5-2019

Effects of Global Warming on Gonadal Functions, Cellular Apoptosis, and Oxidative Stress in the American Oyster

Sarah B. Nash
The University of Texas Rio Grande Valley

EFFECTS OF GLOBAL WARMING ON GONADAL FUNCTIONS, CELLULAR APOPTOSIS, AND OXIDATIVE STRESS IN THE AMERICAN OYSTER (*CRASSOSTREA VIRGINICA)*

A Thesis

by

SARAH B. NASH

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2019

Major Subject: Ocean, Coastal, and Earth Sciences

EFFECTS OF GLOBAL WARMING ON GONADAL FUNCTIONS, CELLULAR APOPTOSIS, AND OXIDATIVE STRESS IN THE AMERICAN OYSTER (*CRASSOSTREA VIRGINICA)*

A Thesis
by
SARAH B. NASH

COMMITTEE MEMBERS

Dr. MD Saydur Rahman
Chair of Committee

Dr. Jude Benavides
Committee Member

Dr. Abdullah Faiz Rahman
Committee Member

May 2019

ABSTRACT

Nash, Sarah B., Effects of Global Warming of Gonadal Functions, Cellular Apoptosis, and Oxidative Stress in the American Oyster (*Crassostrea virginica)*. Master of Science (MS), May, 2019, 62 pp., 24 figures, references 83 titles.

Global warming due to climate change is predicted to intensify the heat stress in marine and coastal organisms affecting their development, growth and reproductive functions. In this study, I analyzed gonadal development, heat shock protein 70 (HSP70), nitotyrosine protein (NTP) and dinitrophenyl (DNP) expressions, cellular apoptosis, and extrapallial fluid (EPF) conditions in American oyster. Oysters were placed in six aquaria and exposed to control (24^{o}C), medium (28^{o}C), and high (32^{o}C) temperatures for one week. Higher temperatures significantly decreased the number of eggs and sperm. EPF protein concentrations declined compared to control. In contrast, EPF pH and HSP70 expression in gonad increased after heat-exposure, consistent with increased gonadal apoptosis. The enhanced apoptosis in gonads of heat-exposed oysters was associated with increased NTP and DNP expressions in gametes. Collectively, these results suggest that higher temperatures drastically increased oxidative stress leading to increased cellular apoptosis, which subsequently decreased gonadal functions in American oyster.

DEDICATION

To my family and friends for encouraging and supporting me and to my supervisor for inspiring and motivating me.

ACKNOWLDGEMENTS

I wouldn’t have been able to carry out this research alone. I want to specially thank Dr. MD Saydur Rahman for all his help throughout this process. Without his dedicated work ethic, insight, and guidance along the way this project wouldn’t have been possible. Thank you to all of those who have helped me with my research, especially Jackson Johnstone, Eleazar Hernandez, Krista Ruppert, Brittney Lacy, and Omar Vasquez. I would also like to thank UTRGV for allowing me to use their laboratory facility as well as Texas Parks & Wildlife for permitting me to collect oysters at South Padre Island. My thanks also go out to all of those who helped me from Dr. Rahman’s laboratory and UTRGV.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Global Climate Change

The average global temperature sets new high records and continues to drastically increase at a rapid rate (Root et al., 2003; Yuan et al., 2018). This increase in global temperature, primarily due to anthropogenic emissions of greenhouse gases, impacts marine and coastal environments, as well as the organisms that live there (Root et al., 2003). In the last 100 years of collecting climate data, temperatures have risen by 0.6^{o}C, and observations suggest there have been increases in the incidence of temperature extremes (Dale et al., 2001; Root et al., 2003). According to future temperature projections by 2100, there will be a 4-and-a-half-degree Celsius increase in the earth's average temperature (Morris et al., 2018; Yuan et al., 2018). This increase in global temperature, referred to as global warming, has numerous detrimental effects in marine environments.

Impacts from intensified infrared radiation penetrating the ocean’s surface causes major concern due to the various environmental stressors interacting in marine ecosystems; consequently, marine organisms are directly influenced by these surrounding environmental factors (Bonaventura et al., 2011). Rising atmospheric temperatures are directly correlated with carbon dioxide levels (IPCC, 2007; Ramos et al., 2007; Doney et al., 2009). When infrared radiation gets absorbed by carbon dioxide and other greenhouse gas pollutants in the atmosphere

instead of escaping to outer space, also known as the greenhouse gas effect, this traps heat and causes the surface of the earth to warm (Snyder et al., 2009; MacMillan, 2016). For the last 500,000 to 600,000 years, carbon levels have remained between a low of 180 ppm during glacial periods and a high of 280 ppm during interglacial periods (Root et al., 2003). Carbon dioxide levels started increasing in the 1700's and began to skyrocket during the 1950's (Yuan et al., 2018). Records from 2016 show levels as high as 405 ppm in Mauna Loa (Morris et al., 2018). Carbon dioxide levels have nearly doubled over the past 300 years, and if the increasing trend continues there will be a 3 ppm increase in carbon dioxide every year (Yuan et al., 2018). This significant increase in environmental carbon dioxide is primarily caused by anthropogenic activities such as burning of fossil fuels, coal and oil extractions, changing land use (example: deforestation) and burning, and even from cement production (Yuan et al., 2018).

Anthropogenic influences on the environment have introduced many environmental stressors, including hypoxia from runoff of pesticides and herbicides into the oceans, and ocean acidification from increasing carbon dioxide levels. Ocean acidification is the decrease in pH of the ocean often caused by increasing carbon dioxide levels in the atmosphere that get dissolved into the oceans waters (Portner, 2008). Seawater acts as a buffer to prevent broad swings of pH when acids or bases are introduced, but even the slightest change in pH of the ocean can have tremendous effects on marine life (Garrison, 2007). These slight changes in pH have negative impacts on the rates at which reef-building corals form as well as shell-forming organisms such as snails, clams, and oysters, and have been proven to reduce calcification rates of these shell-forming organisms (Doney et al., 2009). These natural corals and calcium carbonate organisms begin to erode their hard-skeleton destroying habitats which impose a great threat on marine biota (Doney et al., 2009). Drastic impacts on coral reef communities, such as bleaching, as well

as on other marine invertebrates have also been recorded due to ocean acidification and global climate change (Farag et al., 2018).

Global warming can cause many other serious problems including significant sea level rise from melting of ice on land with an average increase of 0.8 meters per year (if sea level rise continues on trend) (Yuan et al., 2018). This could cause numerous tremendously detrimental effects on coastal comminutes, such as frequent storms and rapid beach erosion, as well as habitat loss that would prove fatal for many species.

Global climate change also alters the frequency, duration, intensity, and timing of forests (Dale et al., 2001). Increases in human caused outbreaks of forest fires from global climate change cause millions in damage annually (Dale et al., 2001). An average of 450,000 hectare per year are impacted from forest fires causing over 261 million in damages (Dale et al., 2001). These forests are also affected by drought, introduced species, insect and pathogen outbreaks, hurricanes, windstorms, ice storms, and landslides all caused, or influenced by, global warming and global climate change (Dale et al., 2001).

Other factors that may also arise from global warming, including increases in new pests on agricultural farms, forests, and cities from heat waves, as well as heavy flooding which may cause allergy, asthma, and other diseases (MacMillan, 2016). Climate and other geographical characteristics influence the frequency of pathogenic microorganism's emergence. Diseases correlated with change in temperature are shown to comprise 4.6% of all environmental hazards and risks (Akil et al., 2014). There is a strong positive correlation between high temperature and *Salmonella*, a pathogenic becteria, infection due to their increasing rate of replication with warmer temperatures (Akil et al., 2014). Furthermore, the warming in the southern United States may increase *Salmonella* infections' rates (Akil et al., 2014). One study revealed a linear

association between temperature and number of reported cases of *salmonella* in 10 European countries (Kovats et al., 2004). As Earth warms, more areas of the planet will become suitable habitats for bacterial growth, increasing not only the risk of contracting disease, but the rate and number at which diseases are spreading.

Heat Stress and Roles of Heat Shock Proteins

Even though impacts from intensified UV radiation penetration causes major concerns in marine environments, marine bivalves such as oysters are tolerant to extreme temperatures, but are vulnerable to environmental stressors, such as oxidative stress; this affects the gonadal functions possibly leading to abnormal growth and cellular apoptosis, or programed cell death, during gonadal maturation (Bonaventura et al., 2011; Saeed-Zidane et al., 2017). The rate of change in temperature is highly important and has greater effects than the temperature itself (Shumway, 1996). For example, the slower the rate of temperature increase, the lower the upper lethal temperature (Shumway, 1996). Additionally, oysters can be killed from either exposure to short-term high temperatures and/or long-term low temperatures (Shumway, 1996).

Heat shock proteins (HSPs) are key chaperone proteins that protect the body under thermal stress (Li et al., 2007). These key proteins also serve as regulators of normal cellular function, while assisting in proper folding, assembly, and transport of other fundamental proteins (Ueda and Boettcher, 2009). If heat shock such as global warming is added to the environment, the HSP expression and mRNA levels increase (Ueda and Boettcher, 2009). Changes in the HSP expressions have been linked to major developmental changes such as gametogenesis, embryogenesis, and metamorphosis for several species (Ueda and Boettcher, 2009). If high temperatures become too extreme then protein denaturation occurs (Li et al., 2007). Multiple

subunits of HSPs such as HSP 70, HSP 40, and HSP 90 bind together with the heat shock factor (HSF), and enter the nucleus of a cell where they bind with other HSPs increasing their protein expression (Giudice et al., 1999).

Heat stress has been shown to affect larval and early spat development (Udea and Boettcher, 2009), which could lead to inhibitions in settlement and metamorphosis, as well as impaired growth and reproductive functions in oyster. Therefore, the motivation behind this study is the desire to further understand molluscan heat stress and its impacts on reproductive fitness.

Cellular Apoptosis and Oxidative Stress

Apoptosis can be described as programed cells death and is involved in many physiological processes (Krammer, 1998). It is a natural and regulated form of cell death to eliminate unwanted cells (Goedken et al., 2005). Eliminating unwanted, damaged, and senescent cells, such as those that have been through morphological changes including cell shrinkage, cell surface blebbing, fragmentation, and nuclear chromatin condensation, aids in early defense, elimination of cells infected by pathogens and proper maintenance of tissue integrity (Goedken et al., 2005). Once cells have initiated apoptosis, or programed cell death, this results in *de novo* gene expression of caspase and additional enzymes responsible for cellular dissolution (Goedken et al., 2005). It is known that apoptosis can be activated by environmental pollutants and stressors in vertebrates, and that the mitochondria plays a central role in this intrinsic cell death pathway (Sokolova et al., 2004). The opening of a channel, called the mitochondrial permeability transition pore, releases cytochrome *c*, a small hemeprotein, into the cytoplasm, which then interact with apaf-1 and procaspase-9 to form apoptosome, a large quaternary protein (Bratton

and Salvesen, 2010). Apoptosome activates effector caspases like caspase-3, also known as a master enzyme in apoptosis, which eventually leads to DNA degradation and other intracellular reactions that accumulate in killing the cell (Gosling, 2015). Most studies of apoptosis and its role as a defense mechanism come from organisms ranging from mammals to nematodes, but few studies document apoptosis in shellfish (Goedken et al., 2005). For this reason, it is essential to understand the impacts on apoptosis in ovarian and testicular tissues of the American oyster under elevated temperatures.

Oxidative stress can be described as the imbalance towards the pro-oxidant sides of the antioxidant homeostasis (Dalle-Donne et al., 2003). This means that oxidative stress occurs in a state when oxidation exceeds the antioxidant system due to the loss of balance between them (Yoshikawa and Naito, 2002). Although oxidative stress is a naturally occurring process in all organisms that plays an important role in physiological adaptation and the regulation of intracellular signal transduction, as well as activating stress responses that are beneficial to organisms, increases of oxidative stress can damage cells permanently by allowing the oxygen free radicals to attack molecules such as lipids, proteins, and DNA which triggers the intrinsic pathway of apoptosis (Yoshikawa and Naito, 2002; Slimen et al., 2014). While most atoms are composed of a central nucleus with orbiting pairs of electrons, free radicals are the atoms and molecules that have unpaired electrons forming pairs with other electrons making them unstable and highly reactive (Yoshikawa and Naito, 2002). Free radicals along with other reactive oxygen species (ROS, e.g., single oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radicals) and reactive nitrogen species (RNS, e.g., peroxynitrite, and nitric oxide) cause severe oxidative damage in cells/tissues during environmental stress (Slimen et al., 2014; Di Meo et al., 2016).

Even though oxidative stress is necessary, an overproduction of ROS alters homeostasis and induces stress situations in animal bodies (Sengupta and Bhattacharjee, 2016).

It is known ROS and RNS are generated in response to environmental stress (Ryter et al., 2006). As a result, it is crucial to observe the how increasing temperatures are influencing ROS and RNS production in the gonadal cells and tissues of the American oyster. Many different types of stress including heat and cold stress lead to overproduction of ROS (Slimen et al., 2014). A previous study revealed elevated temperature induces the formation of ROS, leading to an increase in oxidative damage to DNA (Bruskov et al., 2002). The results proved that heat, a permanent environmental factor, activates oxygen and leads to generation of ROS, and this heat-induced ROS has in fact caused damage to DNA (Bruskov et al., 2002). Heat stress has also been shown to increase superoxide anion levels occurring at specific sites of the electron transport chain and decrease superoxide dismutase (SOD, an enzyme catalyzes the dismutation of the superoxide radical) mRNA levels, cytoplasmic SOD proteins and enzyme activity leading to ROS generation (Slimen et al., 2014). Additionally, oxygen can react with other radicals such as nitric oxygen producing peroxynitrite, a powerful RNS (Slimen et al., 2014).

Oyster

Bivalves such as mussels and oysters are simple marine organisms and are an excellent indicator species to environmental stressors because they act as biomonitors of the health of the waterways. Environmental stress such as warming waters can directly affect the distribution of bivalves, who shift poleward to inhabit areas within their metabolic temperature tolerance (Root et al., 2003). Studies have proven a shift in distribution northwards of blue mussel (*Mytillus edulis)* by more than 350 km (Jones et al., 2010). Since global temperatures have significantly

risen in the last 50 years, this distribution change is primarily caused by intolerance of adults to high summer temperatures (Zippay and Helmuth, 2012). Other consequences of global warming on marine invertebrates include changes in predator prey interactions (Freitas, 2011), immune response (Matozzo et al., 2011), and growth (Petes et al., 2007; Beukema et al., 2009).

Oysters are an intertidal bivalve, so they are constantly exposed to extreme environmental fluctuations from immersion and/or emersion of tidal waves making them highly tolerant to extreme environmental conditions (Shumway, 1996). During spawning season, oysters use more energy when heat stress is intensified to the environment, so they compromise their thermal tolerance and immune status which makes them easily subjected to increases in mortality rates (Li et al., 2007). Post spawning oysters have a much greater mortality rate in water temperature greater than 44^{o}C and therefore, a much less tolerance of higher temperatures than the pre-spawning oysters (Li et al., 2007).

