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# Effects of Elevated Temperature on 8-Hydroxy-2'-Deoxyguanosine Expression, DNA Damage and Cellular Apoptosis in the American Oyster (*Crassostrea virginica*)

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# EFFECTS OF ELEVATED TEMPERATURE ON 8-HYDROXY-2'-DEOXYGUANOSINE EXPRESSION, DNA DAMAGE AND CELLULAR APOPTOSIS IN THE AMERICAN OYSTER (*CRASSOSTREA VIRGINICA*)

A Thesis

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## ABSTRACT

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Global temperature increases due to anthropogenic activities. The effects of rising temperature are well documented in aquatic organisms. The American oyster is an ideal shellfish species to study on global warming and oxidative DNA damage. In this study, we observed the effects of high temperature on heat shock protein-70 (HSP70), 8-hydroxy-2'-deoxyguanosine (8-OHdG), ssDNA, dsDNA, caspase-3 (CASP3), BAX and γH2AX, a molecular marker of DNA damage, protein expressions in gills of oysters. Immunohistochemical results showed that elevated temperatures (28 and 32$^{o}$C) significantly increased HSP70, 8-OHdG, dsDNA, γH2AX, BAX and CASP3 protein expressions in gills of oysters compare to control (24$^{o}$C). *In situ* TUNEL assay showed a significant increase in apoptotic cells in gills. Extrapallial fluid (EPF) glucose level also increased; however, EPF protein concentration decreased in heat exposure oysters. Collectively, these results suggest that heat shock driven oxidative stress induces DNA damage which may lead to decreased physiological functions in oysters.

DEDICATION

To my family and parents for supporting and inspiring me and to my advisor for inspiring me and supervising my work.

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# CHAPTER I

# INTRODUCTION

## Climate Change and Global Warming

Earth is habitable for all living organisms due to its position relative to the sun (Karl and Trenberth, 2003). The solar energy received by the upper atmospheric boundary, only 31% of the energy is reflected by surface of the earth and clouds (Karl and Trenberth, 2003). The rest of the solar energy is absorbed by atmosphere, land, and ocean. This energy then re-radiates back to atmosphere as a form of infrared radiation (Kiehl and Trenberth, 1997). This is called greenhouse effect. Various atmospheric gases, such as water vapor ($H_2O$), carbon dioxide ($CO_2$), ozone ($O_3$), methane ($CH_4$), and nitrous oxide ($N_2O$), seem to responsible to this natural phenomenon (Kiehl and Trenberth, 1997). The global trend of greenhouse gas emission has risen from 0.5 to 1% per year during the past recent decades (Marland et al., 2002). The atmospheric $CO_2$ has increased since the preindustrial period, and there was sudden and recorded increased of atmospheric $CO_2$ from 1950 to date which had never been seen in the preindustrial period (Lu¨thi et al., 2008). These greenhouse gases trap reradiated solar energy coming from earth, and thus, generate a substantially warmer condition (Karl and Trenberth, 2003). In regional level, human activities such as urbanization, agricultural extension, greatly impact the climate (Bonan, 1999; Dolman et al., 2003). Large scale of deforestation and resulting desertification in certain parts of

the world, such as Amazon and Sahel, are the imprints of human influence on regional climate change (Charney, 1975; Hahmann and Dickinson, 1997).

The overall temperature of the earth's atmosphere has increased by 0.8$^{o}$C in the past century and 0.2$^{o}$C per decade in the past 30 years (Hansen et al., 2006). Expanded future temperature projections predict that the earth's average temperature will increase by 4 and ½$^{o}$C by the year 2100 (Morris et al., 2018; Yuan et al., 2018). Much of this increased heat has been absorbed by sea surface resulted warming of ocean top 700 meters water temperature (Levitus et al., 2017). The global sea surface water temperature has increased by 0.7$^{o}$C since the industrial period (Feely et al., 2009). It is predicted to increase by 2-3$^{o}$C at the end of 21$^{st}$ century (Bindoff et al., 2007). In addition, atmospheric temperature increases resulted the change of global climatic conditions. Greenland lost its ice sheet by 54±14 gigatons per year during 1993 to 1994 and 1998 to 1999 which is equivalent to a 0.15 mm sea level rise in a year (Alley et al., 2005). The northern hemisphere has also decreased its winter snow cover over the past few decades (Robinson et al., 2014). Moreover, the global sea level rose in 8 inches in the last century and has increased double during the last two decades and continuously increasing each year (Nerem et al., 2018).

