteria have also been known to cause disease in oysters. The etiological agent causing juvenile oyster disease in cultured oysters <25 mm in height is caused by an unknown *Proteobacteria* designated CVSP (Boettcher 1999). Diseases such as Hinge Ligament Erosion can be linked to *Cytophaga sp.* (Dungan et al 1988) in adult Pacific

oysters, *C. gigas*. *Nocardia* , *Chlamydia*, and *Vibrio anguillarum* have been associated with nocardiosis, chlamydial infections and vibrio infections respectively, (Tubiash 1973, Friedman et al 1991, Renault and Cochennec 1995). Except for the known natural anaerobic bacterial flora of oysters, studies on pathogenic anaerobic bacteria have yet to be performed.

Condition index (CI) can be used to evaluate how changing environments affect oysters (Mercado-Silva 2005). Oysters exhibit distinct seasonal variations with respect to condition index. Oysters had increased condition indices during spring and summer months correlated with gonadal development and a sharp decline in indices were reported after spawning had taken place (Ruddy et al 1975). Oyster condition indices in Virginia estuaries showed a positive correlation with salinity and peaked during periods of rapid temperature change (Austin and Haven 1993). Saoud et al (2000) determined that oysters deployed 40 cm from the bottom in Mobile Bay, Alabama, had a greater mean condition index than oysters deployed on bottom. Poor oyster condition was attributed to oxygen and siltation stress in bottom waters.

Glycogen, the main storage unit of energy for oysters, is an indicator of condition and levels fluctuate throughout the year. Early spring oysters show decreases in levels of glycogen as gametogenesis occurs and increases of glycogen occur during late fall (Chipman 1947, Engle 1951). The main factors in glycogen synthesis can be linked to temperature and food availability (Ruddy et al 1975). Increases in phytoplankton blooms in warm waters produced oysters with 109% higher carbohydrate concentration than control oysters (Ruddy et al 1975). Glycogen stores in two marine bivalves the green mussel, *Perna viridis* and the estuarine clam, *Villorita cyprinoides* showed a substantial

decrease after exposure to toxic levels of copper and mercury at 28ºC (Lakshmanan and Nambisan 1985). Hummel et al (1989) demonstrated that when stressed with high temperatures and exposure to air, the blue mussel, *Mytilus edulis* and the cockle, *Cerastoderma edule* converted glycogen to the by-product acetic acid but cockles with high glycogen content did not use glycogen stores for the first 3 to 7 days of the experiment. Babarro and de Zwaan (2001) showed that cockles exposed to near anoxic conditions exhausted energy stores only when there was addition of the antibiotic Chloramphenicol to the incubation.

Fecundity of the eastern oyster can be highly variable due to changes in salinity, temperature, food supply, nutritional status, and stress from disease (Cox and Mann 1992, Thompson et al 1996). Butler (1949) showed decreased fecundity in the eastern oyster related to stress associated with lower average salinities. Disruption of gametogenesis and asynchronous spawning by physical, biological and chemical factors can be linked to low recruitment on oyster reefs in Maryland (Kennedy and Krantz 1982). Warmer water temperatures and subsequent gametogenesis conditions for oysters in Mobile Bay coincide with hypoxic/anoxic events.

This study evaluates the tissues of *C. virginica* before, during, and after an exposure to anoxic conditions for histological lesions, facultative anaerobic bacterial loads, condition index, and glycogen stores. At present, there have been no documented studies on the effects of anoxia/hypoxia on tissues and fecundity of the eastern oyster.

II. MATERIALS AND METHODS

Acclimation

Five hundred hatchery produced oysters, were collected from suspended culture racks located in Bon Secour Bay, Alabama and transported to the Auburn University Shellfish Laboratory on Dauphin Island, Alabama. The oysters were approximately one year of age with a mean size of 48.07 mm. All oysters were cleaned of fouling organisms and stocked into a 2.4 x 0.6 x 0.25 m tank for acclimation. The tank was supplied with flow-through seawater filtered to 35 microns and delivered at a rate of 3.0 L/h/oyster. A chiller unit (1HP, Aqualogic, Inc) maintained temperatures at 28°C. A Hydrolab MiniSonde 4® was placed in the tank to monitor temperature (°C), salinity (ppt), and dissolved oxygen (mg/L).

Experiment

A preliminary ranging experiment was conducted from May 25, 2006 through May 27, 2006 similar to the trial described below, to determine the approximate time to mortality for oysters under anoxic/hypoxic conditions. Results indicated mortality within the first 24 hours and rapidly increasing above 40% after 72 h (n=15) (Table 1).

Sampling intervals were chosen at 24 h, 48 h and 60 h. These intervals were selected to provide sufficiently stressed oysters for sampling but maintain survival to provide adequate numbers of live oysters.

The anoxia challenge began on May 28, 2006. A total of 370 oysters were randomly divided into control and experimental groups. Each group was then divided into sub-groups; Baseline (40 oysters), Control (40 oysters), Control Recovery (40 oysters), Control Fecundity (30 oysters), 24 Hour Interval (30 oysters), 48 Hour Interval (30 oysters), 60 Hour Interval (40 oysters), Experimental Recovery (40 oysters), Experimental Fecundity (30 oysters). The remaining 86 oysters were grouped with the experimental group to ensure live oysters for sampling even if substantial mortality occurred.

Four, 30 gallon aquariums were used for the experimental set up in the laboratory. Two were set up for anoxic conditions and two were set up for normoxic conditions (Figure 1). Aquaria were filled with natural seawater filtered to 1μm and ultraviolet (uv) sterilized. Hypoxic conditions were established in experimental aquaria by bubbling nitrogen through air stones for 24 hours to drive off oxygen before addition of oysters. Nitrogen was supplied by a regulated pressurized nitrogen cylinder. Normoxic conditions were maintained by bubbling air supplied by a Piston Compressor Model SL22 (Aquatic Ecosystems Inc, Apopka, FL). Temperature was maintained at 28 ± 1ºC with a 500 watt Finnex Titanium heater placed in each aquaria.

Oysters were submerged in normoxic and anoxic aquaria at the same time. Oxygen concentrations for the anoxic tank were maintained below 0.1 mg/L at all times.

Temperature (ºC), salinity (ppt), pH, and dissolved oxygen (mg/L) readings were taken every 4-6 hours in both control and experimental tanks by a handheld YSI 85® coupled with a Accumet AR 20 pH/conductivity meter (Fisher Scientific).

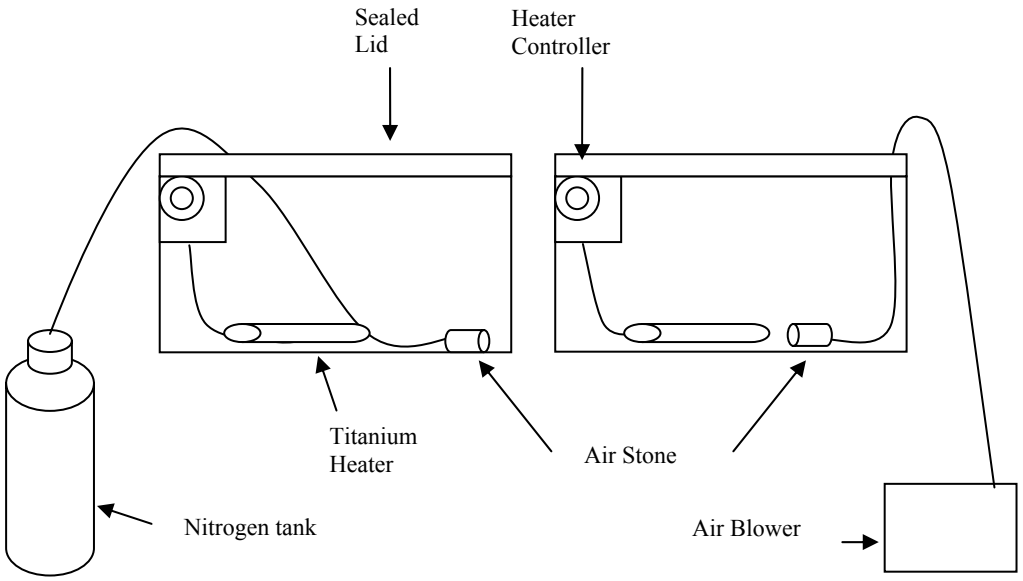

Figure 1. Experimental set-up of tanks used for anoxia trials.

Interval	Mortality
24 hours	1
48 hours	0
72 hours	5

Table 1. Mortality count for preliminary anoxia trial at 28ºC and 25 ppt salinity. (n=15)

Sampling Procedures

The Baseline group of 40 oysters was sampled immediately prior to starting the experiment to determine a baseline for comparison to oysters from the experimental sampling and post recovery sampling. All sampled oysters were measured with Vernier calipers to the nearest 0.1 mm. Ten oysters were used for histological examination, 10 oysters were used for bacterial analysis, 10 oysters were used for glycogen analysis and 10 oysters were used for determination of condition indexes.

After the first 24 h interval, 30 oysters were chosen randomly from the anoxic tank for sampling. The remaining oysters were inspected for mortality. Oysters were considered dead if they remained gaped after handling. Oysters were switched into a new anoxic tank (as described previously) and sealed for another 24 h. The control oysters were also observed for mortality at 24 h and moved to a new aerated tank (as described previously). From the experimental oysters sampled, 10 oysters were used for histological examination, 10 oysters were used for bacterial analysis and the last 10 were used for glycogen determination. Used tanks were emptied, cleaned and refilled with new seawater filtered to 1μm and uv sterilized. The anoxia tank was sealed and nitrogen bubbled through it overnight to drive off all oxygen. The sampling procedure described for the 24 h interval was repeated at the 48 h interval.

At the 60 h interval, 40 experimental oysters and 40 control oysters were sampled. Ten oysters from each control and experimental group were used for histological examination, 10 oysters were used for bacterial analysis, 10 oysters were used for glycogen determination, and 10 oysters were used for determination of condition index. The remaining experimental and control oysters were removed from their respective

tanks and put into a recovery tank (2.4 X 0.6 X 0.25 m) with flow-through seawater filtered to 35 μm and maintained at 25 ± 1ºC by a 1 HP Aqualogic, Inc chiller unit. During the recovery period, a Hydrolab MiniSonde® monitored temperature (ºC), salinity (ppt) and DO (mg/L). After a 4 week recovery period, 40 oysters from the experimental group and 40 oysters from the control group were sampled as described previously for the 60 h interval. Oysters from the recovery period were examined to determine changes in bacteria levels, glycogen levels, condition index, and tissue structure compared to pre-experimental conditions.

The final two groups of oysters, Control Fecundity (30 oysters) and Experimental Fecundity (50 oysters), were used to determine the fecundity of the oysters 18 weeks after the experiment.