Oysters are unique marine shellfish, and are a very popular seafood dish all over the world. The American oyster, *Crassostrea virginica*, is a dioecious, oviparous species of considerable commercial value and one of about 65 species of the genus *Crassostrea* (Hamman, 1969; Eckelbarger and Davis, 1996). Their distribution consists along the East and West coasts of the United States, the East coast of Central America, Northern South America, and some coasts of Europe (Gbif, 2016). Oysters can take in and filter over 50 gallons of water per day, and/or 2.5 gallons of water per hour feeding on phytoplankton and zooplankton (Schilling, 2015). Oysters also provide many benefits to marine ecosystems such as improved water quality, reduced sediment suspension, nutrient uptake and filtration, and even serve as a substrate for hard bottom benthos and a refuge for other biota (Bartol et al., 1999).

Since *Crassostrea* oysters are protandric dioecy, the two sexes are usually separate, but a small percentage of juveniles might show hermaphroditism, meaning they possess both male and female reproductive organs (Guo et al., 1998). Oysters have the ability to change sexes several times throughout their lifetime depending on environmental, physiological, and nutritional factors, which greatly influences the sex of oysters (Guo et al., 1998). When they discharge their gametes for reproduction, eggs and sperm fertilize, and larvae settle and attach in large numbers to the shells of adult oysters creating 3-dimensional habitats composed of live oysters (Bartol et al., 1999). Their anatomy consists of 2 calcium carbonate shells joined by the abductor muscles, and a mantel that consists of folded tissues that cover and protect the internal organs such as the heart, digestive gland, gill, intestine, stomach, rectum, anus, and gonad (Figure 1) (Eble and Scro, 1996).

Oyster Gonad and Reproduction

Gonads are the reproductive organs present in organisms that produce gametes or sex cells. In female oysters, the reproductive cells are oocyte (immature egg) and ova (mature eggs), while in males the reproductive cells are the spermatogenic cells such as spermatogonia, spermatocytes, spermatids, and sperm (spermatozoa). In adult oysters, gonads are the largest organ before spawning season that form one connective mass around the creases of the digestive gland (Loosanoff, 1942). The gonads begin to develop about eight weeks after attachment to substrate (Coe, 1932). Gonads are formed by gonadal tubules that are folded back to form a cavity for tissue storage in the connective tissue (Franco et al., 2008). The outer part of the tubule is determined by the surrounding myoepithelial cells and acellular matrix, while the inner part is comprised of intragonadal somatic cells associated with germinal lineage (Franco et al., 2008).

During spawning season, the gonads may reach up to 1 cm, but gradually decrease and become much thinner once spawning begins and their gametes are discharged for fertilization (Loosanoff, 1942). These profound changes in gonad index of oyster correlates with the different seasons in the year (Loosanoff, 1942).

The annual reproductive cycle of oysters is influenced by environmental factors such as temperature, salinity, food availability etc. (Fabioux et al., 2005; Parker et al., 2018). For example, Pacific oyster (*Crassostrea gigas* that thrive mainly in temperate regions near the pacific coast) exhibited a seasonal reproduction cycle related to temperature (Fabioux et al., 2005). The active phase of gametogenesis, also known as the growing stage, commenced when water temperatures started to increase. Maturity and spawning were observed in summer once water temperatures reached above 19^{o}C (Fabioux et al., 2005). Salinity of sea water also negatively influences gametogenesis when lower than 30 ppt (Fabioux et al., 2005). Studies also show oysters produce more gametes in eutrophic environments, when food availability is high (Fabioux et al., 2005).

The oyster ovary contains branching acini, where oocytes grow and fully mature (Eckelbarger and Davis, 1996). The acini are surrounded by connective tissues which store nutrients for gametes, and each acinus is submersed in fluid known as the hemocoel, which serves to transport the nutrients to oocytes (Eckelbarger and Davis, 1996). Oocytes begin to grow and develop when they are positioned near the inner acini walls (Eckelbarger and Davis, 1996). Late stages of vitellogenesis take place when differentiation occurs, and the oocytes become stalk shaped then enter the acinus lumen (Eckelbarger and Davis, 1996). During vitellogenesis, yolk proteins are synthesized from both auto synthetic and hetero synthetic processes combining the activities of the Golgi apparatus and the rough endoplasmic reticulum (Eckelbarger and

Davis, 1996). One study revealed that the vascular connective tissue cells are the main source of nutrients for vitellogenesis (Eckelbarger and Davis, 1996).

In male oysters, ripe gonads are comprised of a stream of spermatozoa (Dinamani, 1974). The tails are directed into the lumen that occupies a large portion of the follicle. Outside of the spermatozoa, there are bands that consists of spermatids and spermatocytes, while the outermost band contains a single row of spermatogonia (Dinamani, 1974). During spermatogenesis, primary spermatogonia undergo a repeated process of mitotic division to produce secondary spermatogonia (Franco et al., 2008). Two different spermatogonia are classified during development; the first established are large, scarce, and pale cells that are lined against the base of each tubule, while the second cells that form are smaller, more clustered, and dark (Franco et al., 2008). The first large and pale spermatogonia then become spermatocytes during meiosis (Franco et al., 2008). Spermatids are then produced and separated into flagellated spermatozoa and can measure 25 to 60 µm (Franco et al., 2008). These changes in gonadal index are directly correlated with the different seasons of the year (Loosanoff, 1942). This is primarily due to and influenced by the temperature of sea waters (Loosanoff and Davis, 1952).

The consequences of global warming cause a chain reaction. If temperatures continue to rise, damage will be made not only to the environment and species' habitats, but to animals' internal/physiological functions possibly affecting their development, growth, and reproductive performance (Luo et al., 2016). Cumulative research supports that oysters are an excellent model species in response to climate change (Li et al., 2007). It is very important to understand how environmental factors will influence oyster development, growth and reproduction (Fabioux et al., 2005). Elevated temperatures might hinder gonadal development, impair reproductive functions, and generate DNA mutation sequences which could lead to various defects in oysters.

Damage to reproductive organs could lead to the inability to produce offspring for future generations, declining their population, as well as the imperative benefits they provide to marine ecosystems.

Study Objectives

The main aim of this study was to test the hypothesis that elevated temperatures impairs reproductive functions and increases the heat shock protein expression, cellular apoptosis and oxidative stress in the American oyster. The American oyster is an edible and commercially important marine shellfish in the United States and has been an extensive biological asset for multidisciplinary studies including aquaculture, physiological, ecotoxicological and pathogenic research over the past decade (Shumway, 1996). Although research has been conducted on the biology of oysters due to their considerable commercial value (Kirby, 2004; Humphries et al., 2015), the effects of global warming on oyster reproduction remain unclear. The aims of my research are:

(i) to examine the effects of higher temperatures on gonadal functions,

(ii) to investigate the effects of higher temperatures on heat shock protein expression in gonadal tissues and body fluid conditions,

(iii) to determine the effects of higher water temperature on cellular apoptosis in gonadal tissues, and

(iv) to determine the effects of higher water temperature on oxidative stress in the American oyster.

CHAPTER II

METHODS

Laboratory Experiment

Young adult (~2-3 years old, average shell size: 7.2 cm length, 3.3 cm width, and 4.7 g weight) wild-caught American oysters were collected on the bayside of South Padre Island, transported to the laboratory at UTRGV Brownsville campus, and placed in six glass aquaria (Tetra, Blacksburg, Virginia, capacity: 20 gallons) with recirculating sea water at a constant water temperature of 24^{o}C. Over the period of 3 days, two aquaria temperatures were gradually increased to 28^{o}C and another two aquaria were elevated to 32^{o}C at a continuous rate using digital aquarium heaters (Top Fin, Franklin, Wisconsin). Nearly a Each aquarium was equipped with a recirculating water filter (Tetra, Blacksburg, Virginia) and aerator. Two aquaria were used as a control (24^{o}C). Temperature, pH, and dissolved oxygen levels (pH: ~8.1, salinity: 32-33ppt, dissolved oxygen: 5.75-6.63 mg/L) in each aquarium were measured 3 times a day using a YSI (Yellow Springs Instrument, Yellow Springs, OH, USA). Oysters were fed frozen marine cuisine (San Francisco Bay Brand, Inc., Newark, CA, USA), a nutritious carnivorous diet, every other day. After a week of heat exposure, 60 oysters (10 from each aquarium) were cracked open using a Supreme Oyster Knife (Academy, Brownsville, Texas) and protective gloves to prevent scrapes and cuts on hands. Extrapallial fluid was collected and placed in a 1.5 ml tube and kept in dry ice and then stored in -80^{o}C to later measure the protein content. Extrapallial fluid pH and

protein levels were measured using a portable pH and NanoDrop, respectively. Their shell length, width, and body weight were also recorded. They were then dissected on a dissecting tray using tools such as a blade, scalpel, and scissors. The gonad was collected from each oyster and placed in a histology cassette submerged in a plastic bottle of 4% paraformaldehyde (Acros Organics, Morris, NJ, USA) for histological and immunohistological analyses.

Histological Analysis

Histological analyses for all gonad samples were conducted to determine the sex of each oyster and to observe how the development of eggs and sperm are being affected by high temperatures. Oyster gonad samples were kept in 4% paraformaldehyde for 4-5 days at 4^{o}C to fix the tissue. Once taken out of the paraformaldehyde, each sample was dehydrated with series of ethanol dilutions (50, 75, 95 and 100%) in glass vials for 30 min each. After dehydration, xylene (Fisher Chemical, Hampton, NH, USA) was added to each sample for 30 min, switched with melted paraffin and embedded in Paraplast Plus (m.p. 60-65^{o}C) using plastic rectangular molds and cassettes. The embedded tissue samples were serially sectioned at 7 μm in thickness. Deionized water was added to glass slides before placing tissue samples, slide was then placed on warmer plate and left to dry for 10-15 min then stored in -80^{o}C until histological and immunohistological analyses. To execute histology, the tissue slides were deparaffinized with xylene, rehydrated with series of ethanol dilutions (100, 95, 75, and 50%) and stained with hematoxylin (Sigma-Aldrich, St. Louis, MI, USA) and eosin (Fisher Chemicals). Slides were then mounted using two drops of Cytoseal XYL (Thermo Fisher Scientific, Hampton, NH, USA) and a cover slip then left to dry at room temperature for 30 min. Gonadal sections were then identified as male or female by visualizing the eggs or spermatogenic cells under a microscope.

Histological pictures were taken by a photometrics Cool-SNAP camera (Photometrics, Tucso, AZ, USA) using a light microscope (Nikon Eclipse E600, Nikon, Japan).

Percentage of Ova and Oocyte in Ovary

The percentage of ova (mature eggs) and oocyte (immature eggs) was calculated in ovarian sections for all treatment groups. Each section was marked into equally sized quadrants with a ruler and marker to easily count the numbers of mature and immature eggs per slide. Each slide was individually and carefully observed under a microscope. The ova and oocytes were identified, counted with a tally counter, and recorded for every slide at each treatment group.

Gamete Production

The egg diameter (75 to 100 eggs per female slides) was measured in micrometers using ImageJ software (National Institute of Health, Bethesda, MA, USA) according to Schneider et al. (2012). Briefly, pictures of the slides were taken and uploaded to ImageJ software. The straight-line tool was used to trace a line of 100 μm specifically for 10X images. The scale was then set to "100.00" in the known distance box and "μm" was selected in the unit length box. Feret's diameter, a measure of an objects size along a specific direction, was selected and set to analyze the diameter of each egg. Sperm production in testis of male oysters was measured using ImageJ software (Schneider et al., 2012). Measurements were set to "Area" to measure the total area of sperm produced per image. The data was expressed as the percentage of sperm production for each male oyster.

Immunohistochemical Analysis

Immunohistochemistry is important because it provides a visual representation of the antigen/antibody reaction to the detect target protein expression and its significance. Paraffin embedded tissues were sectioned at 7µm on the microtome machine, deparaffinized with xylene three times for 3 min each, rehydrated with a series of ethanol dilutions (100% 2X, 95%, 75%, and 50%), and rinsed in 1X phosphate buffer saline (PBS, Fisher Chemicals) three times for 10 min. Slides were then blocked with 1% BSA (Bovine Albumin Serum, Fisher Chemicals) and rinsed three times in PBS for 10 min each. Mouse anti-HSP70 (Sigma- Aldrich), mouse anti-NTP (Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit anti-DNP (Thermo Fisher Scientific, Waltham, MA, USA) primary antibodies were added to each slide (antibody dilution 1:100 with 1X PBS) and incubated at 4^{o}C overnight. The negative control slides were incubated with PBS instead of anti-HSP70, anti-NTP, or anti-DNP primary antibodies. After incubations, slides were rinsed with PBS again three times for 10 min each and incubated with anti-mouse (Cell Signaling Technology, Danvers, MA, USA) or anti-rabbit (Southern Biotech, Birmingham, AL, USA) secondary antibody (antibody dilution 1:100 with 1X PBS) for 1 h at room temperature. The immunoreactivity of HSP70, NTP, and DNP expressions was detected using 3,3'-diaminobenzidine peroxidase (DAB, Vector Laboratories, Burlingame, CA, USA) substrate in dark conditions. Slides were then wash with deionized water for 5 min and dehydrated with series of ethanol, sequenced with xylene, and mounted with Cytoseal. The immunoreactive (IR) signals of HSP70, NTP and DNP were captured by a Cool-SNAP camera using a light microscope and the IR signals were estimated using ImageJ software.

Extrapallial Fluid pH Analysis

Extrapallial fluid pH was measured using a pH meter (Oakton Instruments, Vernon Hills, IL, USA). Highly sensitive Oakton pH probe was used to obtain accurate readings of extrapallial fluid pH.

Protein Analysis

Extrapallial fluid protein levels were analyzed using a NanoDrop instrument (Thermo Fisher Scientific) according to the method described by Bradford (1976). Bradford protein assay is an accurate spectroscopic analytical procedure used to measure the extrapallial fluid protein concentrations. Briefly, 5 µl of extrapallial fluid was added in 5 ml of protein assay solution (Bio-Rad, Hercules, CA, USA) and incubated for 5 min at room temperature. A standard curve was made of BSA solution (0, 62.5, 125, 250, 500 and 1,000 µg/ml) to measure extrapallial fluid protein concentrations. Absorbance was read at 595 nm, and the protein concentration was expressed as µg/ml.