Global temperature increases due to anthropogenic greenhouse emission which also affects marine and coastal environments and their biota (Root et al., 2003). The global temperature rising in sea and oceanic environments, pose more prominent harmful effects when compared to the terrestrial environment (Johnstone et al., 2019). The increased sea surface water temperature has documented effects on growth, reproduction, and development in marine organisms (Chua et al., 2013). For example, corals are very sensitive to high temperature, resulting the formation of coral bleaching which has negative impact on marine ecosystem

(Baird et al., 2009; McClanahan et al., 2009). Temperature driven coral bleaching reduces growth and impairs reproductive functions of corals over large spatial scales (McClanahan et al., 2009). High temperature also leads to decreased gamete (e.g., egg and sperm) production in Atlantic sea urchin, *Arbacia punctulata* (Johnstone et al., 2019). In addition, high temperature (24-27$^{o}$C) increases the oxygen consumption rate and toxicity of metals (e.g., cadmium, copper and lead) in zebra mussels (*Dreissena polymorpha*) (Rao and Khan, 2000) and crayfish (*Orconectes immunis*) (Khan et al., 2006). Moreover, high temperature reduces reproductive functions as well as induces cellular apoptosis and oxidative stress in the gonads of American oyster, *Crassostrea virginica* (Nash and Rahman, 2019; Nash et al., 2019). Therefore, it is envisaged that heat stress directly influences on growth, reproduction and development of marine invertebrates.

## Heat Stress and Heat Shock Proteins

Heat shock proteins (HSPs) are chaperon proteins those help in stabilizing of protein folding by binding with non-native proteins (Kiang and Tsokos, 1998; Aghdassi et al., 2007). HSPs are a superfamily and response to a wide variety of environmental stress such as heat stress, cold shock, hypoxia etc. (Mikami et al., 2004). Several subunits such as HSP20, HSP60, HSP70, HSP90, and HSP110 are the best studied/understood HSPs in terrestrial and aquatic organisms (kiang and Tsokos, 1998). Among them, HSP70 is the most prominent and highly characterized stress protein (Bierkens, 2000; Ahamed et al., 2009). HSP70 is a highly conserved protein and found to be expressed in all organisms (Evans et al., 2010). The elemental form of HSP70 can be found in a trace amount in unstressed cells (Kiang and Tsokos, 1998). In addition, HSP70 protects the body from any environmental stress such as temperature, toxicant, infections

and proliferations (Oberringer et al., 1995). HSP70 can induce wound healing in human endothelial tissue by upregulating the protein (Oberringer et al., 1995). Thus, HSP70 is considered as a potential biomarker in terrestrial and aquatic organisms during environmental stress (Ait-Aissa et al., 2000; Siddique et al., 2008; Ahamed et al., 2009).

## Heat Stress and 8-Hydroxy-2'-Deoxyguanosine Regulation

8-hydroxy-2'-deoxyguanosine (8-OHdG) is the modified base of DNA and is considered as one of the best characterized base modifications (Min, 2005). 8-OHdG is the result of oxidative DNA damage when deoxyguanosine, a component of DNA, is oxidized (Domijan and Peraica, 2008). Out of 20 different oxidative adducts that have been characterized, 8-OHdG is considered as the major product of oxidative DNA damage (Domijan and Peraica, 2008). Elevated amount of 8-OHdG in patients demonstrated characteristic sign of Alzheimer's disease, diabetes, cancers (Inaba et al., 2011). Accumulation of 8-OHdG in DNA may increase the chance of stable mutation occurrence and development of disease state (Howard et al., 1998). 8-OHdG is a type of oxidative damage which is produced in the body either spontaneously or by various factors (Arima et al., 2006). 8-OHdG can be destroyed or metabolized with inflammation (Witherell, 1998). In addition, 8-OHdG can be excreted from the body through urine without any metabolism (Domijan and Peraica, 2008). Moreover, 8-OHdG can be metabolized to purine derivatives such as xanthine, hypoxanthine etc. (Fox, 1981; Witherell et al., 1998). Ren et al. (2011) found 8-OHdG was significantly associated with secondary ambient air pollutants. Huang et al. (2012) have shown that heat stress increases 8-OHdG production in urine of boiler tenders. Recently, Gleason et al. (2017) found that heat stress increases 8-OHdG formation in rocky

intertidal mussel (*Mytilus californianus*, a marine bivalve). Therefore, it is very important to understand the effects of elevated temperature on cellular 8-OHdG regulation in aquatic organisms (Ren et al., 2011).