Histology

Shell height was measured to the nearest 0.1 mm for all oysters sampled for histology. Oysters were then shucked and wet meats were weighed to the nearest 0.1 g. Whole oyster bodies were preserved in 2 parts Davidsons fixative: 1 part ambient seawater at 25 ppt for 24 h. After 24 h the samples were transferred to 70% isopropyl alcohol (Howard and Smith 1983). Each animal was placed in a separate container, labeled with the sampling interval and the date it was sampled. All samples were transported to the Auburn University Department of Fisheries Southeastern Cooperative Fish Disease for histological processing laboratory in Auburn, Alabama. The 70 samples were dehydrated and infused with paraffin wax using a Sakura Tissue-Tek® VIP. Samples were then mounted and embedded in paraffin on cassettes using a Tissue-Tek®

TEC™ 5 embedding console station to stabilize samples for use on a microtome and embedded in paraffin. A portion of each oyster located at the junction of the labial palps and the gills (Howard and Smith 1983) was sliced into 5 μm sections using a microtome. Three sections of each oyster were produced for each of two slides. Sections were mounted on slides and one was stained with Hematoxylin and Eosin stain and the other with Giemsa stain. Cover slips were affixed to the slides using Permount® and allowed to dry overnight before inspection under the microscope.

Tissues stained with Harris' Hematoxylin and Eosin were examined for normal tissue structure in control oysters and affected tissues in experimental oysters. Tissues stained with Giemsa were examined for pathogens, specifically bacteria. The gills, digestive diverticula and mantle tissue were examined for lesions under a compound microscope. Digestive tubules were measured using techniques described by Winstead (1995). Each oyster section was divided into four quadrants and then three randomly selected digestive tubules were measured in each quadrant. Two sets of measurements perpendicular to each other were taken from each tubule. The first set measured the external diameter (A_1, A_2) and the second set measured the internal diameter (B_1, B_2) of the digestive tubule. The sum of the two sets of internal diameter measurements were then divided by the sum of the two external diameter measurements to get the oyster tubule ratio. The following formula was used to calculate tubule ratio for the baseline, 24 h, 48 h, 60 h and 60 h control intervals (Winstead 1995):

$$\text{Tubule Ratio} = \frac{B_1 + B_2}{A_1 + A_2}$$

Bacteriology

The following procedure was used at each interval sampled to analyze bacterial loads. Each oyster was scrubbed under fresh running water with a wire brush, measured with Vernier calipers to the nearest 0.1 mm for height, and then wiped with 70% isopropyl alcohol to sterilize the area. Oysters were shucked using aseptic technique (APHA 1970) into a sterile tared homogenizer cup. The shucking knife was then cleaned with 70% isopropyl and set aside for the next sample. The wet meat weight was recorded to the nearest 0.01 g then 10 ml of sterile 25 ppt seawater was added to the sample. Parafilm® was secured onto the top of the homogenizer cup and the sample was homogenized for approximately 30 sec using a commercial blender on high speed. A sterile disposable 1 ml pipette was used to extract 1 ml of homogenized sample and place it into a labeled, sterilized 15 ml centrifuge tube with 9 ml of sterile 25 ppt seawater. The tube was capped, labeled with the sample number and placed under a sterile UV hood for plating. The remainder of the homogenate was discarded. The homogenizer cup was then washed thoroughly with water then 70% isopropyl and rinsed with sterile 25 ppt seawater in preparation for the next sample. Plating of samples began after all oysters were homogenized and aliquoted to their respective tubes. Each interval had a specific plating dilution based on expected number of bacteria. Expected bacterial numbers were determined by prior sampling of oysters in a preliminary experiment. Marine Agar and Brain Heart Infusion Agar with 1% NaCl were both chosen for plating bacteria due to their non-selective properties. Also Brain Heart Infusion Agar was recommended by the Rapid ANA II system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) that was used to identify bacteria.

Each oyster dilution was plated by placing 0.1 ml on each of four Marine Agar plates and two Brain Heat Infusion with 1% NaCl plates and spread with a sterilized glass rod (Buck and Cleverdon 1960). Three Marine Agar plates and one Brain Heart Infusion with 1% NaCl plate were incubated in an anaerobic environment using BD Bio-Bag™ Environmental Chambers Type A (Becton, Dickinson and Company, MD). Anaerobic chambers were used to isolate facultative anaerobic bacteria for total body counts, isolation and identification. The remaining Marine Agar and Brain Heart Infusion with 1% NaCl plate were incubated simultaneously in aerobic conditions. A simultaneous incubation was used to determine if there was a difference between bacterial counts in an aerobic environment versus an anaerobic environment. During each plating session, 1 Marine Agar plate and Brain Heart Infusion with1% NaCl were left open in the hood and incubated to account for any potential contamination.

Colony forming units (CFU) were counted and recorded for each plate after 24-48 h (as per recommended by Anaerobic Chamber Type A directions) of incubation in an oven at 31ºC. Individual colonies were chosen from the anaerobic incubated plates for further isolation and identification based on morphological properties of the colony. Only the most abundant colonies were used for identification. Rapid ANA II (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) was used to identify the bacteria along with a gram stain, Catalase test, Oxidase test and Indole test.

Condition Index

Condition indexes were determined using the method described by Crosby and Gale (1990). Oysters were cleaned thoroughly under running fresh water with a wire

brush then dried for 30 min in the air. Heights were recorded to the nearest 0.1 mm and whole body weights to the nearest 0.01 g were recorded for each oyster. Oysters were then frozen until processing. At the time of processing, oysters were shucked and meats and shell were placed into separate tared, labeled weighing tins. Oysters shell and tissue were put on racks in a drying oven (80°C) for 48 h. After drying, dry soft tissue weight and dry shell weight were recorded to the nearest 0.01 g. The following formulas were used to calculate condition index (CI)(Crosby and Gale 1990):

Internal shell capacity = Total whole live weight - Dry shell weight

$$CI = \frac{\text{Dry soft tissue wt (g)} \times 1000}{\text{Internal shell cavity capacity (g)}}$$

Glycogen Analysis

Each oyster was measured for height to the nearest 0.1 mm then shucked into labeled, tared Nasco Whirl-Pak bags and weighed to the nearest 0.01 g. Oysters were promptly put into the freezer after their weights were recorded. The frozen oysters were lyophilized with a Labconco lyophilizer (Labconco Co, Kansas City, MO) in separate bags for glygogen analysis (Keppler and Decker 1974). For each oyster, 0.25 g of lyophilized tissue was homogenized with 12.25 ml Perchloric acid for 30-45 sec. An aliquot of the resulting homogenate (0.2 ml) was transferred into 2 labeled centrifuge tubes. The first tube was hydrolyzed by adding 0.1 ml of Potassium Hydrogen Carbonate and 2.0 ml of Amyloglucosidase. This tube was incubated in a shaker bath (40°C) for 2 h. At the end of the two hour incubation, 1.0 ml of Perchloric Acid was added to the centrifuge tube to stop the hydrolysis and then centrifuged for 15 min. The second tube of homogenate (blank) was promptly centrifuged for 15 min. A 0.05 ml aliquot of

supernatant was dispensed from both the hydrolysis and blank tube into respective small volume cuvets along with 1.0 ml of ATP solution. Addition of the ATP solution causes an oxidation of glucose-6-phosphate which is directly related to the amount of glycogen in the sample. After 5 min., both samples were read with a spectrophotometer at 340 nm. When all glucose-6-phosphate is oxidized and it is read with a spectrophotometer the resultant number is deemed extinction of hydrolysis 1 (E_{1H}) and extinction of blank 1(E_{1B}). Into both cuvetts 0.005 ml Hexokinase solution was then added and mixed well to determine the amount of glucose formed during the hydrolysis. After a period of 15 min both cuvets were read with a spectrophotometer at 340 nm to give extinction of hydrolysis 2 (E_{2H}) and extinction of blank 2 (E_{2B}). To calculate the final glycogen content of each oyster, the following formulas were employed (Keppler and Decker 1974):

Formula 1 $(E_{2H}) - (E_{1H}) = \Delta E_H$

Formula 2 $(E_{2B}) - (E_{1B}) = \Delta E_B$

Formula 3 $\Delta E_H - \Delta E_B$ = Glycogen content (μmole glycosyl units/g dry tissue weight)

Fecundity

Female oysters were processed for fecundity values by the method described by Cox and Mann (1992). Height was recorded to the nearest 0.1 mm for all oysters. Oysters were then shucked and wet meats were placed into tared weighing tins and weights were recorded to the nearest 0.01 g. A sample of gonadal tissue was examined under a microscope for sex determination. Female oysters were placed into a clean

homogenizer cup with 10 ml of sterile ambient seawater (25 ppt). The blender cup was covered with Parafilm® and blended on high for 45 sec. The homogenate was sieved first through a 100 μm mesh then through a 40 μm mesh. The liquid passing through the 53 μm containing oyster eggs was retained in a 1 L beaker. The blender cup and sieves were also rinsed into the same 1 L beaker. Additional seawater (25 ppt) was added to bring the volume to 1 L. Total number of eggs was determined by mixing the beaker thoroughly, taking a 1 ml aliquot and counting the eggs under a compound microscope using a Sedgewick Rafter. Three replicate 1 ml aliquots for each sample was taken for consistency. The fecundity of each oyster was calculated by multiplying the mean number of eggs/ml by the total volume in the sampled beaker.

Statistics

Statistical analyses were performed using Statistical Analysis System (SAS) version 9.1. Bacteriological counts, condition indexes and glycogen analysis measurements were averaged at each sampling interval for each group. Bacteriological samples were $\log_{10}$ transformed prior to analysis. Data was analyzed using one way Analysis of Variance (ANOVA) with Tukey's Studentized Range (HSD) test for determination of differences among groups with a significance level of $P < 0.05$.

III. RESULTS

The 500 oysters used in the anoxia experiment had a mean (± 1SE) height of 48.05 mm ± 0.30. There were no mortalities found in the control group throughout the experiment. A total of 24 oysters were found dead in the experimental tanks for the duration of the experiment (Table 2). Dissolved oxygen in the control tank ranged from 5.70 mg/L to 6.20 mg/L while temperature was from 27.8 ºC to 28.5 ºC (Figure 2). Dissolved oxygen levels in the experimental tank ranged from 0.03 mg/L to 0.09 mg/L. Temperature ranged from 27.5 ºC to 28.2 ºC (Figure 3). Salinity for both experimental and control tanks was stable at 24 ppt and 25 ppt respectively. Mean pH levels in control and experimental tanks were 7.92 ± 0.10 (Figure 4) and 7.77 ± 0.14 (Figure 5), respectively.

Microscopic examination determined mantle tissue for baseline oysters was normal with non-ciliated tall columnar to cuboidal epithelial cells with microvilli on the shell side and simple, ciliated cuboidal cells on the pallial side. Normal vesiculated Leydig tissue cells connect the shell side and pallial side mantle tissue (Figure 6). Oysters examined at the 24 h, 48 h, and 60 h interval all showed signs of stress exhibited in the mantle tissue. Mantle epithelium remained in tact with columnar epithelial cells on the shell side of the tissue and ciliated cuboidal cells on the pallial side of the tissue. Connective tissue in the mantle was slightly broken down at the 24 h interval and showed

a progressive deterioration through to the 60 h interval (Figure 6). Congestion of hemolymph sinuses along with movement of hemocytes into the mantle tissue were observed in the 24 h oysters (Figure7). Leucocytes were observed in mantle Leydig tissue at the 48 h and 60 h intervals (Figure 6).