In situ TUNEL Assay for Apoptosis

Apoptosis of gonadal tissues was detected using *in situ* Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) detection kit (Promega, Madison, WI, USA). The procedures carried out for apoptosis was followed according to manufactures instructions. Briefly, paraffin-embedded tissues were sectioned at 7 µm sections on a rotary microtome (Leica, Weltzar, Germany) and placed on a superfrost plus glass slide (Fisher Scientific). Sections were then deparaffinized with xylene and rehydrated with a series of ethanol. Deparaffinized tissue sections were incubated with proteinase K (20 mg/ml) for 15 min

at room temperature, and endogenous peroxidase was quenched by treatment with 3% H_2O_2 in PBS for 5 min at room temperature. Sections were then incubated with TdT enzyme at 37^{o}C for 1 h in a humidified chamber. After stopping TdT enzymatic reaction, sections were rinsed in PBS, incubated with anti-digoxignenin conjugate for 30 min, and stained with peroxidase substrate for 5 min. Sections were then counter stained with 0.5% (weight:volume) methyl green for 10 min, rinsed with deionized water, dehydrated with series of ethanol, sequenced with xylene, and mounted with Cytoseal. The integrated intensity of apoptotic cell bodies in ovarian and testicular tissues was measured using ImageJ software.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (GraphPad, San Deigo, CA, USA) and StatView (SAS Institute, NC, USA) computer software. All results shown the mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Fisher's Protested Least Significant Difference (PLSD) tests were used to analyze the experimental data for multiple comparisons. Student's *t*-test was used to compare with unpaired means. A P value <0.05 was considered statistically significant.

CHAPTER III

RESULTS

Effects of Heat Stress on Gonadal Development

Ovarian functions were significantly attenuated in oyster exposed to high temperature (32ºC) for one week compared to control (24ºC) which was associated with a reduction in the percentage of fully-grown egg (mean ova: 64.8% at 24ºC and 43.3% at 32ºC, Figures 2, 3). The diameter of eggs in oysters were also significantly reduced after exposed to medium (28ºC) and high temperatures (control: 34.85±0.82 µm, medium temperature: 30.21±0.53 µm, and high temperature: 28.03±0.55 µm) (Figure 4).

There was a marked decrease in spermatogenesis and the production of fully grown spermatogenic cells compared to control, medium, and high temperature groups (Figures 5, 6). The outcome of oysters exposed to high temperature (32ºC) significantly impaired testicular functions, leading to a declined percentage of sperm production compared to controls (mean sperm production: 63% at 24ºC, 57% at 28ºC, and 50% at 32ºC) (Figure 6).

Effects of Heat Stress on Heat Shock Protein Expression

Heat shock protein 70 (HSP70) is a key chaperone protein in protecting the body under stressed conditions and regulating normal cellular functions (Udea and Boettcher, 2009). For this reason, I measured the HSP70 expression in oyster ovarian and testicular tissues. Through

immunohistochemical analysis, HSP70 expression was detected in oocytes (immature egg) and ova (mature egg), and seminiferous tubules of spermatogenic cells in oyster ovary and testis, respectively (Figures 7, 9).

Immunohistochemical detection of HSP70 in ovary and testis sections showed the immunoreactive intensity of HSP70 appeared to be stronger in both medium and higher temperature (28, and 32^{o}C) treatment groups (Figures 7, 9). The immunostaining intensity of HSP70 in ovaries increased around 3-fold in medium and ~7-fold in higher temperatures compared to controls (Figure 8). The immunoreactive intensities of HSP70 in testicular tissues were also significantly increased in medium and high temperature groups compared to controls (Figure 10). Negative control sections were used to reveal nonspecific binding and false positive results. No immunohistological signals were detected in the negative sections of ovaries and testes (Figures 7A, 9A).

Effects of Heat Stress on Extrapallial Fluid Conditions

Since extrapallial fluid is an important body fluid that regulates many physiological functions in marine invertebrates (Robertson, 1939; Mangum and Shick, 1972), I analyzed the biochemical compositions (total protein, pH, and glucose levels) of extrapallial fluid in oysters. Oysters exposed to higher temperatures significantly decreased extrapallial fluid protein concentrations compared to controls (control: 719.5±106.4 μg/ml, medium temperature: 556.8±65.8 μg/ml, and high temperature: 452.7±52.9 μg/ml, Figure 11A). Contrarily, EF pH tended to increase from control to medium and high temperatures (control: 6.56±0.04, medium temperature: 6.7±0.03, and high temperature: 6.71±0.06, Figure 11B).

Effects of Heat Stress of Cellular Apoptosis

Apoptosis is a natural process that occurs as part of an organism's development and growth (Gervais et al., 2015). Here, I observed how higher temperatures affect cellular apoptosis in oyster reproductive cells (egg and sperm). Female oysters exposed to the control temperature (24^{o}C) displayed few apoptotic cells while those exposed to medium and high temperatures exhibited a large number of apoptotic bodies/cells in ovaries (Figure 12). The intensity of apoptotic cells in the ovaries was measured using ImageJ software. The intensity was significantly higher in medium (28^{o}C) and high (32^{o}C) temperatures compared to controls (Figure 13).

Similarly, oysters exposed to medium and high temperatures displayed a considerable amount of apoptotic bodies in spermatogenic cells and seminiferous tubules compared to controls, and the intensity of apoptotic cells was significantly higher in medium and high temperatures compared to control (Figures 14, 15).

Effects of Heat Stress on Nitrotyrosine Protein Expression

Nitrotyrosine protein (an indicator of reactive nitrogen species, RNS) expression was detected in ovarian and testicular tissues of oysters in control (24^{o}C), medium (28^{o}C), and high (32^{o}C) temperatures through immunohistochemical analysis. Nitrotyrosine protein expression was significantly elevated in ovarian and testicular tissues of oysters exposed to medium and high temperatures compared to control (Figures 16, 18). The intensity of nitrotyrosine protein in ovaries was increased by ~2.7-fold in medium temperature and ~3.5-fold in high temperature compared to control (Figure 17). The immunoreactive intensity of nitrotyrosine protein in

spermatogenic cells also significantly increased in medium (~6.6-fold) and higher (~20-fold) temperatures compared to controls (Figure 19).

Effects of Heat Stress on Dinitrophenyl Protein Expression

Dinitrophenyl (DNP) is a biomarker of protein oxidation for protein carbonyl (PC, an indicator of reactive oxygen species; Simon, 1953). To elucidate the possible relationship of heat stress and ROS levels, immunohistochemical analysis was conducted to examine the DNP expression in oyster gonadal tissues. DNP expression in ovarian and testicular tissues significantly increased in both medium and high temperatures, displaying a very strong immunoreactive signal at 32^{o}C compared to controls (Figures 20, 22). Immunoreactivity intensity of DNP protein was also measured using ImageJ software. Results showed a much greater intensity in ovarian and testicular tissues of oysters exposed to 28^{o}C and 32^{o}C compared to 24^{o}C (Figures 21, 23).

CHAPTER IV

DISSCUSSION

From this research, I found that elevated temperature attenuates ovarian and testicular development, intensifies HSP70 expression, increases apoptosis, and most importantly, increases nitrotyrosine and dinitrophenyl (biomarkers for ROS and RNS) in gonadal tissues of American oysters. The influence of seasonal temperatures on gonadal development, photoperiodic cycles, and perpetual winter conditions on gametogenesis including the initiation of gonadal development, maturation and gamete resorption have previously been studied in oysters (Fabioux et al., 2005); however, many questions remain unanswered in reproductive impairment of oysters during environmental stress. In this study, I described the effects of high temperature on gonadal functions, cellular apoptosis, and oxidative stress in the American oyster.

The results of my study clearly demonstrate that exposure to medium and/or high temperatures (28 and 32^{o}C) for one-week decreases the number of fully-grown oocyte (ova) by nearly 1.5-fold and diameter of ova ~1.25-fold. The production of sperm in oysters also significantly decreases by over 1.26-fold. Effects of high temperature on gametogenesis have been reported in other bivalves (Sastry et al., 1971; Chávez-Villalba et al., 2011; Gosling, 2015). In the Pacific oyster, the speed of oocyte development significantly increases with temperatures between 16 and 22^{o}C, but the growing speed of oocytes quickly decreases at 25^{o}C compared to 22^{o}C exposure for four weeks (Chávez-Villalba et al., 2011). In blue mussel, *Mytilus edulis*, the lower and upper lethal temperature are 0 and 23^{o}C, respectively, and the optimum temperature

for gametogenesis is between 2 and 15^{o}C (Gosling, 2015). Temperature also influences the oocyte growth by regulating the transfer of nutrient reserves to the gonads in the Atlantic bay scallop, *Argopecten irradians* (Sastry et l., 1971). Collectively, these results suggest that the elevated sea water temperature from global climate change imposes a great threat on gametogenesis in the American oyster and other marine organisms. I can conclude there are potential intermediates blocking the physiological and molecular responses inhibiting the development and production of gametes in the American oyster.

Most marine invertebrates do not contain blood in their internal organs; however, they carry similar fluids that act in the same manner (Chapman and Newell, 1956; Trueman, 1966; Allam, 1998; Allam and Paillard, 1998). Bivalve's hearts consist of a single muscular ventricle and two auricles; hemolymph flows from the auricles into the ventricle, which then contracts to force the hemolymph into a single vessel (Johansen and Martin, 1962; Gosling, 2015). Most bivalves have open circulatory systems, with hemolymph in the sinuses (Gosling, 2015). From the sinus's hemolymph is transported to the kidneys for purification. Extrapallial fluid can be described as hemolymph in most marine invertebrates and plays various important roles in marine bivalve physiology including gas exchange, osmoregulation, nutrient distribution, waste elimination, and internal defense (Gosling, 2015). Hemolymph acts as a fluid skeleton by providing a temporary sternness to organs such as labial palps, foot, siphons, and mantel (Allam et al., 2000; Gosling, 2015). Hemocytes are the cells which make up the hemolymph that floats in a colorless plasma (Gosling, 2015). Since hemocytes move freely out of the sinuses into surrounding tissues, the mantel cavity, and gut lumen and are not confined to a hemolymph system, it is applicable to think that these cells play key roles in physiological processes such as internal defense, nutrient digestion and transport, excretion, tissue repair, shell formation, and

biomineralization (Allam et al., 2000; Lau et al., 2017). Alterations of extrapallial fluid composition restricts immune defense, shell repair, homeostatic maintenance, and most importantly the transport of important nutrients to cells hindering the growth, development, and production of gametes in the reproductive organ (Lau et al., 2017). That being the case, it is important to analyze the body fluid (extrapallial fluid) conditions in oysters under heat stress. An important finding of my study is that elevated water temperature decreases extrapallial fluid, an important body fluid that regulates physiological functions, pH levels in the American oyster. In normal conditions, extrapallial fluid pH is naturally acidic, but my results indicate that heat stress shifts extrapallial fluid pH from acidic (~6.5) towards neutral (~6.7) by over a 1-fold increase. Oyster exposed to high temperature significantly decreases extrapallial fluid protein concentrations by approximately 37% from control (24^{o}C) to high (32^{o}C) temperature. Therefore, it is envisaged that the decrease in extrapallial fluid protein concentrations which were accompanied by the significant decrease in gamete production. This suggests that the extrapallial fluid pH and protein are the most important factors that lead to influence the gonadal development and/or functions in oysters.

HSPs are important proteins when it comes to protecting the body from environmental stress (Li et al., 2007). HSPs are not only serve as regulators of internal functions, but they are also chaperone proteins that assist in folding, assembly, and transport of other essential proteins (Udea and Boettcher, 2009). They also play vital roles in apoptosis, immune system, and cellular defense aiding in the removal and refolding of protein destroyed by environmental stress which in turn increases HSPs expression and mRNA levels in cells and tissues (Udea and Boettcher, 2009). HSPs (e.g. HSP70, HSP72, HSP77) expression has also been linked to developmental changes during embryogenesis as well as early spat development in the American oyster (Udea

and Boettcher, 2009). The results from the immunohistochemical analysis showed an increase of HSP70 expression in follicles/eggs and spermatogenic cells in the American oyster (Figures 12-15). The intensity of HSP70 expression increased around ~2-4-fold (Figures 8, 10). This significant increase in HSP70 expression in oysters' gonadal tissues could lead to extensive developmental changes in gametogenesis, embryogenesis, and metamorphosis in oysters (Ueda and Boettcher, 2009).

Cellular apoptosis is an orderly sequence of events that leads to complete disassembly of the cell which may result in DNA fragmentation and DNA damage (Steinert, 1999). Apoptosis can be activated by environmental pollutants and stressors, therefore one study looked at the effects of DNA damage in the Mediterranean mussel from sediment contaminants in the San Diego Bay (Steinert, 1999; Sokolova et al., 2004). Consequently, Steinert discovered increases in DNA damage was due to the induction of apoptosis (Steinert, 1999). In a similar study, mussels were exposed to a variety of toxicants increasing apoptotic contribution to DNA damage in cells (Steinert, 1996). Steinert (1996) also observed that under certain circumstances apoptosis was the major contributor to DNA damage. One of the most interesting discoveries of my study is the tendency of apoptotic cells in ovarian follicles, eggs, and spermatogenic cells to increase in oysters exposed to high temperature (Figures 12, 14). It is plausible to conclude increases in HSP70 expression and changes in extrapallial compositions promote heat-induced cell death or apoptosis. Induction of cellular apoptosis in gonadal tissues suggests this may cause DNA damage and/or DNA methylation (Ueda and Boettcher, 2009; Gavery and Roberts, 2010). Therefore, future research will be focused on global DNA methylation in gonads of oysters exposed to high temperature. Additionally, to improve this research, future work will examine the long term effects of high temperature exposure compared to the short term impacts.

RNS and ROS are free radicals displayed in many physiological functions that often damage cells (Donaght et al., 2014). It has been shown that environmental stress increases ROS and RNS production resulting in oxidative stress (Donaght et al., 2014). Although they have both beneficial and detrimental effects in vertebrates, some mechanisms and pathways of ROS and RNS only exist in invertebrates (Donaght et al., 2014). It has also been shown when marine bivalves are exposed to environmental stress, they protect themselves by increasing peroxynitrite, a RNS (Torreilles and Romestand, 2001). It is formed by the combustion of nitric oxide and a superoxide radical that reacts with tyrosine residues of proteins yielding 3-nitrotyrosine (Torreilles and Romestand, 2001). I observed the nitrotyrosine protein (NTP, a biomarker of RNS) expression in oyster gonadal tissues exposed to control (24^{o}C), medium (28^{o}C), and high (32^{o}C) temperatures. Observations revealed a much greater expression of NTP in oysters exposed to 28^{o}C and 32^{o}C. After measuring the integrated density of each image using ImageJ software, results confirmed a significant increase of NTP levels with rising temperatures in both ovarian and testicular tissues, respectively. NTP intensity increased by ~3.5-fold in female and ~20-fold in males from control to high temperatures. These results suggest that increases in NTP expression from elevated temperature could be responsible for cellular apoptosis and oxidative stress in oysters.