## Heat Stress and DNA Strand Breaks

High temperature and/or heat shock promotes reactive free radicals (e.g., reactive oxygen species, ROS; and reactive nitrogen species, RNS) and induces DNA damage in cells and tissues in vertebrates (Tuul et al., 2013). Chen et al. (1997) found DNA damage occur after cerebral ischemia and perhaps work through both apoptotic and non-apoptotic pathways. Generally, non-apoptotic or oxidative DNA damage occurs at early stages in neuronal nuclei and requires oxygen-derived free radicals actively act upon DNA (Chen et al., 1997). Single-stranded DNA (ssDNA) originates/arises during DNA replication, recombination, and repair (Marintcheva et al., 2008). Non-apoptotic DNA damage involves single-strand breaks (SSBs) or base modifications (Nguyen et al., 1992). The anti-ssDNA antibody is recognized the short base sequences of ssDNA molecules after double-stranded DNA (dsDNA) molecule being irreversibly denatured and formed randomly coiled compact structure (Yamamoto et al., 1994). The double-strand breaks (DSBs) are biomarkers of apoptotic cell death due to their apoptotic morphology (Wylie, 1980). Notably, anti-dsDNA antibody easily recognizes the double helix structure of DNA molecule and access to its surface polydeoxyribose-phosphate atoms (Yamamoto et al., 1994). Both ssDNA and dsDNA have antigenic properties; those bind with the antibodies usually found in circulation and cells/tissues (Bhagavan, 2002).

## Heat Stress and γH2AX Expression

γH2AX is a subfamily of H2A histone protein family which is central to nucleosome chromatin and DNA structure, and it represents 2-25% components of the H2A complement in vertebrates (Rogakou et al., 1998). High temperature causes the formation of nuclear foci and phosphorylation of histone γH2AX at serine 139 in human HeLa cells (Tuul et al., 2013). γH2AX formation is a quick and highly sensitive cellular response to the radiation during dsDNA breakage indicating a sign of DNA double-strand breaks (Rogakou et al., 1998). Tumminello and Fuller-Espie, (2013) reported that heat stress decreases viability and increases ROS production and phosphorylation of H2AX in coelomocytes of European nightcrawler (*Eisenia hortensis,* a medium-small earthworm). Therefore, γH2AX is a key player in the repair process of DNA damage and is considered a molecular biomarker for DNA break/damage in animals (Kuo and Yang, 2008; Mah et al., 2010; Whittemore et al., 2019).

## Heat Stress, Cellular Apoptosis, BAX, and Caspase-3 Expressions

Apoptosis is considered as programmed cell death, or controlled autodigestion cell death induced by acute cellular impairment (Thompson, 1995). However, incorrect apoptosis induces a range of human diseases including cancer, AIDS, neurodegenerative diseases (Thompson, 1995). Activation of apoptosis in cells and tissues involves two mechanisms; intrinsic or mitochondria mediated and extrinsic or death receptor-mediated pathways (Kepp et al., 2007). Scaffidi et al. (1998) found that mitochondrial pathway mediates the receptor-mediated mechanism on certain cells. Recently, Sleadd et al. (2014) found that sub-lethal heat stress induces apoptosis in common Antarctic fish (also called emerald rock cod, *Trematomus bernacchii*). In response to