Baseline oysters had healthy digestive tubules. Tubules consisted of the three normal cell types which consist of dark staining basophilic cells (non-flagellated basophil cells and flagellated basophil cells) and digestive cells or secretory absorptive cells (Figure 10). Digestive cells formed a simple columnar epithelium between the crypts composed of flagellated and non-flagellated basophil cells (Weinstein 1995). The crypts rest on a thin basement membrane composed of muscle fibers. Normal connective or Leydig tissue can be observed between the digestive tubules (Figure 9). Digestive tubule cells transformed from columnar cells into squamous cells at the 24 h interval with an increased mean tubule ratio of 0.25 ± 0.02 in the baseline interval to 0.62 ± 0.02 in oysters from the 60 h interval (Figure 8, 11). The 24 h, 48 h, and 60 h intervals had mean digestive tubule ratios of 0.63 ± 0.02, 0.61 ± 0.02 and 0.63 ± 0.02 respectively, which were not significantly different. The mean digestive tubule ratio of control oysters at 60 h measured 0.46 ± 0.02 and was significantly lower than the experimental intervals. Sloughing of the basophil cells into the lumen of the digestive tubules was evident at 24 h (Figure 9). At 48 h necrosis of the digestive tubule could be seen with leucocytic infiltration of the digestive tubule cells (Figure 9). Notable deterioration of tubule epithelium was seen at the 60 h interval (Figure 9). Giemsa stain performed on digestive tubule tissue showed bacterial infiltration of the lumen and digestive cells of 24 h, 48 h, and 60 h oysters (Figure 10). At the 24 h interval 3 out of the 10 oysters sampled had

positively staining bacteria. Both at the 48 h interval and the 60 h interval 2 out of 10 oysters sampled showed signs of bacterial infection. Digestive tubule ratios at control recovery and experimental recovery intervals were not significantly different (Figure 11) Experimental recovery tubules were regenerated to baseline condition with small lumens and tall columnar epithelial digestive cells (Figure 13) and showed no signs of bacterial infection.

Gills were normal at the baseline interval with ciliated frontal and lateral cells along with goblet cells. Filaments had normal slender lacunar cells forming the hemolymph sinus (Figure 11). Gill tissue remained normal throughout the experiment. No notable change was seen in the lacunar cells, frontal or lateral ciliated cells (Figure 12, 15).

Control recovery and experimental recovery oysters exhibited necrosis of Leydig tissue in the mantle and digestive gland. Shadows of the outlines of lightly staining Leydig tissue could be seen in expermental recovery oysters with some leukocytes still present (Figure 13).

Mean $\log_{10}$ transformed facultative anaerobic bacterial count for test oysters from the 60h interval was 5.87±0.08 CFU/ml (mean ± SE). This was significantly higher than counts for the baseline (5.05±0.07), 24 h (4.94±0.11), 48 h (5.60±0.09), and 60h (5.69±0.06 CFU/ml) control intervals. Both control recovery and experimental recovery oysters had significantly lower than baseline, 24 h, 48 h, 60 h and control 60 h oysters (Figure 16). A total of 43 isolates were taken for identification. The 3 main anaerobic bacteria isolated throughout the experiment were *Clostridium sp* (53%), *Propionibacterium sp* (12%), and *Actinomyces sp* (9%) with the remaining 26% being

unidentifiable gram negative rods. Heterotrophic aerobic counts throughout the experiment had a slight increasing trend. The baseline and 24 h interval were significantly lower than the 60 h interval with values of 5.23 ± 0.12, 5.40 ± 0.18 and 6.38 ± 0.07 CFU/ml, respectively. The other intervals, control recovery and experimental recovery, had no significant difference with means of 4.44 ± 0.40 and 4.58 ± 0.43 CFU/ml (Figure 17).

Condition indices were not significantly different between baseline oysters with a mean of 59.0±2.6 and test oysters at the 60 h interval with a mean of 57.4 ± 1.4. There was also no significant difference between the baseline oysters and the 60 h control oysters which had a mean of 55.9 ± 2.2. There was however a significant difference between experimental oysters at the 60 h interval and experimental recovery oysters with a mean of 45.4 ± 0.79. Control oysters at 60 h interval and control recovery oysters with a mean of 45.2 ± 2.3 were also significantly different (Figure 18).

Mean glycogen levels for test oysters from the 24 h, 48 h and 60 h intervals were 100.4± 23.2, 47.0± 15.2, and 64.5± 13.0 μmoles glucosyl units per g dry weight, respectively. Mean glycogen levels from test oysters did not differ from the baseline with a mean of 71.6± 18.2 μmoles glucosyl units/g dry weight. No significant difference was seen between the control recovery oysters and experimental recovery oysters 32.4 ± 8.76 and 27.8 ± 7.25 (Figure 19).

At 18 weeks post experiment only 5 oysters out of the 43 surviving experimental oysters were female while only 2 out of the 28 surviving control oysters were female. The

experimental oysters had a mean of 11640 ± 3363 eggs/g wet weight and control oysters had a mean of 14235 ± 3124 eggs/g wet weight. No statistical operations could be performed due to low female to male ratios.

Time (h)	Mortality
24	2*
48	7
60	15

Table 2. Mortality of experimental oysters subjected to anoxic water (0 to 60 h).

*Denotes 1 oyster may have died prior to study. (n=15)

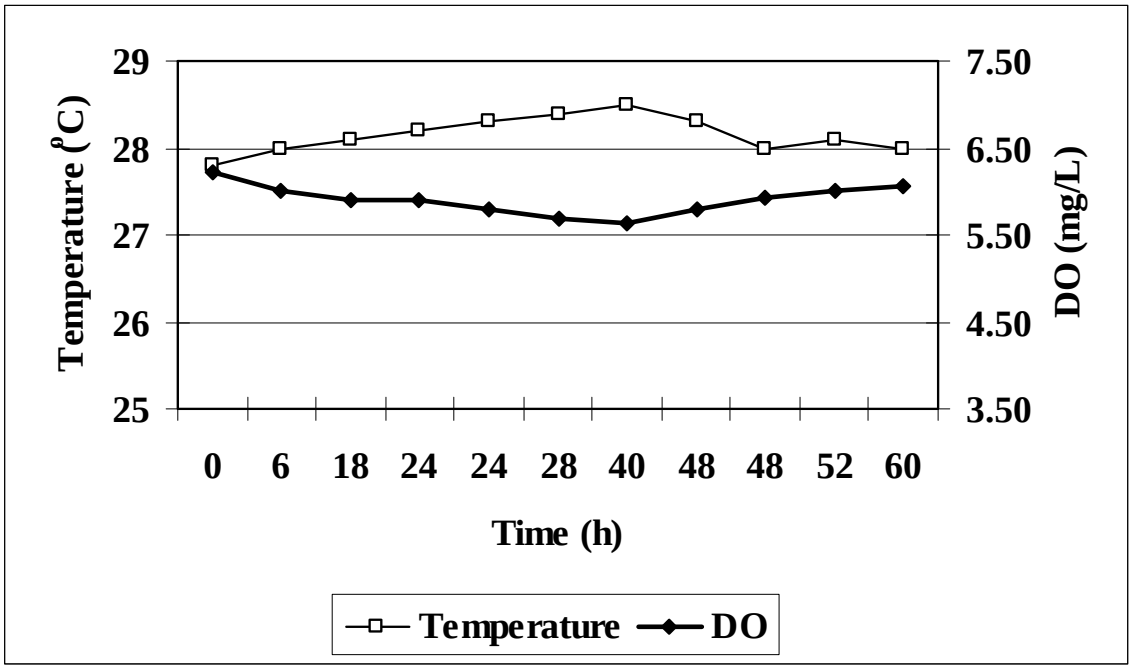

Figure 2. Temperature (°C) and dissolved oxygen (mg/L) for control tanks (0 to 60 h).

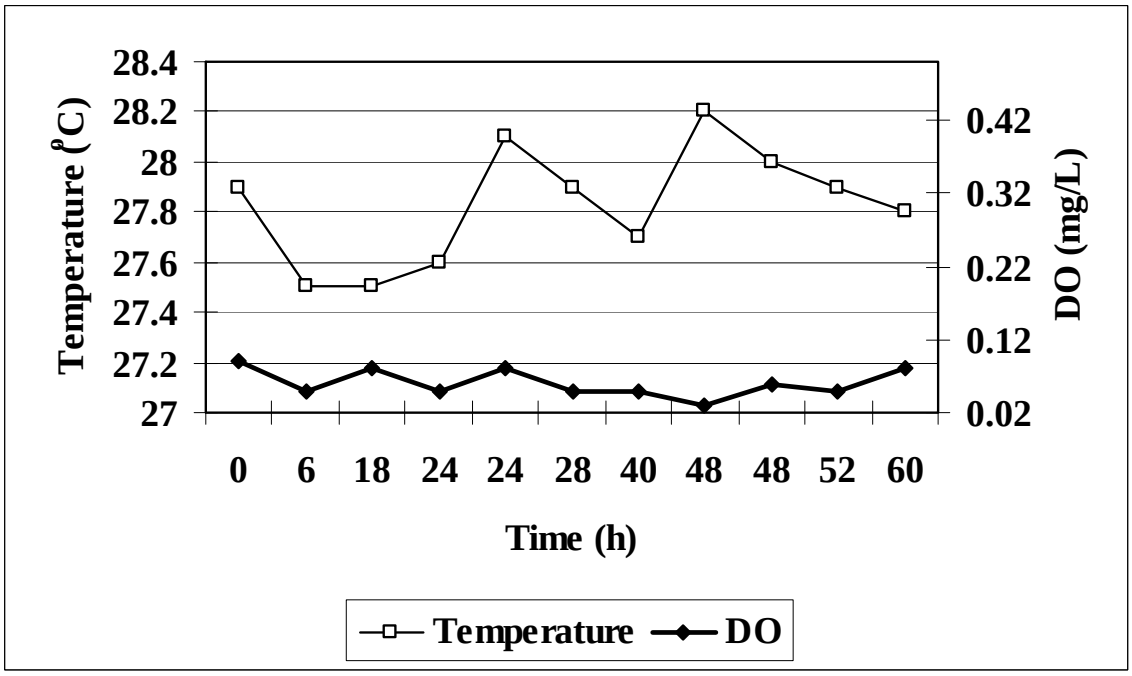

Figure 3. Temperature (°C) and dissolved oxygen (mg/L) for anoxic tanks (0 to 60 h).