Dinitrophenyl (DNP, a biomarker for ROS) is known to stimulate several adaptive cellular stress-response signaling pathways in neurons and research has found that numerous DNP-amino acids can induce delayed and immediate type hypersensitivity of DNP specificity (Frey et al., 1969; Geisler et al., 2017). Mitochondrial uncoupling proteins or UPCs are induced by bioenergetic challenges that provide resistance to metabolic and oxidative stress (Geisler et al., 2017). While mitochondrial uncoupling is a normal physiological process that mediates

cellular responses to environmental challenges, high doses of DNP can exhibit serious adverse effects (Geisler et al., 2017).

Recent research on three different types of bivalve species, including the Mediterranean mussel (*Mytilus galloprovincialis*), the mud cockle (*Katelysia rhytiphora*), and the Pacific oyster (*Crassostrea gigas*) compared the response to temperature change at three different temperatures, 15, 20, and 25^{o}C by measuring the total hemocyte count (THC), phagocytosis, the activity of antioxidant enzymes such as superoxide dismutase (SOD, an enzyme that catalyzes the dismutation of superoxide radical) and catalase (CAT, an enzyme in protecting the cell from oxidative damage), and ROS (Rahman et al., 2019). Results showed a rapid increase in SOD, CAT, and ROS activities with increasing temperatures among the Mediterranean mussel and mud cockle, while the Pacific oyster, only increased SOD and ROS levels occurring at temperatures between 20 and 25^{o}C (Rahman et al., 2019). I discovered that DNP was expressed in oyster gonadal tissues exposed to all temperatures and observed a tendency for DNP expression to increase with elevated temperatures in both female and male oysters (Figures 20, 23). After measuring the integrated intensity of DNP expression in female and male gonadal tissues, results revealed a significant difference between control, medium, and high temperatures. Around 1.2-fold increase in DNP intensity in ovarian follicles in female oysters and ~10.5-fold increase in spermatogenic cells in male oysters from control to high temperatures. Collectively, these results show increases in DNP expression from elevated temperature could be the limiting factors responsible for apoptosis and oxidative stress in mussels and oysters.

The fact that increases in NTP or DNP indicate increases of RNS or ROS, is consistent with the proposed mechanisms gonadal functions play during heat stress, such as increases in extrapallial fluid pH and cellular apoptosis, and decrease in total protein concentrations of

extrapallial fluid. The present results show the upregulation of NTP and DNP, (biomarkers of heat induced cellular reactive free radicals such as RNS and ROS, respectively) promote cellular apoptosis and oxidative damage, and plays an important role in gamete injury as well as the inhibition of gonadal functions and development. Therefore, extrapallial fluid is proposed as an important mediator of heat-induced down-regulation of gonadal development through the upregulation of HSP, NTP, and DNP expressions, accelerating the rate at which cellular apoptosis, programmed cell death occurs in oysters.

Conclusion

This study reports, to the best of my knowledge, the first clear findings on the impacts of elevated temperatures on gonadal function, extrapallial fluid conditions, cellular apoptosis, and oxidative stress in the American oyster. This research not only progressed my knowledge on the effects of higher temperatures in oyster reproductive development, but also on the implications that are associated with damage from increasing temperatures such as increased HSP expression, apoptosis, and oxidative stress in marine mollusks (Figure 24). Collectively, my results propose higher temperatures from global warming have a negative impact on ovarian and testicular development as well as reproductive performances in American oysters. Seawater conditions such as temperature, biotic factors, and seasonality all play vital roles under the influence of gonadal development in marine biota. The American oyster carries many unique characteristics that are highly beneficial to marine ecosystems making them an excellent indicator species to these climatic changes. Since oysters are both commercially and economically important, it is crucial to fundamentally understand this species and their development. The rise of mean global temperatures will hinder reproductive functions and gonadal development resulting in

reproductive failure that may be passed on from generation to generation. The significance of this research highlights how the influence of elevated temperatures leads to decline reproductive functions which could have long term impacts on population and ecological levels facing severe economic consequences.

REFERENCES

Akil, L., Ahmad, H. A., & Reddy, R. S. (2014). Effects of Climate Change on Salmonella Infections. *Foodborne Pathogens and Disease*, *11*(12), 974–980.

Allam, B. (1998) The Role of Bivalve Extrapallial Fluids in Immunological Defense: The Case of Brown Ring Disease in the Manila Clam, *Ruditapes philippinarum*. PhD thesis, Universite de Bretagne Occidentale, Brest, France.

Allam, B, & Paillard, C. (1998). Defense factors in clam extrapallial fluids. *Diseases of Aquatic Organisms*, *33*, 123–128.

Allam, Bassem, Paillard, C., & Auffret, M. (2000). Alterations in hemolymph and extrapallial fluid parameters in the Manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio tapetis*. *Journal of Invertebrate Pathology*, *76*(1), 63–69.

Bartol, I. K., Mann, R., & Luckenbach, M. (1999). Growth and mortality of oysters (*Crassostrea virginica*) on constructed intertidal reefs: effects of tidal height and substrate level. *Journal of Experimental Marine Biology and Ecology*, *237*(2), 157–184.

Beukema, J., Dekker, R., & Jansen, J. (2009). Some like it cold: populations of the tellinid bivalve *Macoma balthica* (L.) suffer in various ways from a warming climate. *Marine Ecology Progress Series*, *384*, 135–145.

Bonaventura, R., Zito, F., Costa, C., Giarrusso, S., Celi, F., & Matranga, V. (2011). Stress response gene activation protects sea urchin embryos exposed to X-rays. *Cell Stress & Chaperones*, *16*(6), 681–687.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.

Bratton, S. B., Salveson, G. S. (2010). Regulation of the Apaf-1-caspase-9 apoptosome. *Journal of Cell Sciences 123*(19):3209-3214.

Bruskov, V. I., Malakhova, L. V., Masalimov, Z. K., & Chernikov, A. V. (2002). Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. *Nucleic Acids Research*, *30*(6), 1354–1363.

Chapman G., Newell G. E., & Cannon H. G. (1956). The role of the body fluid in the movement of soft-bodied invertebrates - II. The extenstion of the siphons of *Mya arenaria* L. and *Scrobicularia plana* (da Costa). *Proceedings of the Royal Society of London B – Biological Sciences*, *145*(921), 564–580.

Chauvaud, L., Patry, Y., Jolivet, A., Cam, E., Le Goff, C., Strand, Ø., & Clavier, J. (2012). Variation in size and growth of the great scallop Pecten maximus along a latitudinal gradient. *PloS ONE*, *7*(5), e37717.

Chávez-Villalba, J., Soyez, C., Huvet, A., Gueguen, Y., Lo, C., & Moullac, G. L. (2011). Determination of gender in the pearl oyster *Pinctada margaritifera*. *Journal of Shellfish Research*, *30*(2), 231–240.

Coe, W. R. (1932). Development of the gonads and the sequence of the sexual phases in the California oyster (*Ostrea lurida*), *Bulletin of the Scripps Institution of Oceanography,* 3, 119-139.

Dale, V. H., Joyce, L. A., Mcnulty, S., Neilson, R. P., Ayres, M. P., Flannigan, M. D., & Wotton, B. M. (2001). Climate Change and Forest Disturbances. *BioScience*, *51*(9), 723.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *329*(1–2), 23–38.

Di Meo, S., Reed, T. T., Venditti, P., & Victor, V. M. (2016). Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxidative Medicine and Cellular Longevity*. 1-44.

Dinamani, P. (1974). Reproductive cycle and gonadial changes in the New Zealand rock oyster *Crassostrea glomerata*. *New Zealand Journal of Marine and Freshwater Research*, *8*(1), 39–65.

Donaghy, L., Hong, H.-K., Jauzein, C., & Choi, K.-S. (2015). The known and unknown sources of reactive oxygen and nitrogen species in haemocytes of marine bivalve molluscs. *Fish & Shellfish Immunology*, *42*(1), 91–97.

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean Acidification: The Other CO2 Problem. *Annual Review of Marine Science*, *1*(1), 169–192.

Eckelbarger, K. J., & Davis, C. V. (1996). Ultrastructure of the gonad and gametogenesis in the eastern oyster, *Crassostrea virginica*. I. Ovary and oogenesis. *Marine Biology*, *127*(1), 79–87.

Eble A., F., & Scro, R. (1996) "General Anatomy." *The Eastern Oyster: Crassostrea Virginica*, by Victor S. Kennedy et al., Maryland Sea Grant College, 19-71.

Fabioux, C., Huvet, A., Le Souchu, P., Le Pennec, M., & Pouvreau, S. (2005). Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. *Aquaculture*, *250*(1–2), 458–470.

Fabry, V. J., Seibel, B. A., Feely, R. A., & Orr, J. C. (2008). Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science*, *65*(3), 414–432.

Farag, M. A., Meyer, A., Ali, S. E., Salem, M. A., Giavalisco, P., Westphal, H., & Wessjohann, L. A. (2018). Comparative metabolomics approach detects stress-specific responses during coral bleaching in soft corals. *Journal of Proteome Research*, *17*(6), 2060–2071.

Franco, A., Heude Berthelin, C., Goux, D., Sourdaine, P., & Mathieu, M. (2008). Fine structure of the early stages of spermatogenesis in the Pacific oyster, *Crassostrea gigas* (Mollusca, Bivalvia). *Tissue & Cell*, *40*(4), 251–260.

Freitas, V. (2011). Climate induced changes in estuarine predator-prey systems: a DEB approach. *Vrije Universiteit, Amsterdam*. 1-182.

Frey, J. R., de Weck, A. L., Geleick, H., Lergier, H. (1969). The immunogenicity of dinitrophenyl amino acids. *Journal of Experimental Medicine,* 1123-1143.

Garrison, T. (2007). *Oceanography: an invitation to marine science* (Sixth edition.). Belmont, CA: Thomson Brooks/Cole. 1-640.

Gavery, M. R., & Roberts, S. B. (2010). DNA methylation patterns provide insight into epigenetic regulation in the Pacific oyster (*Crassostrea gigas*). *BMC Genomics*, *11*(1), 483.

GBIF.org. (2016). GBIF Occurrence Download https://www.gbif.org/species/2286079

Gerisler, J. G., Marosi, K., Halpern, J., Mattson, M. P. (2017). DNP mitochondrial uncoupling, and neuroprotection: A little dab'll do ya. *Alzheimer's & Dementia*, *13*(5), 582-591.

Gervais, O., Renault, T., & Arzul, I. (2015). Induction of apoptosis by UV in the flat oyster, *Ostrea edulis*. *Fish & Shellfish Immunology*, *46*(2), 232–242.

Giudice, G., Sconzo, G., & Roccheri, M. C. (1999). Studies on heat shock proteins in sea urchin development. *Development, Growth & Differentiation*, *41*(4), 375–380.

Goedken, M., Morsey, B., Sunila, I., Dungan, C., & Guise, S. D. (2005). The effects of temperature and salinity on apoptosis of *Crassostrea virginica* hemocytes and *Perkinsus marinus*. *Journal of Shellfish Research*, *24*(1), 177–183.

Gonçalves, C., Martins, M., Costa, M. H., & Costa, P. M. (2018). Development of a method for the detection of polystyrene microplastics in paraffin-embedded histological sections. *Histochemistry and Cell Biology*, *149*(2), 187–191.

Gosling, E. (2015). *Marine Bivalve Molluscs*. John Wiley & Sons. 1-537.

Guo, X., Hedgecock, D., Hershberger, W. K., Cooper, K., & Jr, S. K. A. (1998). Genetic determinants of protandric sex in the pacific oyster, *Crassostrea gigas* Thunberg. *Evolution*, *52*(2), 394–402.

Hammen, C. S. (1969). Metabolism of the oyster, *Crassostrea virginica*. *Integrative and Comparative Biology*, *9*(2), 309–318.

Humphries, A. T., & Peyre, M. K. L. (2015). Oyster reef restoration supports increased nekton biomass and potential commercial fishery value. *PeerJ*, *3*, e1111.

IPCC. (2007). Climate change 2007: The physical basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 996.

Johansen, K., & Martin, A. W. (1962). Circulation in the cephalopod, Octopus Dofleini. *Comparative Biochemistry and Physiology*, *5*(3), 161–176.

Jones, S. J., Lima, F. P., & Wethey, D. S. (2010). Rising environmental temperatures and biogeography: poleward range contraction of the blue mussel, *Mytilus edulis* L., in the western Atlantic: *Mytilus edulis* poleward range contraction. *Journal of Biogeography*, *37*(12), 2243–2259.

Kennedy, V. S., Newell, R. I. E., Eble, A. F., & Maryland Sea Grant College. (1996). *The eastern oyster: Crassostrea virginica*. College Park, MD: Maryland Sea Grant College.

Kirby, M. X. (2004). Fishing down the coast: Historical expansion and collapse of oyster fisheries along continental margins. *Proceedings of the National Academy of Sciences*, *101*(35), 13096–13099.

Kovats, R. S., Edwards, S. J., Hajat, S., Armstrong, B. G., Ebi, K. L., & Menne, B. (2004). The effect of temperature on food poisoning: a time-series analysis of salmonellosis in ten European countries. *Epidemiology & Infection*, *132*(3), 443–453.

Krammer, P. H. (1998). CD95(APO-1/Fas)-Mediated Apoptosis: Live and Let Die. In F. J. Dixon (Ed.), *Advances in Immunology* (Vol. 71, pp. 163–210). Academic Press.

Lau, Y.-T., Sussman, L., Pales Espinosa, E., Katalay, S., & Allam, B. (2017). Characterization of hemocytes from different body fluids of the eastern oyster *Crassostrea virginica*. *Fish & Shellfish Immunology*, *71*, 372–379.

Li, Y., Qin, J. G., Abbott, C. A., Li, X., & Benkendorff, K. (2007). Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas* : an explanation for summer mortality in Pacific oysters. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *293*(6), R2353–R2362.

Loosanoff, V. L. (1942). Seasonal gonadal changes in the adult oysters, *Ostrea virginica*, of Long Island Sound. *Biological Bulletin*, *82*(2), 195–206.

Loosanoff, V. l., & Davis, H. C. (1952). Temperature requirements for maturation of gonads of northern oysters. *The Biological Bulletin*, *103*(1), 80–96.

Luo, M., Li, L., Xiao, C., Sun, Y., & Wang, G.-L. (2016). Heat stress impairs mice granulosa cell function by diminishing steroids production and inducing apoptosis. *Molecular and Cellular Biochemistry*, *412*(1–2), 81–90.

MacMillan, A. (2016). Global warming 101. NRDC**.**

Mangum, C. P., & Shick, J. M. (1972). The pH of body fluids of marine invertebrates. *Comparative Biochemistry and Physiology Part A: Physiology*, 42(3), 693–697.

Morris, P. J., Swindles, G. T., Valdes, P. J., Ivanovic, R. F., Gregoire, L. J., Smith, M. W. Bacon, K. L. (2018). Global peatland initiation driven by regionally asynchronous warming. *Proceedings of the National Academy of Sciences*, *115*(19), 4851–4856.

Munari, M., Matozzo, V., & Marin, M. G. (2011). Combined effects of temperature and salinity on functional responses of haemocytes and survival in air of the clam Ruditapes philippinarum. *Fish & Shellfish Immunology*, *30*(4), 1024–1030.

Parker, L. M., O'Conner, W. A., Byrne, M., Dove., M., Coleman, R. A., Portner, H. O., Scanes, E., Virtue, P., Gibbs. M., & Ross, P. M. (2018). Ocean acidification but not warming alters sex determination in the Sydney rock oyster, *Saccostrea glomerate. Proceedings of the Royal Society: B.* 285, 20172869.

Petes, L. E., Menge, B. A., & Murphy, G. D. (2007). Environmental stress decreases survival, growth, and reproduction in New Zealand mussels. *Journal of Experimental Marine Biology and Ecology*, *351*(1), 83–91.

Portner, HO. (2008). Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series. 373*:203-217.

Rahman, M. A., Henderson, S., Miller-Ezzy, P., Li, X. X., & Qin, J. G. (2019). Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel Mytilus galloprovincialis and mud cockle Katelysia rhytiphora. *Fish & Shellfish Immunology*, *86*, 868–874.

Ramos, J. B. e, Biswas, H., Schulz, K. G., LaRoche, J., & Riebesell, U. (2007). Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer Trichodesmium. *Global Biogeochemical Cycles*, *21*(2).

Robertson, J. D. (1939). The inorganic composition of the body fluids of the three marine invertebrates. Journal of Environmental Biology. 16: 387-397.

Root, T. L., Price, J. T., Hall, K. R., Schneider, S. H., Rosenzweig, C., & Pounds, J. A. (2003). Fingerprints of global warming on wild animals and plants. *Nature*, *421*(6918), 57–60.

Ryter, S. W., Kim, H. P., Hoetzel, A., Park, J. W., Nakahira, K., Wang, X., & Choi, A. M. K. (2006). Mechanisms of cell death in oxidative stress. *Antioxidants & Redox Signaling*, *9*(1), 49–89.

Sengupta, S., & BhattacharjeeS. A. (2016). Dynamics of protein tyrosine nitration and denitration: A Review. *Journal of Proteomics & Genomics*, *1*(1).

Saeed-Zidane, M., Linden, L., Salilew-Wondim, D., Held, E., Neuhoff, C., Tholen, E., & Tesfaye, D. (2017). Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. *PLoS ONE*, *12*(11), e0187569.

Sastry, A. N., & Blake, N. J. (1971). Regulation of gonad development in the bay scallop, *Aequipecten irradians* lamarck. *The Biological Bulletin*, *140*(2), 274–283.

Schilling, D.R. (2015). Adult oysters filter fifty gallons of water per day. Industry Tap.

Schneider, C. A., Rasband, W. S., Elicein, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nature Methods*. 9, 671-675.

Shumway, S.E. (1996). Natural environmental factors. In: V.S. Kennedy, R.I.E. Newell and A.F. 467-513.

Simon, E. W. (1953). Mechanisms of dinitrophenol toxicity. *Biological Reviews*, *28*(4), 453–478.

Slimen, I. B., Najar, T., Ghram, A., Dabbebi, H., Mrad, M. B., & Abdrabbah, M. (2014). Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. *International Journal of Hyperthermia*, *30*(7), 513–523.

Snyder, C. S., Bruulsema, T. W., Jensen, T. L., & Fixen, P. E. (2009). Review of greenhouse gas emissions from crop production systems and fertilizer management effects. *Agriculture, Ecosystems & Environment*, *133*(3–4), 247–266.

Sokolova, I. M., Evans, S., & Hughes, F. M. (2004). Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *Journal of Experimental Biology*, *207*(19), 3369–3380.

Steinert, S. A. (1996). Contribution of apoptosis to observed DNA damage in mussel cells. *Marine Environmental Research*, *42*(1–4), 253–259.

Steinert, S. A. (1999). DNA damage as a bivalve biomarker. *Biomarkers*, *4*(6), 492–496.

Svitkina, T. M., Neyfakh, A. A., & Bershadsky, A. D. (1986). Actin cytoskeleton of spread fibroblasts appears to assemble at the cell edges. *Journal of Cell Science*, *82*(1), 235–248.

Torreilles, J., & Romestand, B. (2001). In vitro production of peroxynitrite by haemocytes from marine bivalves: C-ELISA determination of 3-nitrotyrosine level in plasma proteins from *Mytilus galloprovincialis* and *Crassostrea gigas*. *BMC Immunology*, *2*(1), 1.

Trueman, E.R. (1966). The fluid dynamics of the bivalve molluscs, Mya and Margatitifera. *Journal of Experimental Biology, 45*:369-382.

Ueda, N., & Boettcher, A. (2009). Differences in heat shock protein 70 expression during larval and early spat development in the Eastern oyster, *Crassostrea virginica* (Gmelin, 1791). *Cell Stress and Chaperones*, *14*(4), 439–443.

Yoshikawa, T., & Naito, Y. (2002). What Is Oxidative Stress? *Oxidative Stress*, 6.

Yuan, Z., Jiang, S., Sheng, H., Liu, X., Hua, H., Liu, X., & Zhang, Y. (2018). Human perturbation of the global phosphorus cycle: changes and consequences. *Environmental Science & Technology*, *52*(5), 2438–2450.

Zippay, M. L., & Helmuth, B. (2012). Effects of temperature change on mussel, Mytilus. *Integrative Zoology*, *7*(3), 312–327.

FIGURES

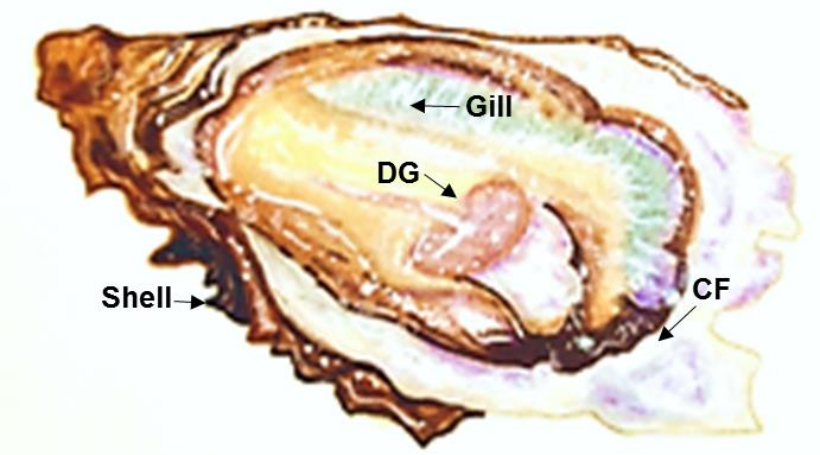

Figure 1. Anatomy (soft body parts: mantle cavity and topography of the internal organs) of the American oyster (*Crassostrea virginica*). DG: digestive gland, EF, extrapallial fluid.

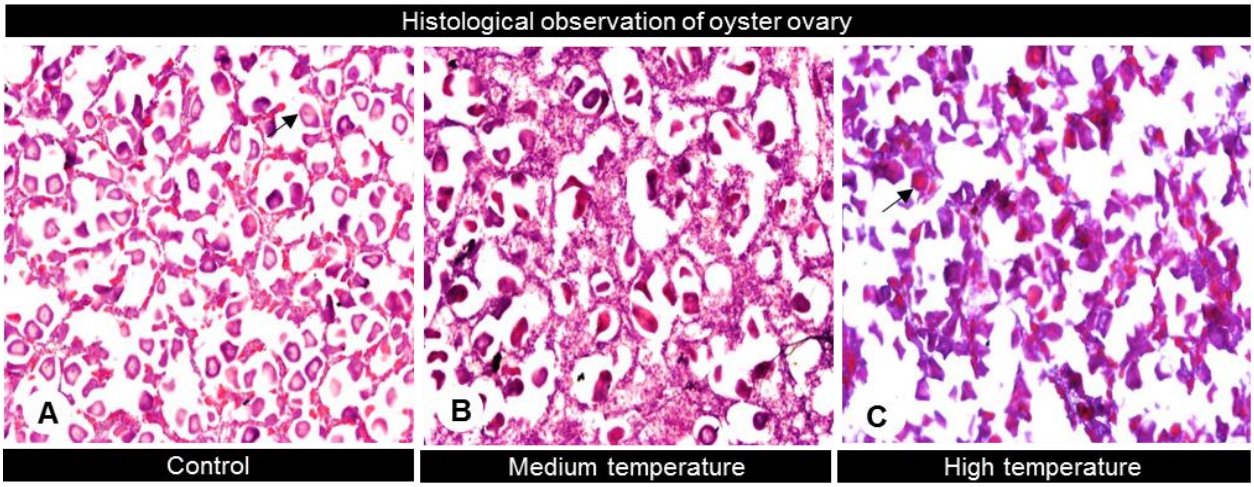

Figure 2. Effects of high temperature on ovarian development of the American oyster. Arrow indicates ova (mature egg). (A) control: 24ºC, (B) medium temperature: 28ºC, and (C) high temperature 32ºC. Magnification = 10x.

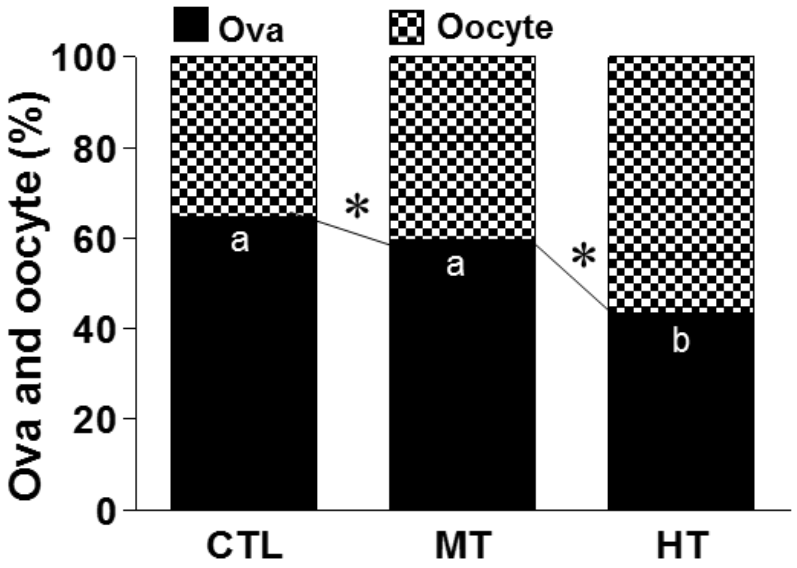

Figure 3. Effects of high temperature on percentage of ova (mature eggs) and oocyte (immature eggs) in ovary of the American oyster. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Asterisk indicates significant difference (Student *t*-test, $*P<0.05$). N = 8-12.

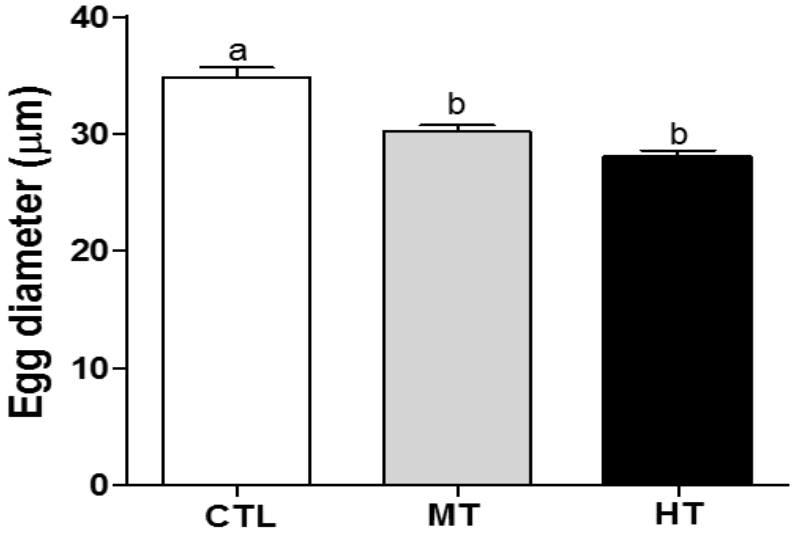

Figure 4. Effects of high temperature on egg (ova) diameter in the American oyster. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Each value represents the mean ± SEM (N = 75-100).

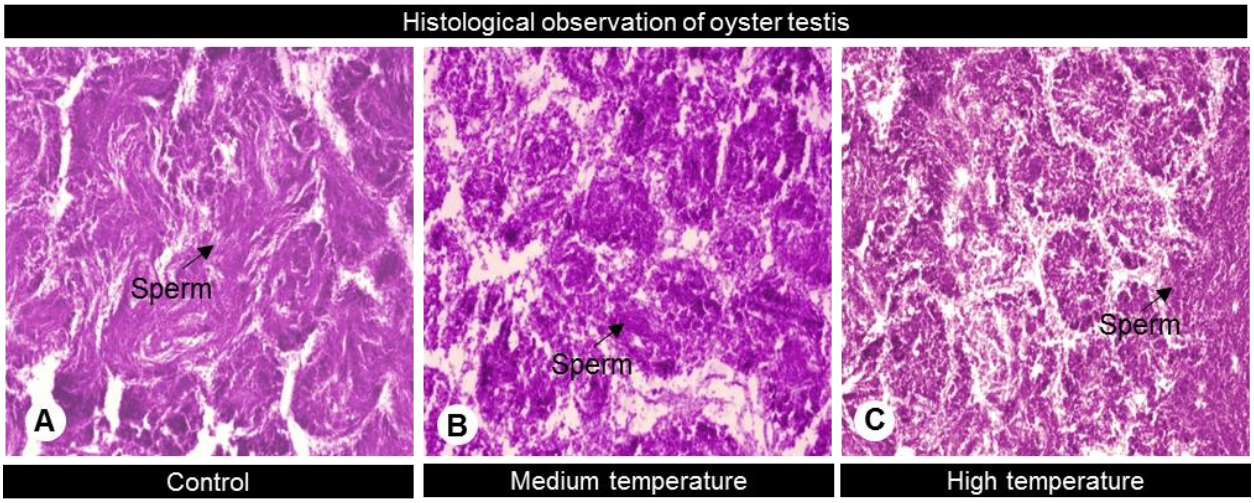

Figure 5. Effects of high temperature on testicular development of the American oyster. Arrow indicates sperm. (A) control: 24ºC, (B) medium temperature: 28ºC, and (C) high temperature 32ºC. Magnification = 10x.