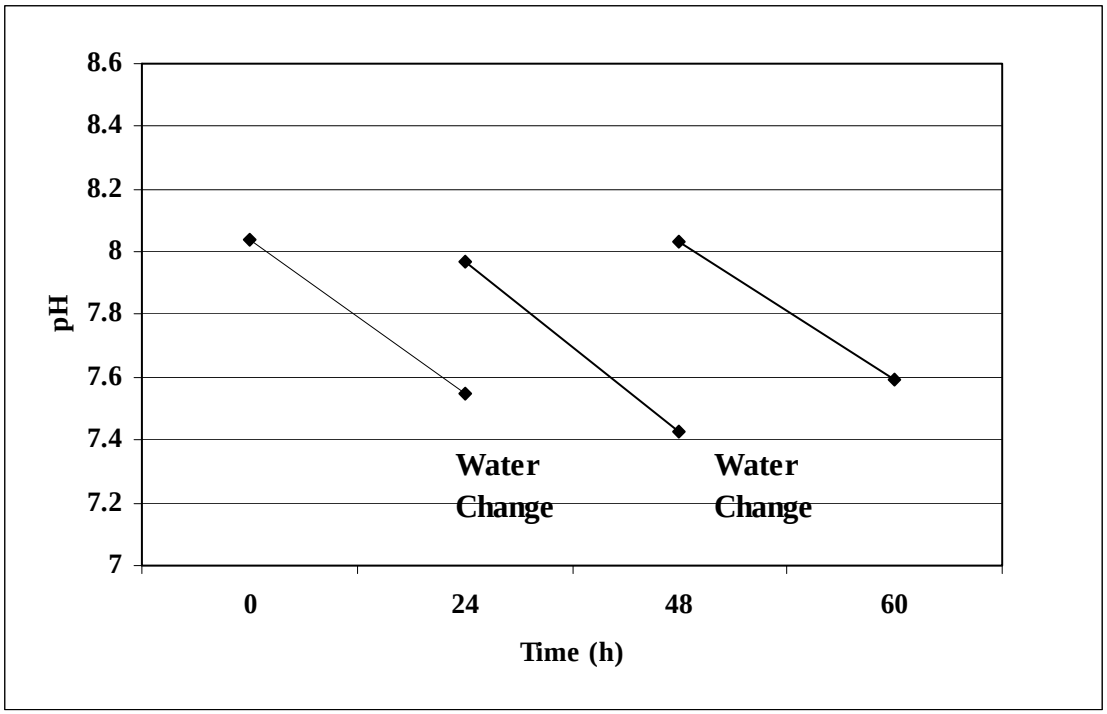

Figure 4. pH in control tanks (0 to 60 h).

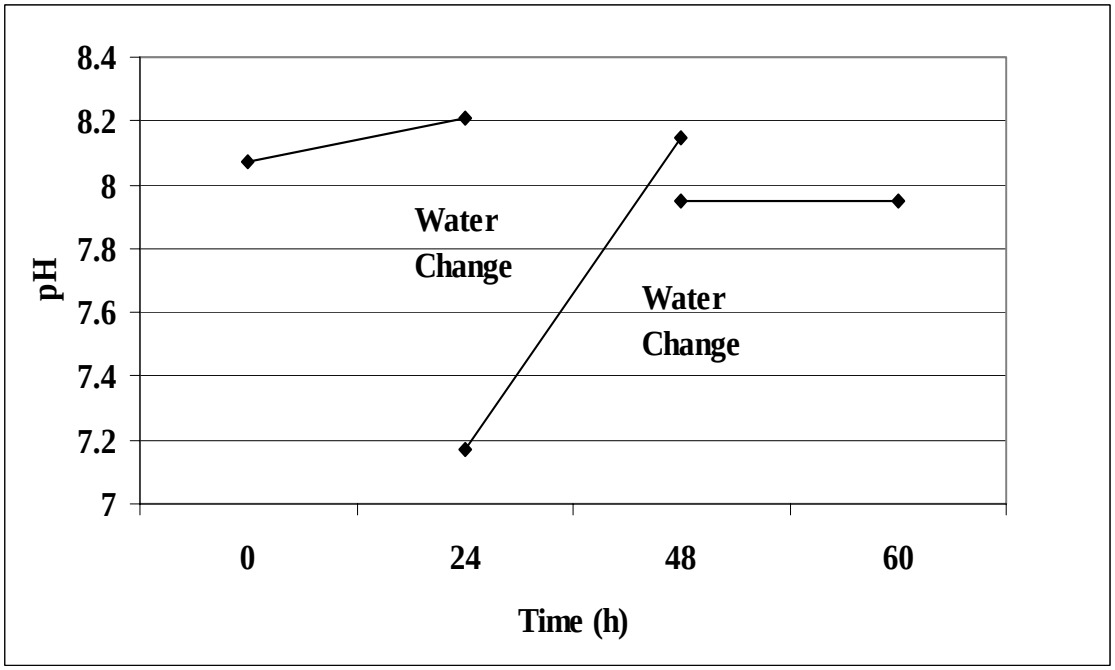

Figure 5. pH in experimental tanks (0 to 60 h)

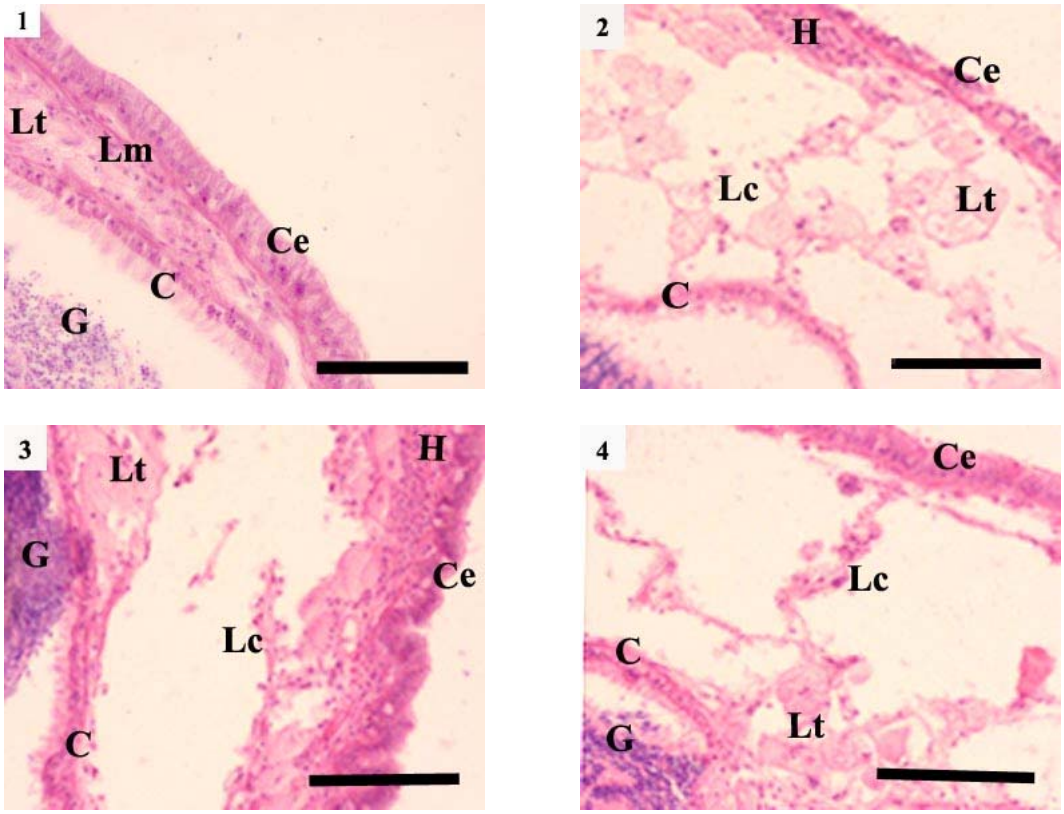

Figure 6. Photomicrograph of a section (5µm) of oyster mantle tissue at 20X. (1) Baseline; (2) 24 h interval; (3) 48 h interval; (4) 60 h interval. Abbreviations: C, ciliated cuboidal cells; Ce, columnar epithelial cells; G, gonad; H, hemocytes; Lc, leucocytes; Lm, smooth muscle; Lt, Leydig tissue. Scale bar = 100 µm.

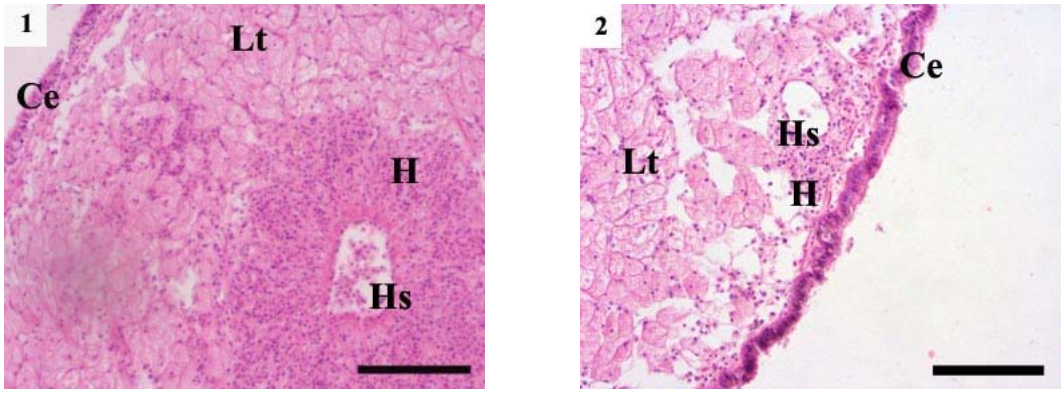

Figure 7. Photomicrograph of a section (5µm) of oyster mantle tissue showing infiltration of hemocytes into the connective tissue at 20X. (1) 24 h interval (2) 60 hr interval. Abbreviations: Ce, columnar epithelium; H, hemocytes; Hs, hemolymph sinus; Lt, Leydig tissue. Scale bar = 100 µm.