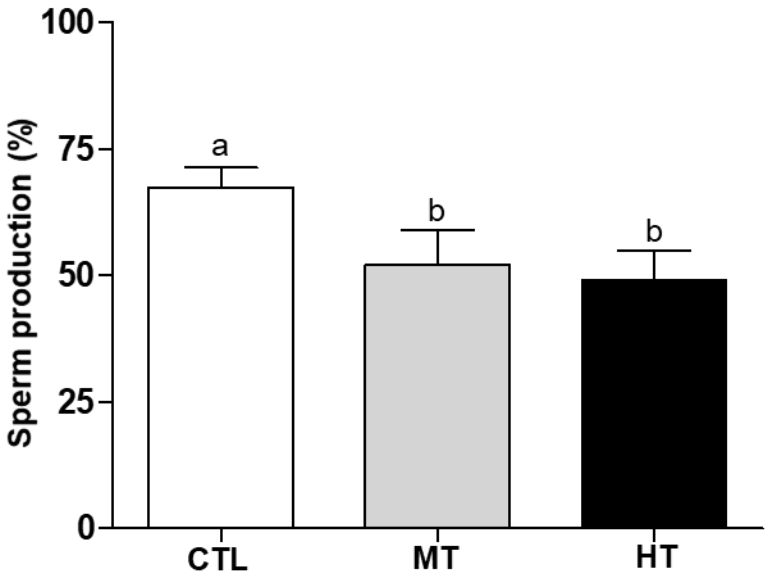

Figure 6. Effects of high temperature on sperm production in the American oyster. Control (CTL): 24ºC, medium temperature (MT): 28ºC, and high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Asterisk indicates significant difference (Student *t*-test, $*P<0.05$). Each value represents the mean ± SEM (N = 6-15).

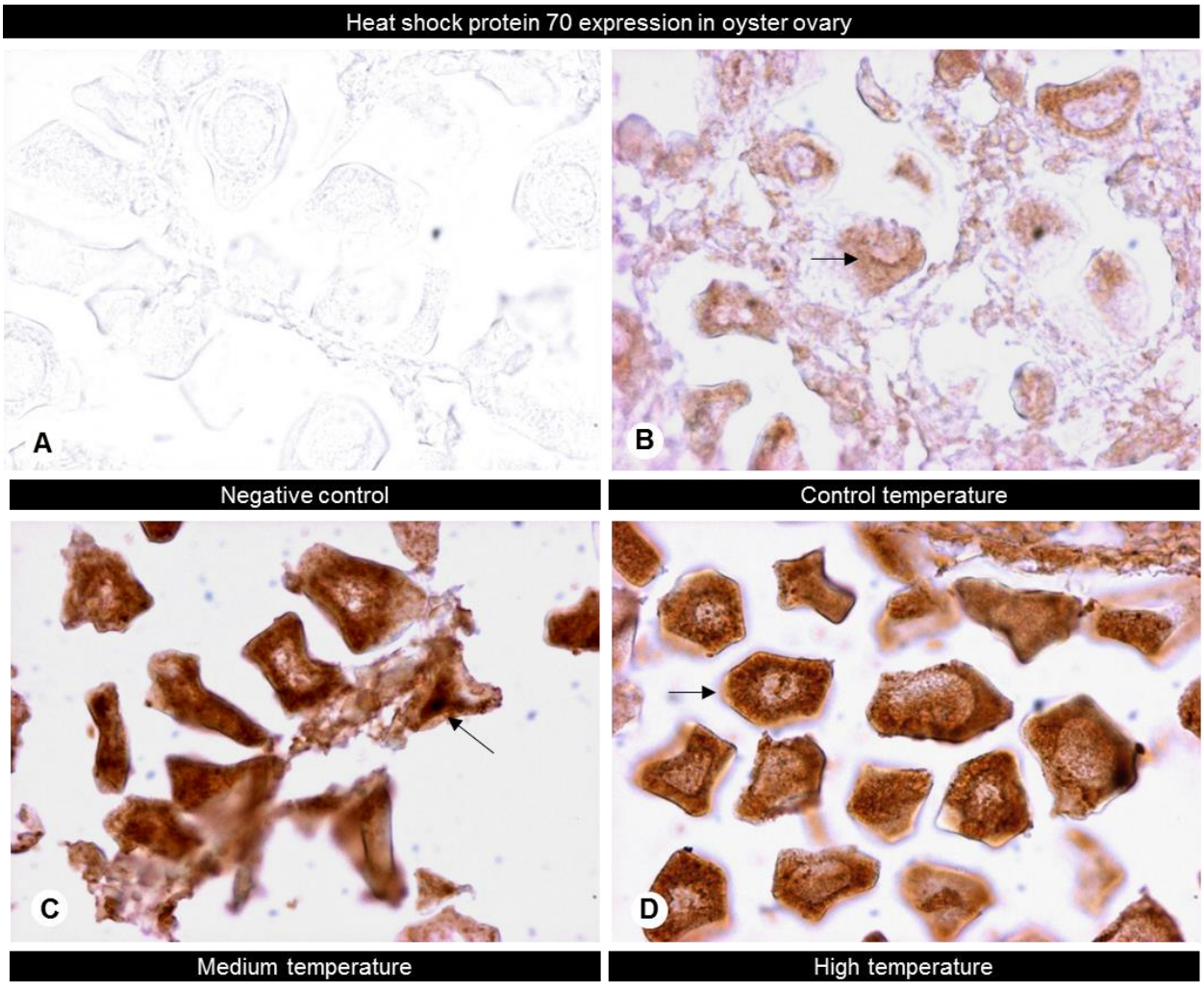

Figure 7. Effects of high temperature on heat shock protein 70 (HSP70) expression in the American oyster ovary. Arrow indicates HSP70 expression in oyster egg (ova). A: negative control of HSP70 expression in ovary. B, control: 24ºC; C, medium temperature: 28ºC; and D, high temperature 32ºC. Magnification 40x.

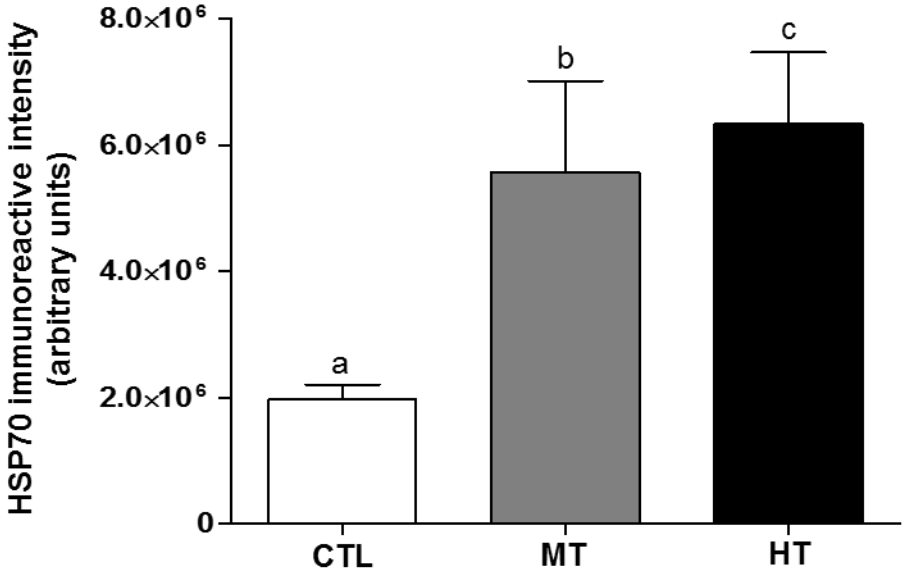

Figure 8. Immunohistochemical analysis of effect of high temperature on heat shock protein 70 (HSP70) levels in the American oyster ovary. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 23-53).

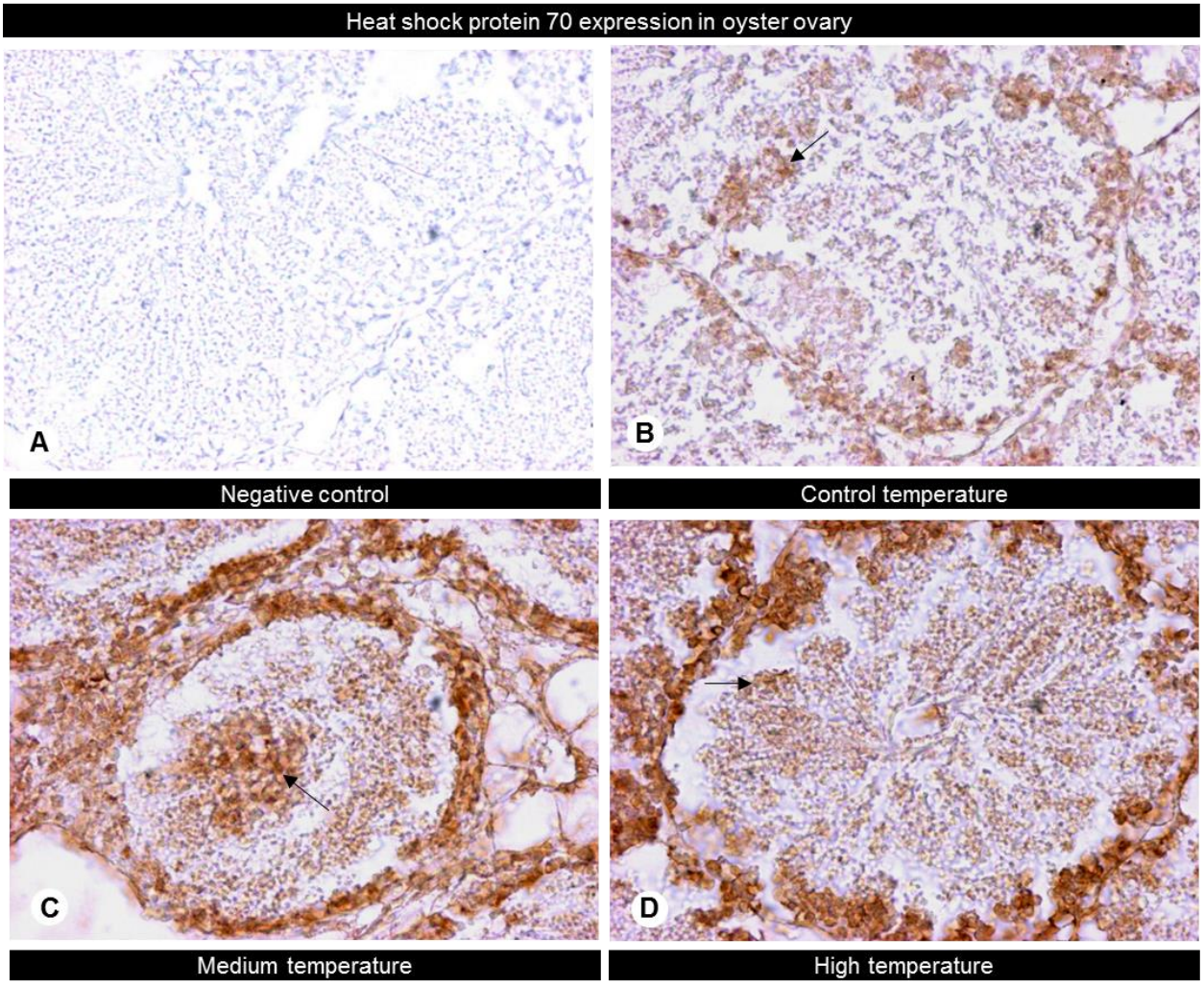

Figure 9. Effects of high temperature on heat shock protein 70 (HSP70) expression in the American oyster testis. Arrow indicates HSP70 expression in spermatogenic cells. A: negative control of HSP70 expression in testis. B, control: 24ºC; C, medium temperature: 28ºC; and D, high temperature 32ºC. Magnification 40x.

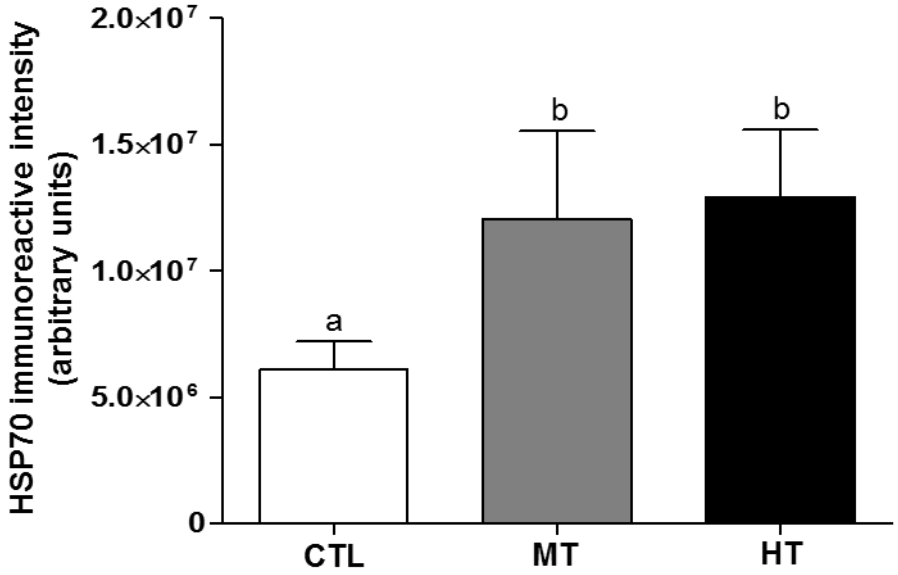

Figure 10. Immunohistochemical analysis of effect of high temperature on heat shock protein 70 (HSP70) levels in the American oyster testis. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity of HSP70 estimated by ImageJ software. Each value represents the mean ± SEM (N = 15-38).

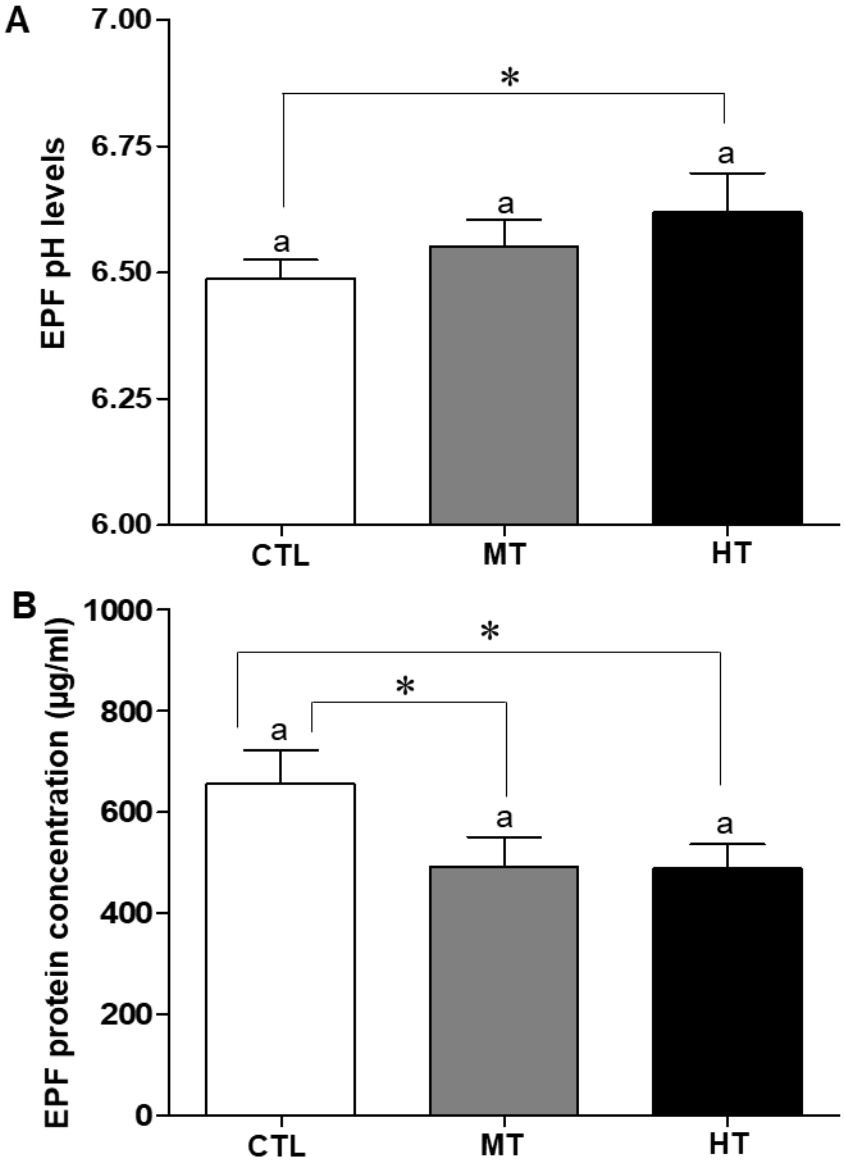

Figure 11. Effects of high temperature on extrapallial fluid (EF) pH and protein concentration in the American oyster. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Asterisk indicates significant difference (Student *t*-test, $*P<0.05$). Results of EF pH and protein concentration from both male and female sexes were combined because they were not significantly different. Each value represents the mean ± SEM (N= 7-29).

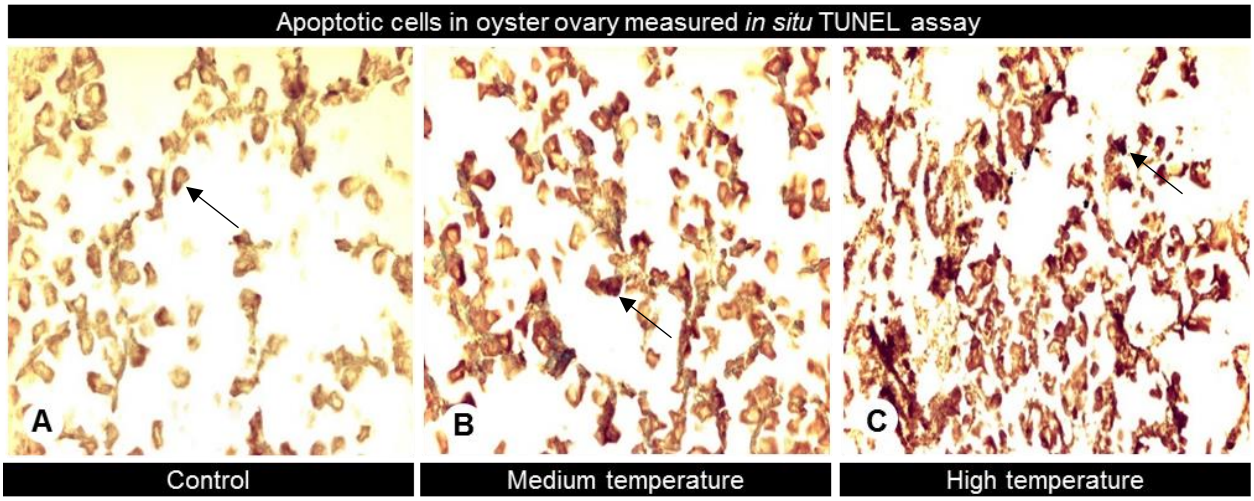

Figure 12. Effects of high temperature on ovarian apoptosis stained with *in situ* TUNEL assay in the American oyster. The presence of apoptotic nuclei shown as brown staining. Control: 24ºC, medium temperature: 28ºC, high temperature : 32ºC. Magnification 10x.

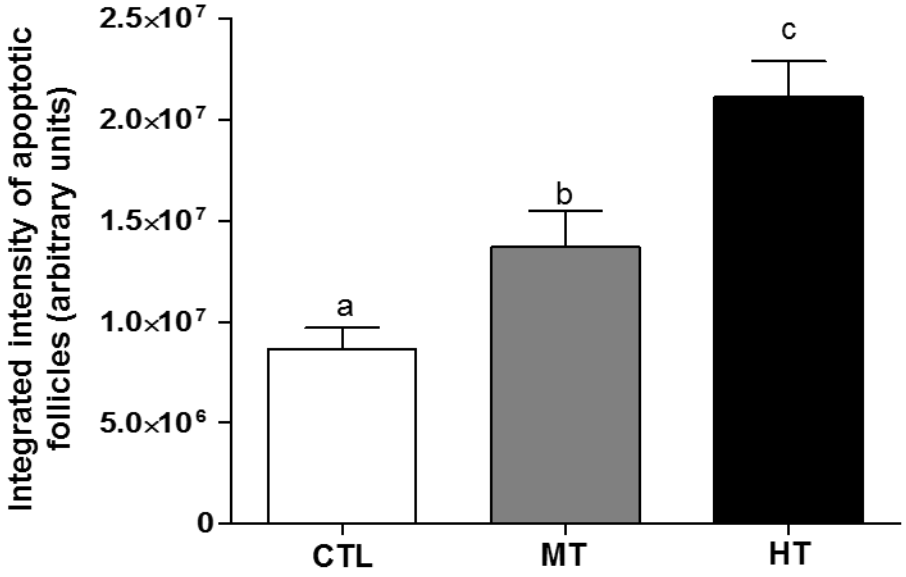

Figure 13. Effects of high temperature on integrated intensity of apoptotic follicles stained with *in situ* TUNEL assay in the American oyster ovary. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Each value represents the mean ± SEM (N = 8-10).

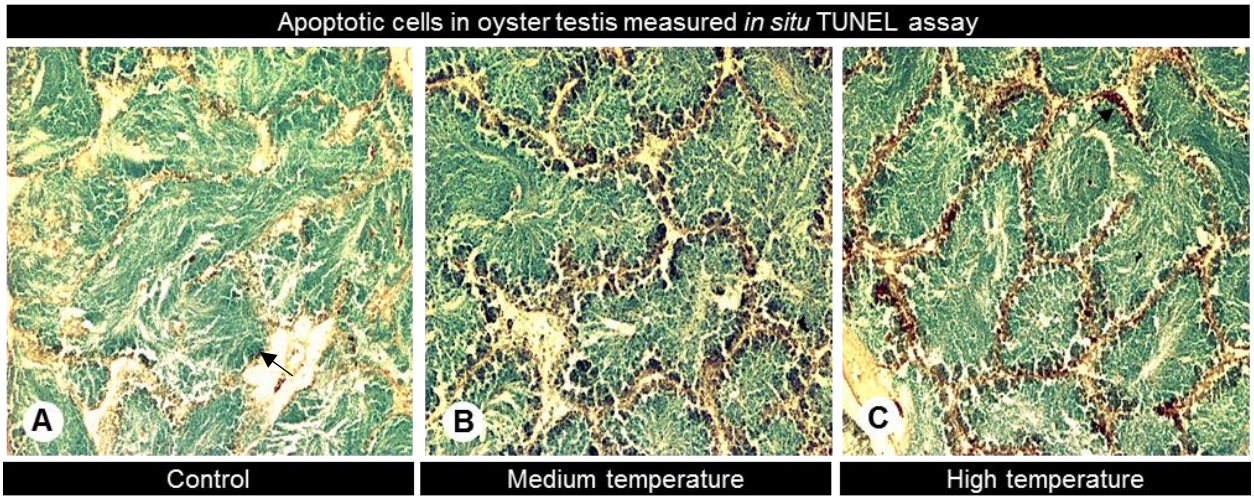

Figure 14. Effects of high temperature on testicular apoptosis stained with *in situ* TUNEL assay in the American oyster. The presence of apoptotic nuclei shown as brown staining. Control: 24ºC, medium temperature: 28ºC, high temperature : 32ºC. Magnification 10x.

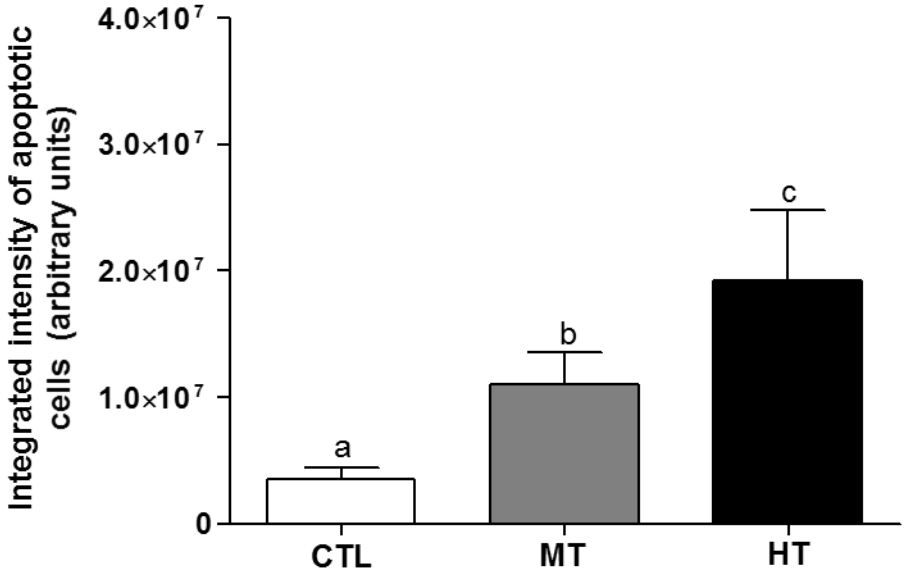

Figure 15. Effects of high temperature on integrated intensity of apoptotic spermatogenic cells stained with *in situ* TUNEL assay in the American oyster testis. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 16-42).

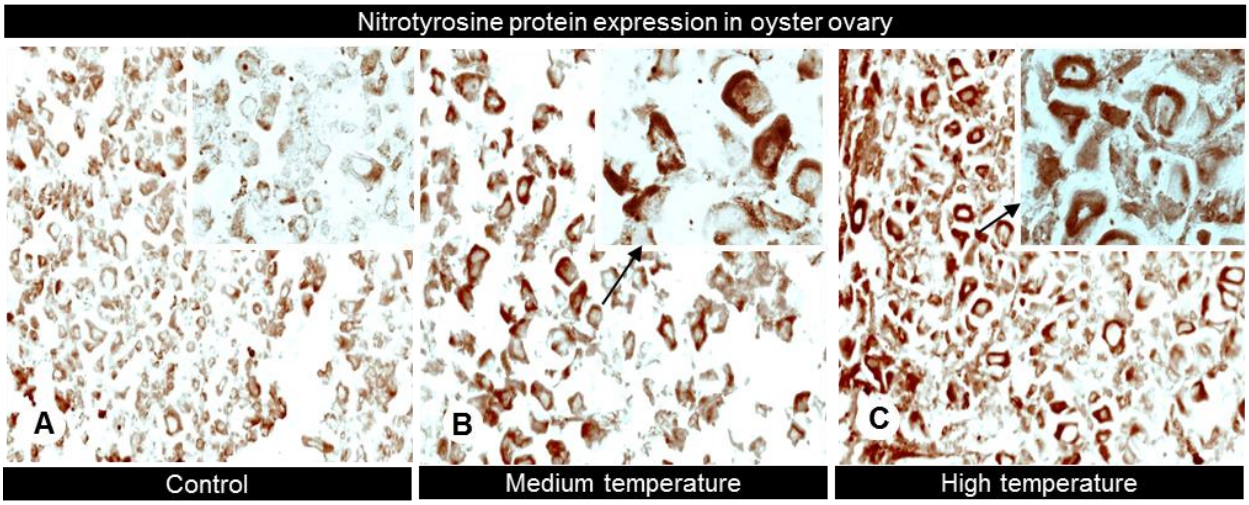

Figure 16. Effects of high temperature on nitrotyrosine protein (NTP, a marker of reactive nitrogen species) expression in ovary of the American oyster. Arrow indicates NTP expression in ova (mature egg). (A) Control : 24ºC; B, medium temperature: 28ºC; and C, high temperature 32ºC. Magnification 10x.

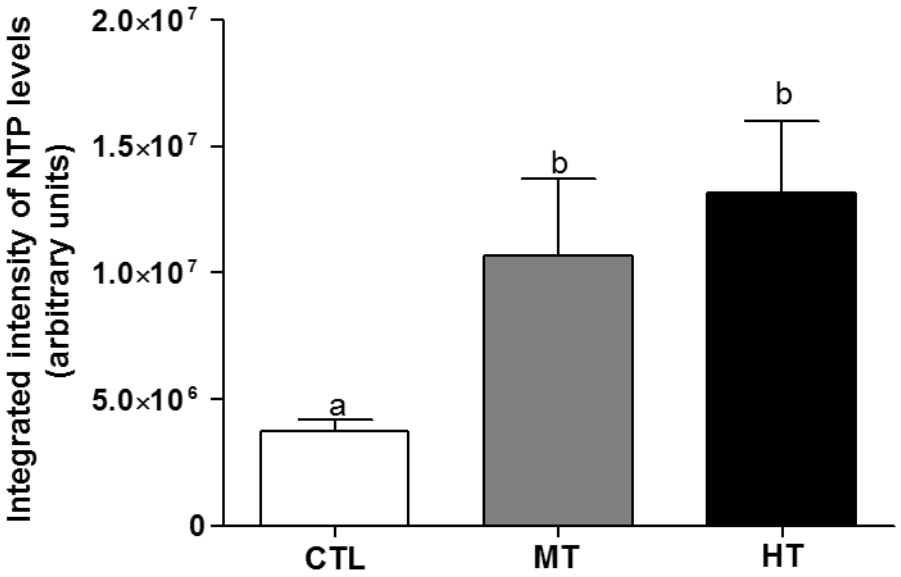

Figure 17. Effects of high temperature on integrated intensity of nitrotyrosine protein (NTP) in the American oyster ovary. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 16-42).