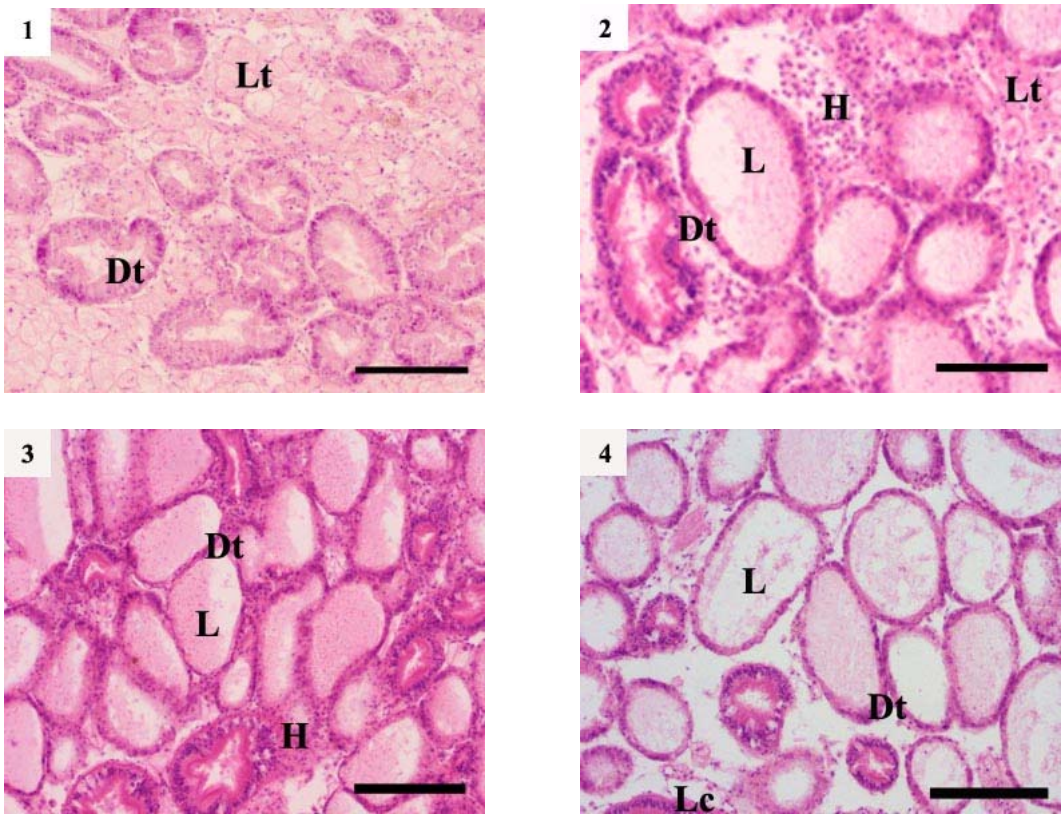

Figure 8. Photomicrograph of 5 μm sections of oyster digestive tubule tissue at 20X showing increased tubule lumen. (1) Baseline; (2) 24 h interval; (3) 48 h interval; (4) 60 h interval. Abbreviations: Dt, digestive tubules; H, hemocytes; L, lumen; Lc, leucocytes; Lt, Leydig tissue. Scale bar = 100 μm

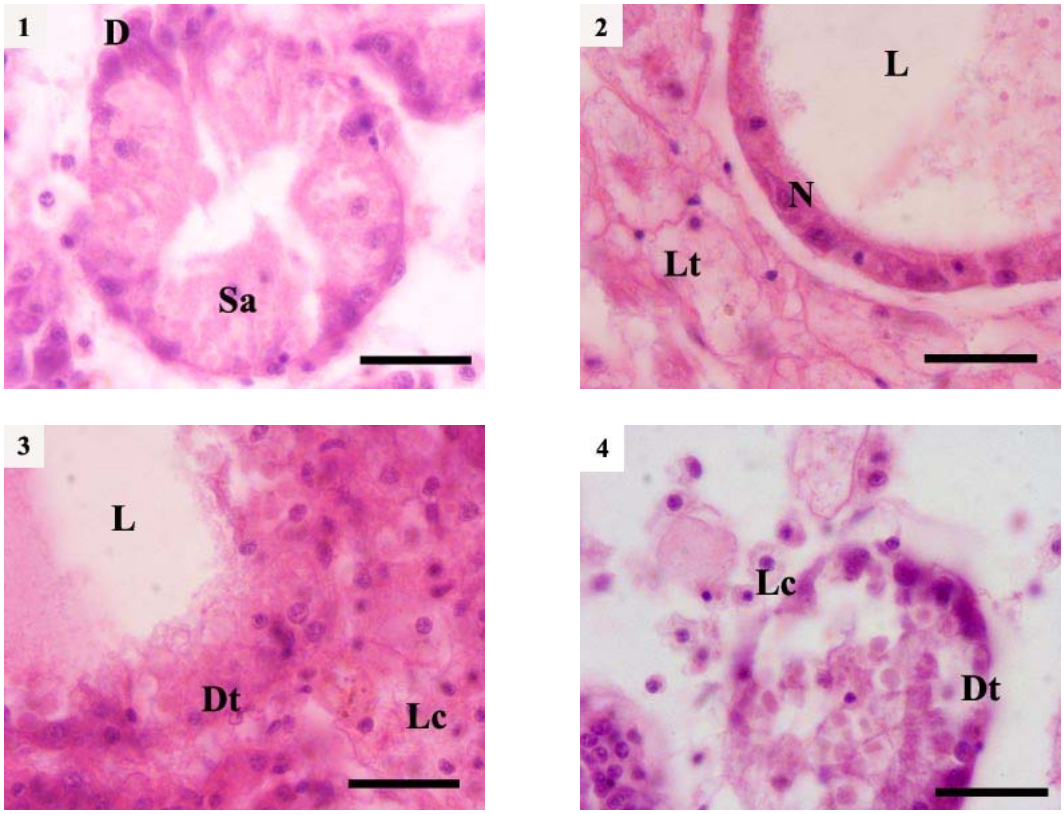

Figure 9. Photomicrograph of digestive tubules at 100X showing necrosis. (1) Baseline; (2) 24 h interval; (3) 48 h interval; (4) 60 h interval. Abbreviations: D, digestive cells; Dt, digestive tubule; L, lumen; Lc, leucocyte; Lt, Leydig tissue; N, non-flagellated basophil cells; Sa, secretory aborptive cells. Scale bar = 50 μm

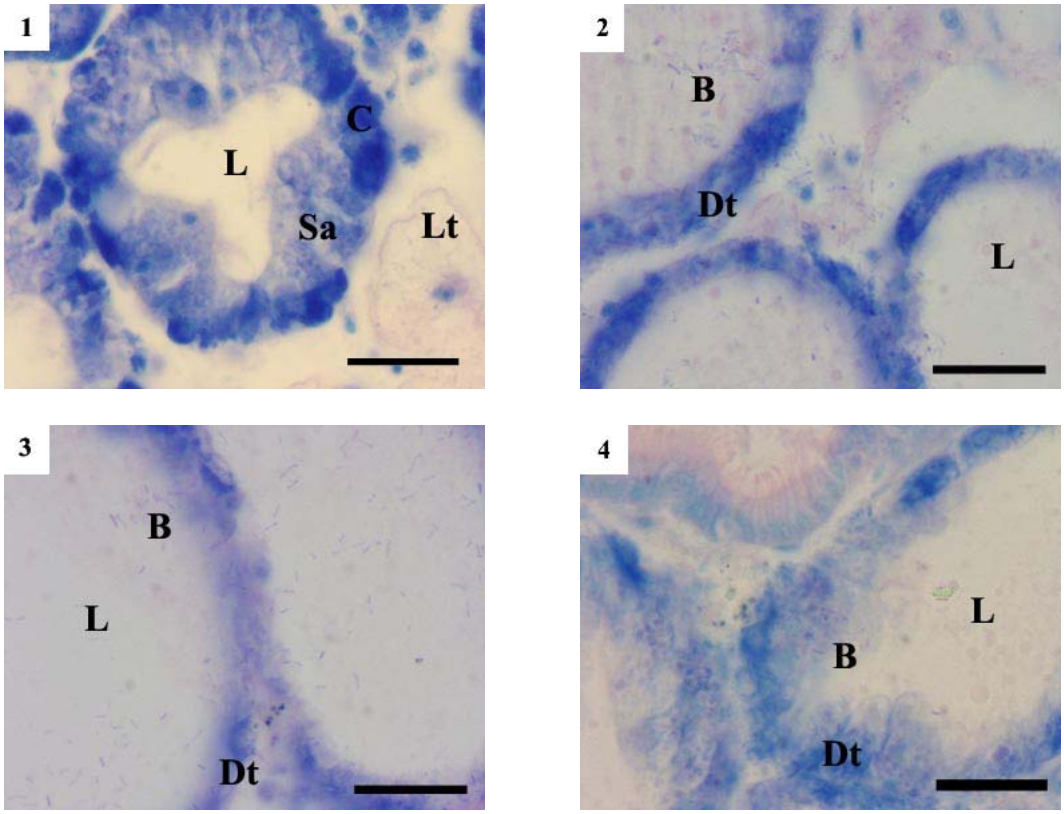

Figure 10. Photomicrograph of Giemsa stained digestive tubules at 100X showing breakdown and bacterial infection. (1) Baseline; (2) 24 h interval; (3) 48 h interval; (4) 60 h interval. Abbreviations: B, bacteria; C, crypts (flagellated basophil cells and non-flagellated basophil cells); D, digestive cells; Dt, digestive tubule; L, lumen; Lt, Leydig tissue; Sa, Secretory absorptive cells. Scale bar = 50 μm

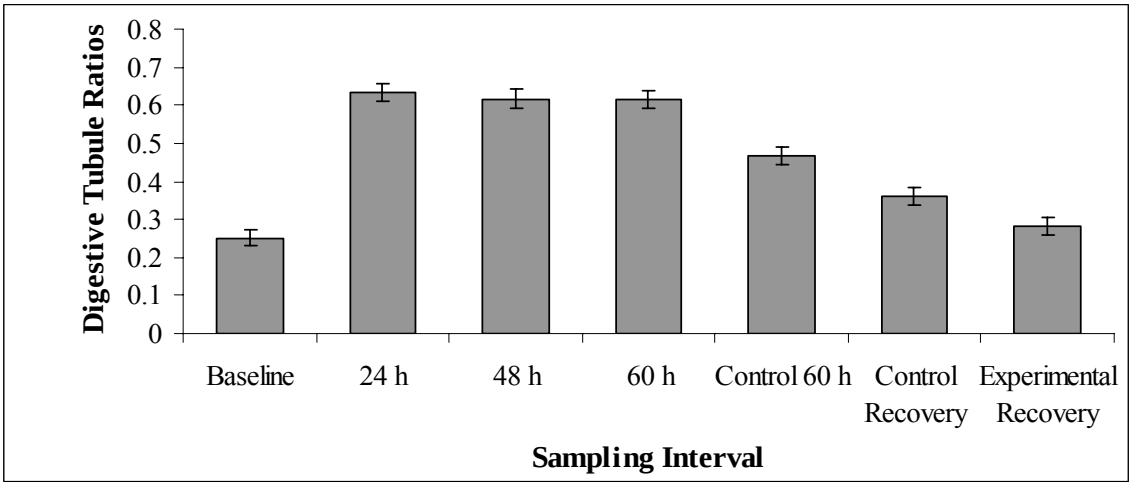

Figure 11. Digestive tubule ratios from oysters exposed to anoxia measured at Baseline, 24 h, 48 h, 60 h, Control 60 h, Experimental recovery, and Control recovery intervals. Error bars represent the standard error at each sampling interval.