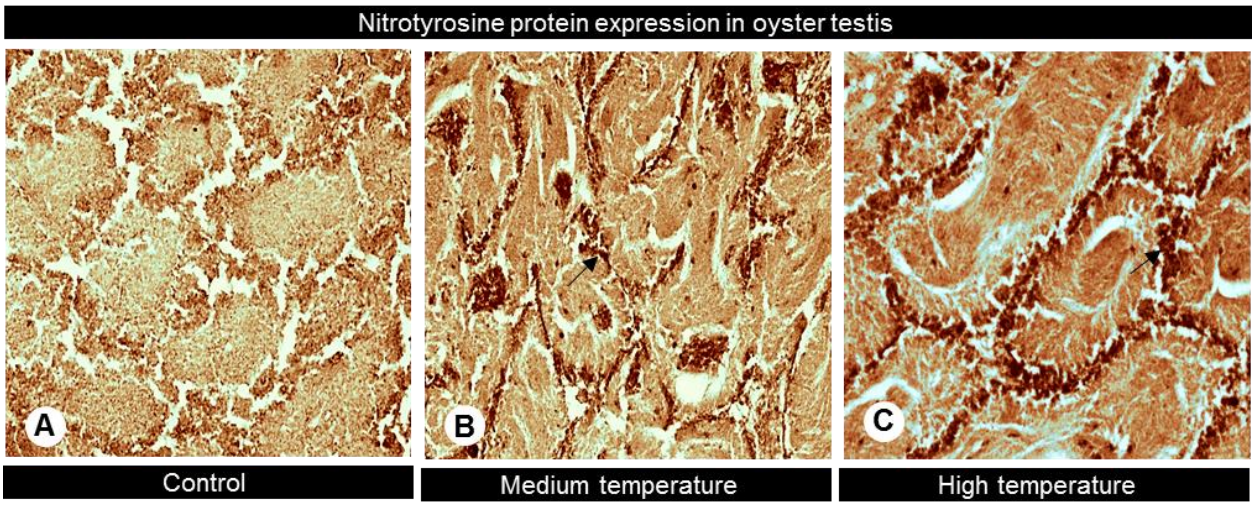

Figure 18. Effects of high temperature on nitrotyrosine protein (NTP, a marker of reactive nitrogen species) expression in testis of the American oyster. Arrow indicates NTP expression in spermatogenic cells. (A) Control : 24ºC; B, medium temperature: 28ºC; and C, high temperature 32ºC. Magnification 10x.

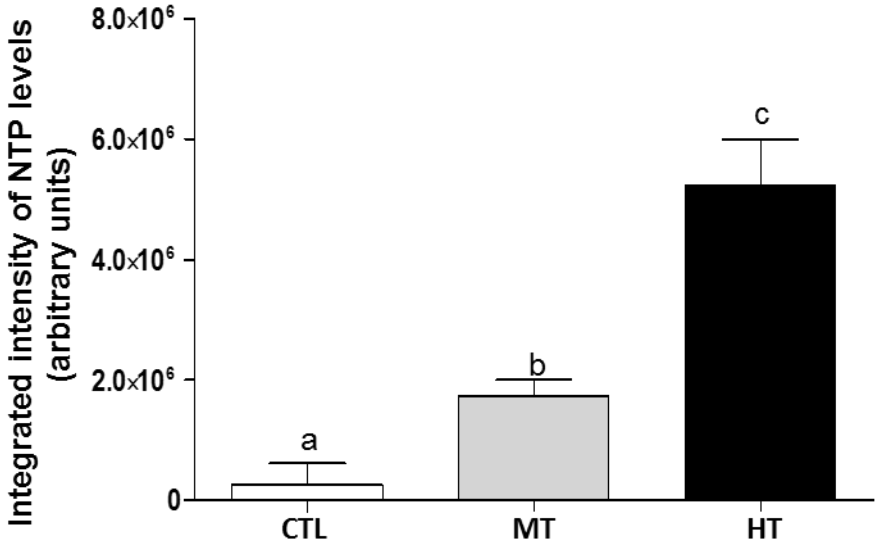

Figure 19. Effects of high temperature on integrated intensity of nitrotyrosine protein (NTP) levels in the American oyster ovary. Control (CTL): 24ºC , medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 38-76).

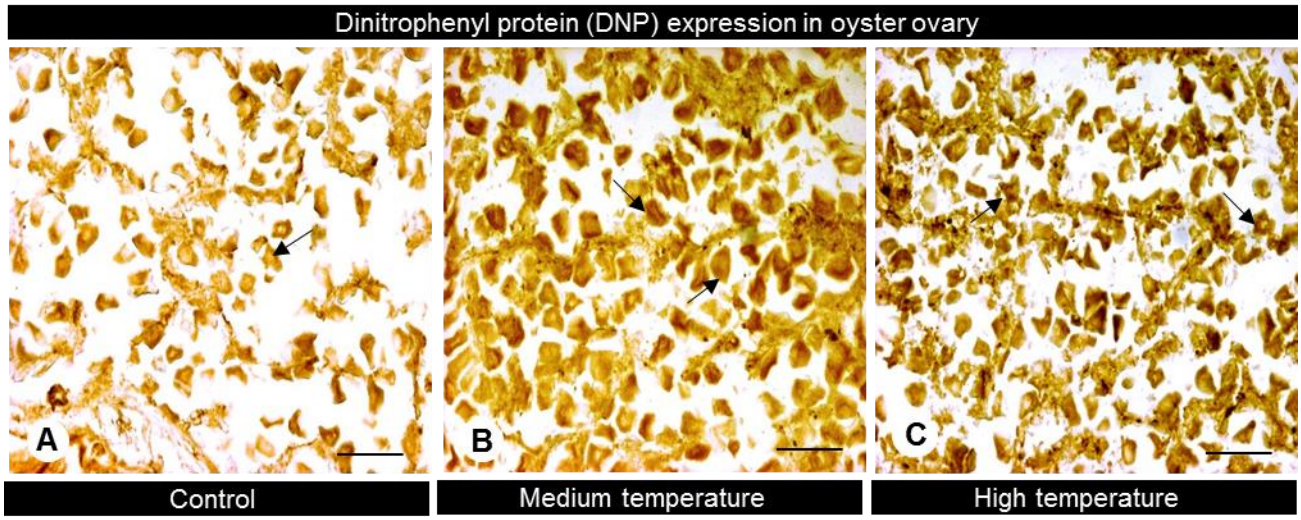

Figure 20. Effects of high temperature on dinitrophenyl (DNP, a marker of protein oxidation) expression in ovary of the American oyster. Arrow indicates DNP expression in eggs. (A) Control: 24ºC; B, medium temperature: 28ºC; and C, high temperature 32ºC. Magnification 10x.

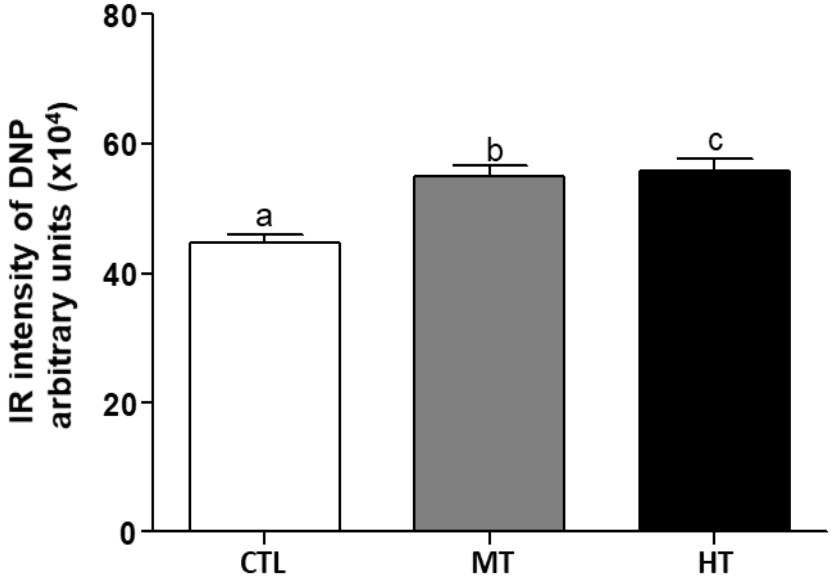

Figure 21. Effects of high temperature on integrated intensity of dinitrophenyl protein (DNP) levels in the American oyster ovary. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 38-76).

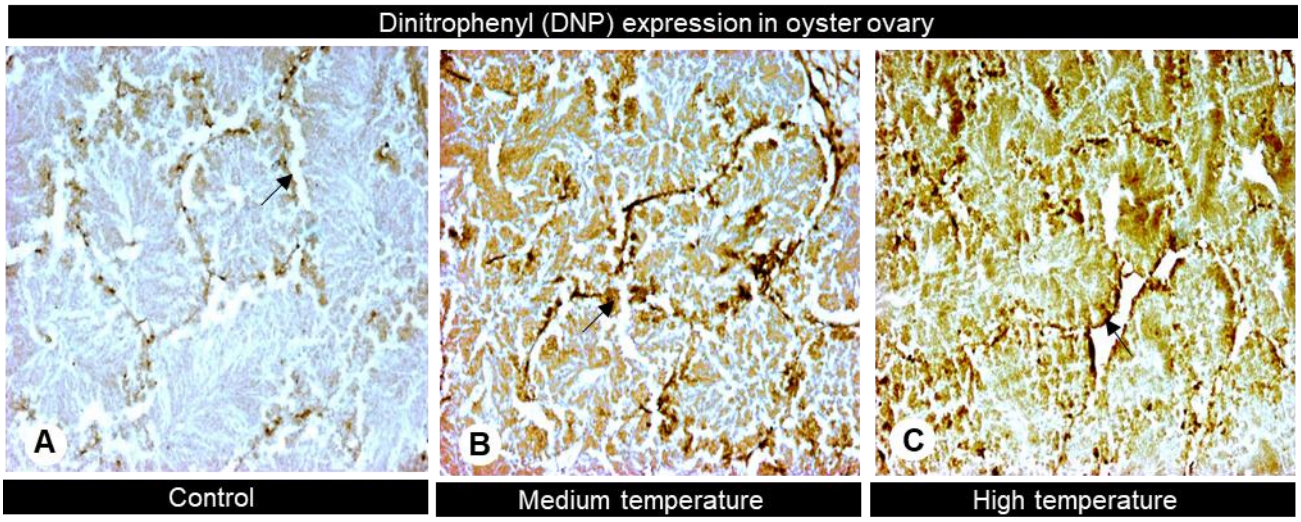

Figure 22. Effects of high temperature on integrated intensity of dinitrophenyl protein (DNP) levels in the American oyster testis. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 38-76).

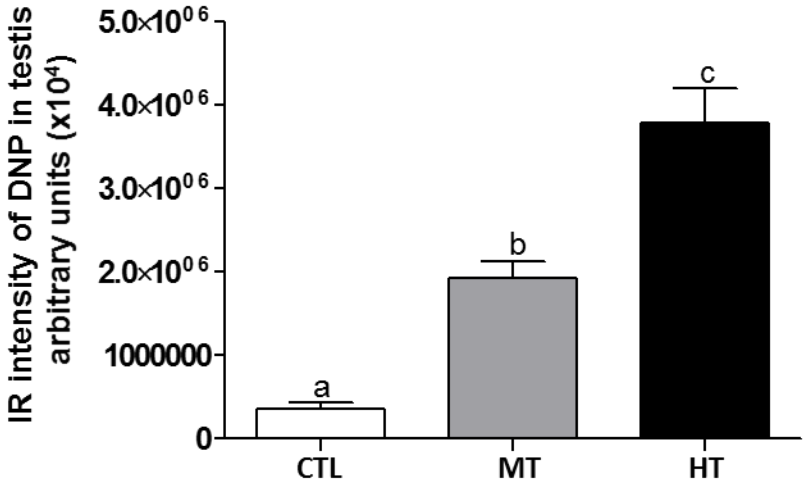

Figure 23. Effects of high temperature on integrated intensity of Dinitrophenol (DNP) levels in the American oyster testis. Control (CTL): 24ºC, medium temperature (MT): 28ºC, high temperature (HT): 32ºC. Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). The integrated staining intensity estimated by ImageJ software. Each value represents the mean ± SEM (N = 48-62).

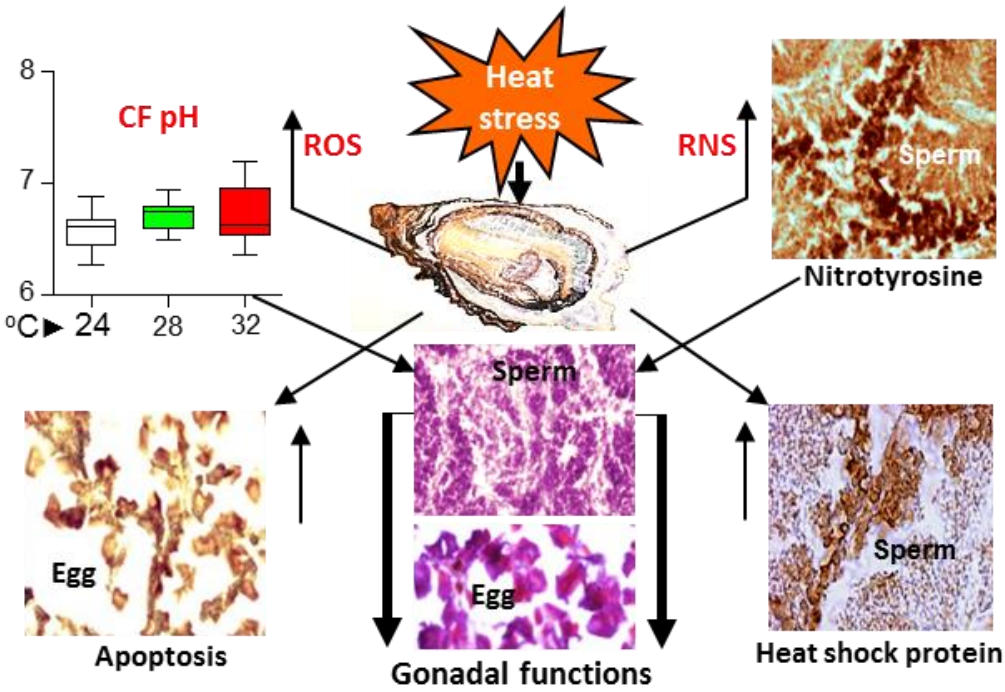

Figure 24. Proposed model of high temperature on reproductive functions in the American oyster. The model shows several pathways through which high temperature could potentially increased cellular apoptosis and oxidative stress (e.g. reactive oxygen species, RNS; and reactive nitrogen species, RNS) and decreased gonad functions.

BIOGRAPHICAL SKETCH

Sarah B. Nash attended the University of Texas Rio Grande Valley and earned a Bachelor's of Science in Environmental Science in December of 2016. During her undergraduate career, Sarah contributed to a range of environmental studies ranging from oceanography to Advanced GIS. After graduating, Sarah began her master's thesis under Dr. Saydur Rahman at the University of Texas Rio Grande Valley in August of 2017. During her graduate career, she was awarded several travel awards to present her research at both national and international conferences including NOAA EPP MSI 9th Biennial Education and Science Forum and PRIMO20. She received her master's degree in of May 2019 in in Ocean, Coastal, and Earth Science. Sarah plans to pursue a Ph.D in August of 2019 in related research.