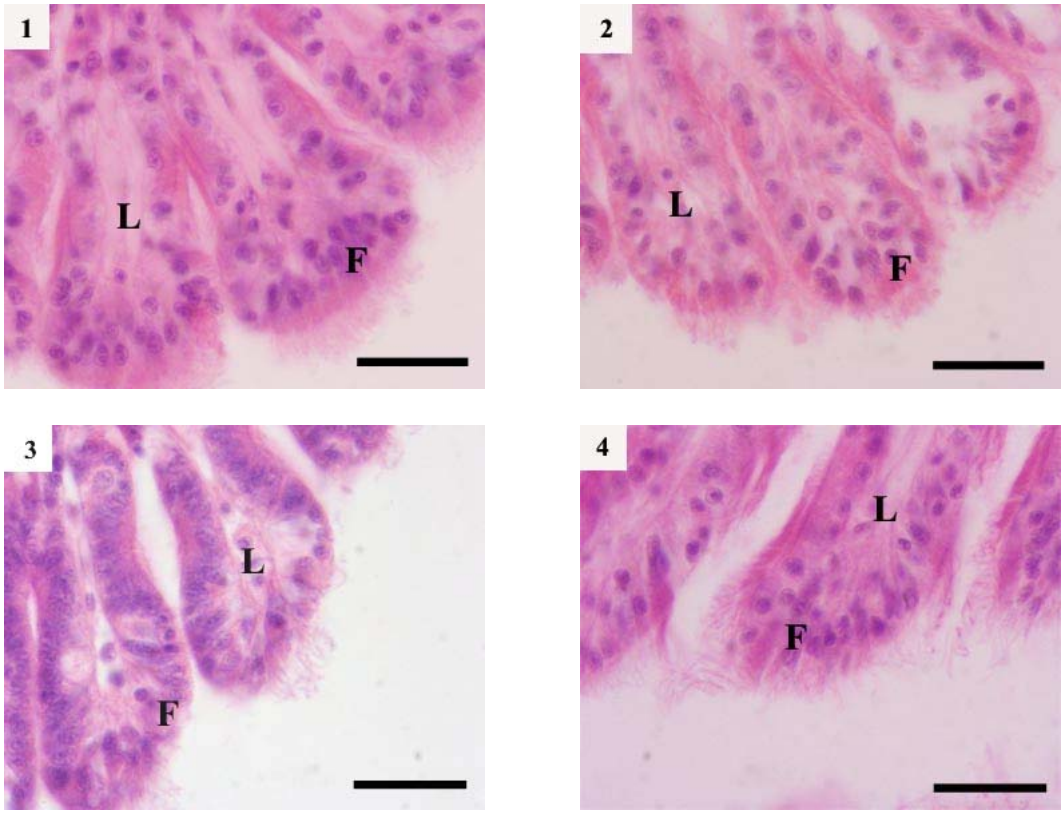

Figure 12. Photomicrograph of 5 μm sections of oyster gills at 100X. (1) Baseline; (2) 24 h interval; (3) 48 h interval; (4) 60 h interval. Abbreviations: F, gill filament; L, lacunar cells. Scale bar = 50 μm.

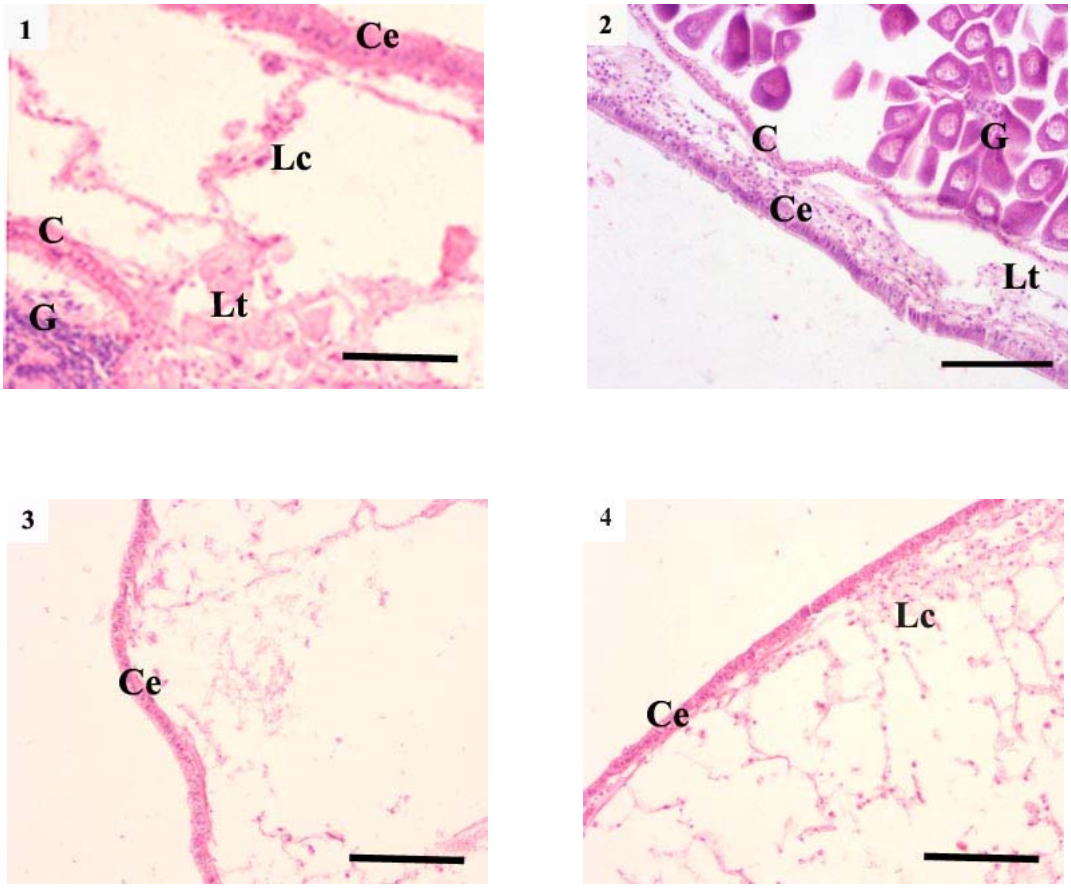

Figure 13. Photomicrograph of 5 μm sections of oyster mantle tissue at 20X. (1) 60h; (2) 60h Control; (3) Experimental Recovery; (4) Control Recovery. Abbreviations: C, ciliated cuboidal epithelia; Ce, columnar epithelia; G, gonad; Lt, Leydig tissue. Scale bar = 100μm.

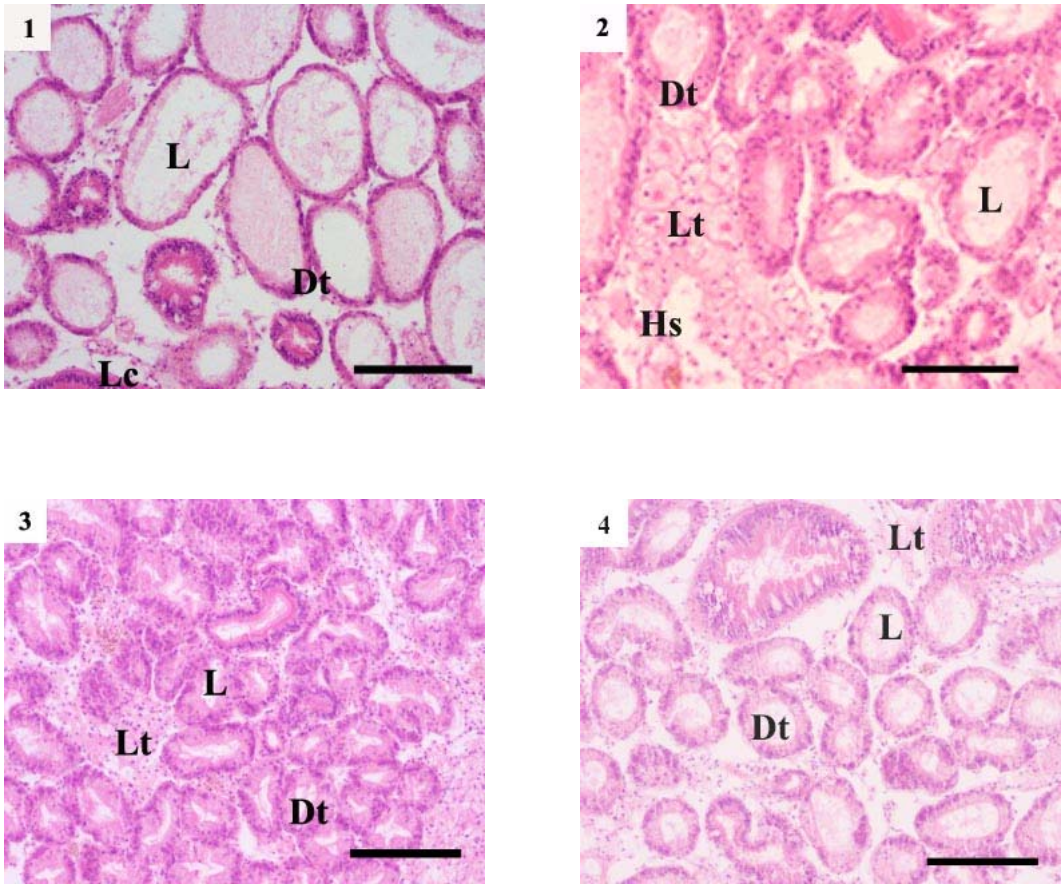

Figure 14. Photomicrograph of 5 µm sections of oyster digestive tubule tissue at 20X. (1) 60 h; (2) 60 h Control; (3) Experimental Recovery, (4) Control Recovery. Abbreviations: Dt, digestive tubule; L, lumen; Lt, Leydig tissue. Scale bar= 100µm.

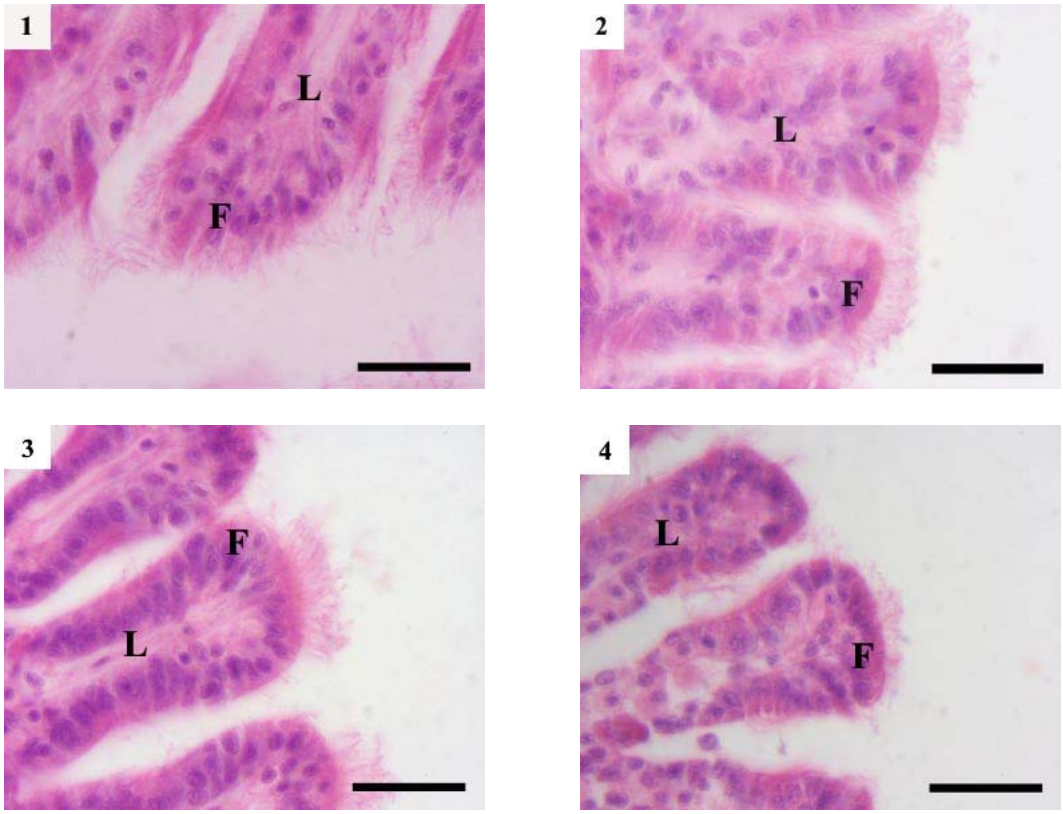

Figure 15. Photomicrograph of 5 µm sections of oyster gill tissue at 100X. (1) 60 h; (2) 60h Control; (3) Experimental Recovery; (4) Control Recovery. Abbreviations: F, gill filament; L, lacunar cell. Scale bar= 50 µm.

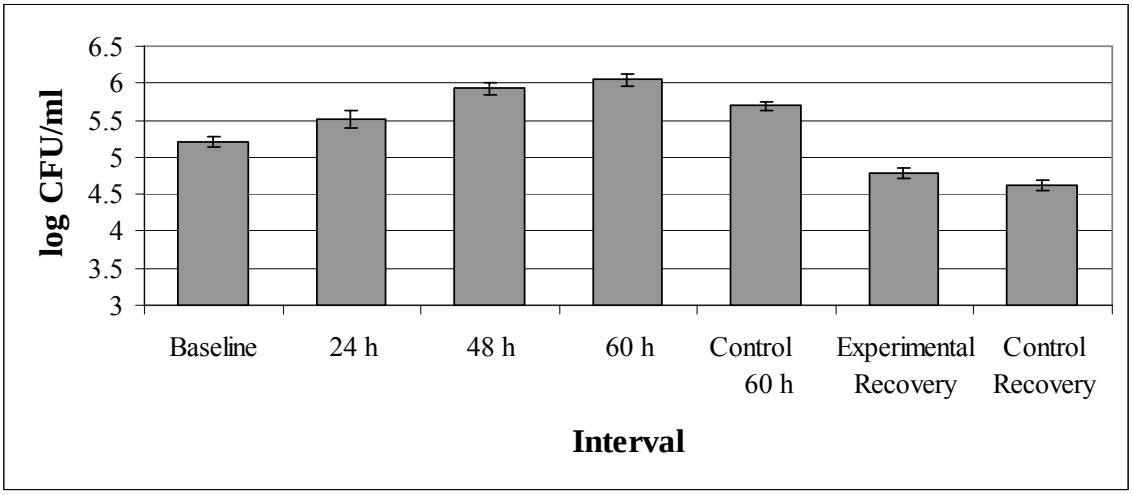

Figure 16. Anaerobic Bacterial counts (CFU/ml) from homogenized oyster meats, $\log_{10}$ transformed at Baseline, 24 h, 48 h, 60 h, Control 60 h, Experimental recovery, and Control recovery intervals. Error bars represent the standard error at each sampling interval.

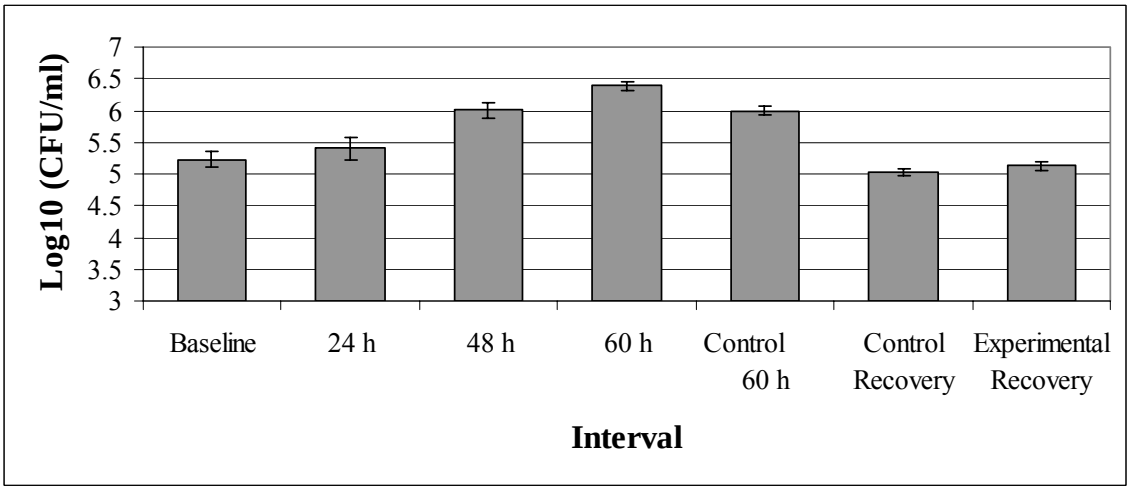

Figure 17. Aerobic counts (CFU/ml) from homogenized oyster meats, $\log_{10}$ transformed at Baseline, 24 h, 48 h, 60 h, Control 60 h, Experimental recovery, and Control recovery intervals. Error bars represent the standard error at each sampling interval.

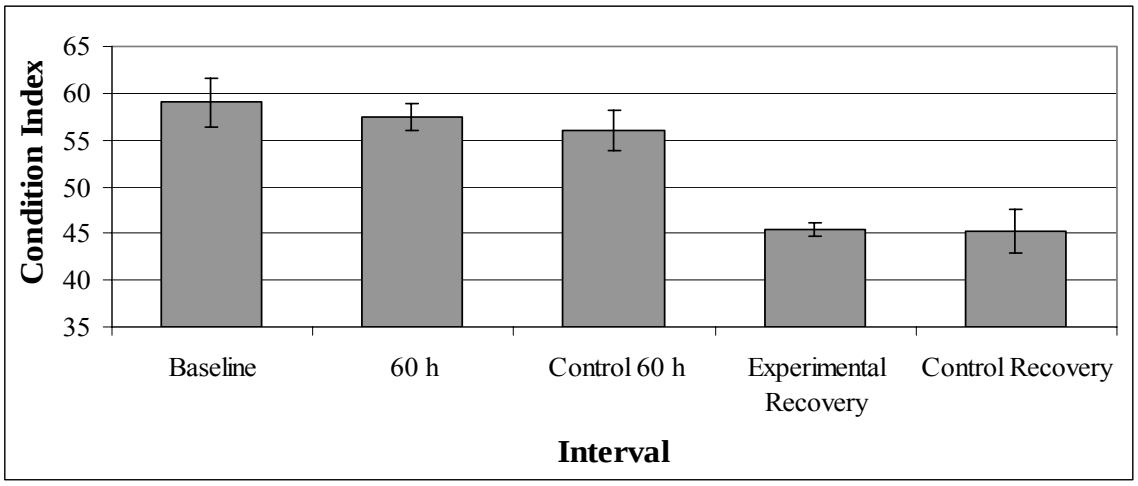

Figure 18. Oyster condition indices of oyster samples at Baseline, 60 hr, Control 60 hr, Experimental recovery, and Control recovery. Error bars represent the standard error at each sample interval.

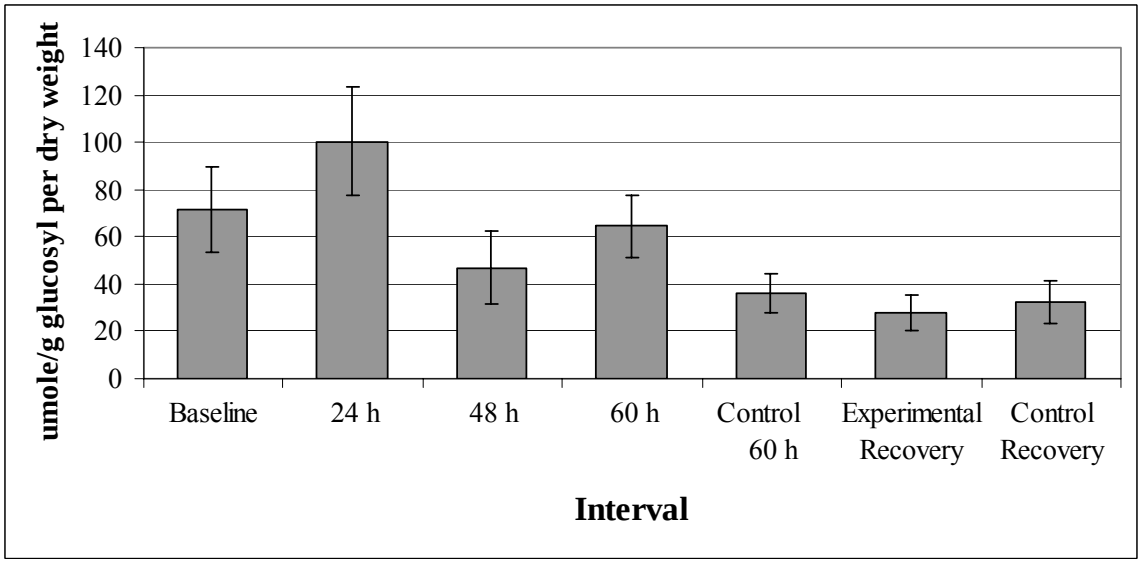

Figure 19. Glycogen levels (μmole glucosyl units per g dry weight) from lyophilized oyster meats at Baseline, 24 h, 48 h, 60 h, Control 60 h, Experimental recovery, and Control recovery. Error bars represent the standard error at each sampling interval.

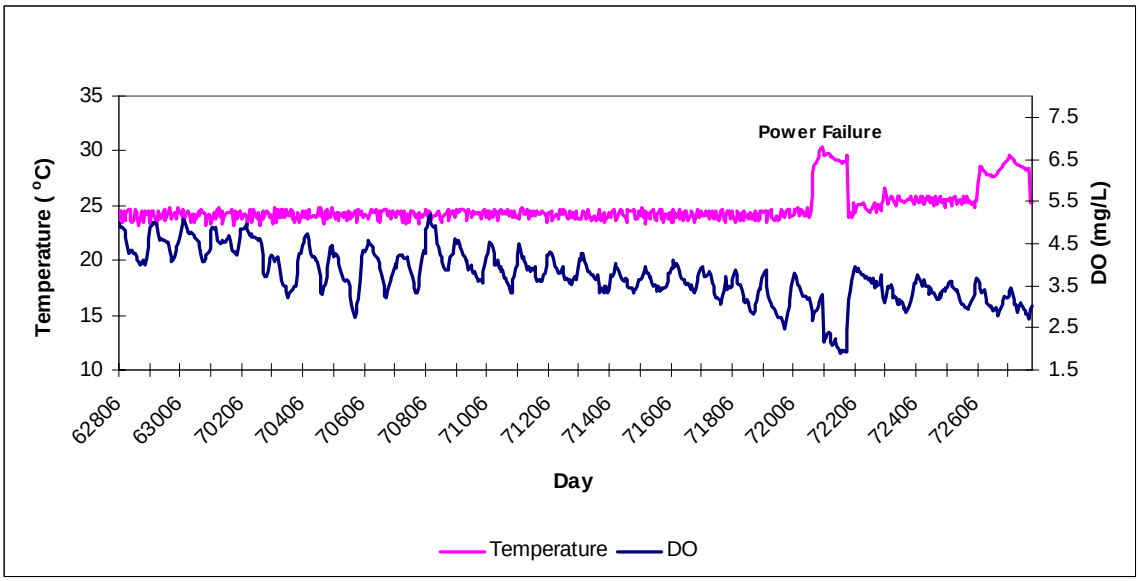

Figure 20. Recovery tank Temperature (ºC) and DO (mg/L) during 4 week post anoxia experiment.

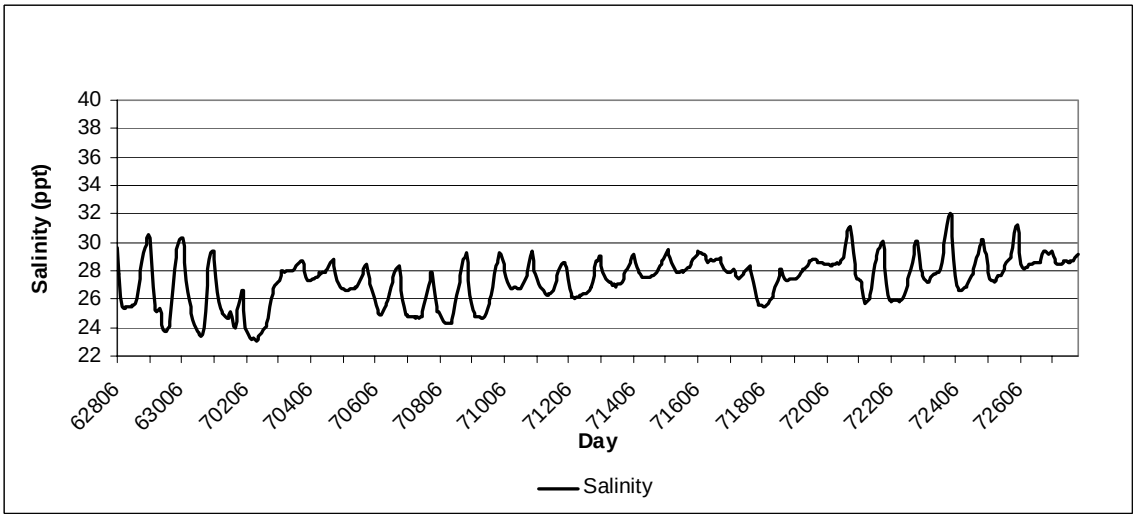

Figure 21. Salinity (ppt) in recovery tank during 4 week post anoxia experiment.

IV. DISCUSSION

Temperature, salinity and dissolved oxygen remained relatively constant for the duration of the experiment in both control and recovery tanks but the pH in the experimental tank experienced a unique drop at the 24 hour water change Both experiment and control tanks were filled at the same time as well as with water from the same source. The control tank had a pH of 7.9 at the 24 h water change sampling while the experimental tank had a pH of 7.2 at the same sampling. There may have been a malfunction in the pH meter at that specific reading or another possibility may be attributed to nitrogen gas being bubbled overnight through the experimental tank. Babarro and de Zwaan (2001) reported seawater with a stable pH of 8.2 after 2 h of nitrogen bubbling through the tank. This absence of a drop in pH provides evidence that the latter hypothesis is probably not a factor.

There was a sharp spike in temperature and drop in dissolved oxygen on July 22, 2006 due to a power failure at the laboratory. The chilling unit attached to the recovery tank as well as the aeration system on the same circuit was out of order for several hours attributing to the large fluctuation in temperature and dissolved oxygen. Incoming seawater was 32.5 ºC according to the Mobile Bay National Estuary Program Environmental Monitoring station at Dauphin Island, AL which concurs with the reading from the Hydrolab® on that particular date.

The results of this experiment indicate that anoxia plays a significant role in deterioration of mantle tissue as well as digestive tubule atrophy in eastern oysters. Although oysters go through distinct changes during feeding and tidal rhythms (Morton 1977), the oysters tested in this experiment were determined to be in a holding or absorptive stage at the baseline sampling with low tubule ratios (Weinstein 1995; Winstead 1995). According to the histological analysis distinct changes in tubule morphology could be seen after 24 h which suggests that oysters were stressed. The significant increase of mean tubule ratio in experimental oysters also gives evidence that oysters were affected by the anoxic conditions. It is interesting that the mean tubule ratio for the experimental recovery oysters was not significantly different from the baseline but the control recovery oysters mean tubule ratio was significantly higher than the baseline oysters. One can speculate that there was a possible tank effect where position in the recovery tank affected the amount of food that was delivered to the two recovery groups thus encouraging one group to develop better than the other. Another point of speculation is the significant increase in the mean tubule ratio for the control 60 h group as compared to the baseline group. This difference may account for the lack of food in both control and experimental tanks during the 60 h experiment.

Starvation has been shown to decrease cell height and tubule lumen ratios in digestive tubules of *C. virginica* (Winstead 1995). Shaw and Battle (1957) also described spherical large lumina with some degeneration of vacuoltated secretory absorptive cells which can be linked to a lack of food during the winter season. Oysters were not fed throughout the 60 hours of the experiment presented here, which could have increased the stress level on the oysters. Starvation cannot account for the apparent inflammatory

response seen in the mantle tissue as well as the digestive tubules. Infiltration of leucocytes into tissue surrounding an effected area denotes an inflammatory response in oysters as described by Pauley and Sparks (1976) when oysters were injected with turpentine. Edema and leukocytic infiltration along with congestion of smaller blood vessels and hemocytes, occurred within 8 hours of injection of turpentine into Leydig cells. The congestion of blood vessels in the mantle tissue of oysters exposed to anoxic conditions along with the appearance of bacteria in digestive tubules at all of the experimental intervals suggests that the mantle and digestive tissue are being adversely affected by bacteria rather than just the product of starvation stress.

Starvation may however be responsible for the deterioration in Leydig tissue seen at the Control recovery and Experimental recovery. Leydig cells store glycogen for oysters (Cheng and Burton 1966). In a starvation event oysters will use their glycogen stores to maintain normal metabolic functions which can explain the reduced Leydig cells in both groups of recovery oysters.

Digestive tubules exposed to anoxic conditions regenerated to normal cuboidal digestive cells. Decreased tubule ratios reflect the regeneration of the digestive cells. Mix and Sparks (1971) observed tubule re-epithelization in *C. gigas* after ionizing radiation treatments. It is curious that the tubules for the experimental recovery oysters had significantly lower tubule ratios than the control recovery oysters. Further studies are warranted to see why this may have occurred.

Proliferation of bacteria during an anoxic event is natural due to the anaerobic breakdown of decaying biomass. Filtered, uv sterilized water was used for both control and experimental tanks leading to the conclusion that bacteria affecting the oysters were

originating from the oysters themselves. The ANOVA analysis indicated that oysters observed in the 60 h had significantly higher anaerobic bacteria counts than the baseline oysters and control oysters. Prior studies show bacterial proliferation and survival times had a negative relationship in *M. edulis* during anoxic incubation (Babarro and de Zwaan 2002).

Oysters did not use their endogenous fuel which corresponds to studies with the cockle by Babarro and de Zwaan (2001) as shown by glycogen measurements. Oysters did not have significantly different levels of glycogen during the experiment but the recovery oysters were significantly different than the experimental oysters. The difference between experimental and recovery oysters may have been due to lack of feed in the recovery tank. During the 4 week recovery period oysters were kept in a temperature regulated to 28ºC tank with flow through water filtered to 35µm. When the filter gathers extraneous particles and becomes clogged it cannot let phytoplankton pass. It is suspected that the oysters were not getting enough nutrition thus they started to use their glycogen supplies to maintain normal metabolism. The same trend was seen in condition index of oysters during and after the experiment which solidifies the hypothesis that they were not getting enough food during recovery to maintain glycogen levels.

Fecundity estimates could not be statistically substantiated due to the low female to male ratio as well as the high proportion of undeveloped oysters in both groups. Oysters are more prone to develop male gonads in their first year due to the rapid multiplication of male germ cells (Galtsoff 1964). Since the oysters used in this experiment were from the last years spawn, it is normal to see such a high ratio of male to females. Coe (1936) found that 7.1 % of first year oysters from Appalachicola Bay, FL

were females in their first breeding season. During the recovery period it has been stated that the oysters were possibly not getting enough feed as well as there was a drop in the temperature from 28 °C to 23 °C for 4 weeks which apparently induced the oysters to reabsorb their gametes. Oysters were then brought up to 28 °C and fed 75 ml of algae paste (Reed Mariculture) for the remaining 14 weeks in flow through. Unfortunately the conditioning of these oysters did not produce enough females to show if anoxia had an effect on fecundity. Baseline oysters had 4 out of 10 females whereas experimental oysters had 5 out of 43 females. It is a possibility that the oysters exposed to anoxic stress developed into a higher percentage of males.

During an anoxic event, oysters down regulate metabolism to limit the amount of glycogen needed to survive. Although the overall condition of the oyster did not change during this short anoxic trial, the oyster's tissues were affected by the anoxia stress. Bacteria found in stressed oyster tissues confirm that oyster defenses are compromised during an anoxic event. It is not clear whether the reactive oxygen intermediates or another line of defense is being affected during anoxia allowing bacteria to invade the oyster tissues. This study suggests that oyster mortality may be due to bacterial infection rather than lack of energy stores.

Further studies using PCR methods to identify bacteria at the 16S ribosomal RNA level would be helpful to single out a single pathogenic strain. Also it would be interesting to see the difference between anoxia tolerant strains of oysters and natural strains of oysters. Another interesting study could involve sex reversal due to anoxic stress and how it affects fecundity at the next spawn.

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