various environmental stimuli, mitochondria releases cytochrome *c* and Smac/DIABLO (pro-apoptogenic factors) results the activation of cellular apoptosis and caspase activity in cells/tissues (Kepp et al., 2007). Caspases are a large family (e.g., caspase 2, -3, -4, -5, -6, -7, -8, -9, -10 etc.) of cysteine proteases and are considered as key mediators of apoptotic pathway (Nicholas and Thornberry, 1997). Among them, caspase-3 (CASP3) plays a key role in apoptotic DNA fragmentation (Wolf et al., 1999). CASP3 is also able to regulate non-apoptotic function in many cells including keratinocytes and lens epithelium (Ishizakai et al., 1998; Weil et al., 1999). In addition to CASP3, the Bcl-2 family proteins such as Bcl-2-associate X (BAX), also play a key role in regulating cellular apoptosis at the level of mitochondrial cytochrome *c* release (Danial and Korsmeyer, 2004). BAX become multimerization and affect the permeabilization of mitochondrial outer membrane (Korsmeyer et al., 2000). Mitochondrial outer membrane permeabilization mediates the release of pro-apoptotic proteins (cytochrome c, Smac/DIABLO) during apoptosis (Kepp et al., 2007). Kepp et al. (2007) found cytochrome *c* is released even in the absence of BAX during Ngo- and cisplatin-induced apoptosis. In addition to this, cytochrome *c* release itself cannot drive the cell towards apoptosis may require the release of other proapoptotic factors from the mitochondria (Kepp et al., 2007). Therefore, BAX protein is considered as a pro-apoptotic protein member under Bcl-2 family and induce mitochondria mediated apoptosis (Green and Reed, 1998; Gross et al., 1999; Wei et al., 2001). BAX gene is also considered as a proto-oncogene and has reported to perform a key role in cellular apoptosis (MacGibbon et al., 1997).

## Heat Stress and Marine Mollusks

Elevated temperature due to seasonal temperature fluctuations and/or long-term effects of global climate change challenges marine ectothermic/poikilothermic organisms (e.g., fish, mollusks etc.). Rising sea water temperature may even pose additional threats to their survival due to their susceptibility to hypoxia, pollution, and pathogen under extreme temperature conditions (Walther et al., 2002). The effects of high temperature and a combination of cadmium like pollutants were previously studied in marine bivalve mollusks such as oyster (Sokolova, 2004; Sokolova et al., 2004; Sokolova et al., 2005; Lannig et al., 2006). Moreover, few studies have addressed the effects of high temperature on reproductive functions in oysters (Nash and Rahman, 2019; Nash et al., 2019). Currently there is no available information on elevated temperature on cellular oxidative DNA damage in marine bivalve mollusks such as clams, mussels, scallops, oysters, etc.

American oyster (*Crassostrea virginica*) is an important marine shellfish species and found along the Atlantic and Gulf coasts of North America (MacKenzie et al., 1997). American oyster is an ectotherm (i.e., body temperature rises and falls along with the surrounding environmental temperature), inhabits in shallow coastal waters and estuaries, and is frequently exposed to variety of environmental stressors such as hypoxia (low dissolved oxygen, DO <2.0 mg/L), pollution, ocean acidification, temperature fluctuations etc. (Lannig et al., 2006). American oyster is an intertidal species and found to survive extreme temperature condition (thermal tolerance) during tidal fluctuations (Shumway, 1996; Nash et al., 2019). Extreme temperature condition force them to use their stored energy to compensate with biochemical demands, ultimately decrease their survival rate as well as increases mortality during the post spawning period (Li et al., 2007; Nash et al., 2019).

American oyster is a suspension feeder that maintains suspended minute particles on its gills (Haven and Morales-Almo, 1970). Oysters effectively retain particle size down to a few microns and 80% of which are easily passed through the gills (Jorgensen and Goldberg, 1953). Particles of this size may play a crucial role in oyster nutrition (Haven and Morales-Almo, 1970). Most of these particles are phytoplankton (e.g., nano and ultra-plankton) which play a crucial role in nutrition for oysters (Haven and Morales-Almo, 1970). The suspension and filter feeding activities let them to change the assemblage of phytoplankton population in an embayment (Newell, 1988; Dame et al., 1986), since densely populated suspension feeder has proved to be significantly important by improving basin-wide water quality and maintaining phytoplankton dynamics (Cloern, 1982; Cohen et al, 1984; Fréchette et al, 1989; Dame, 1996).

Oyster is a bivalve mollusk and filter feeder that filtrates around 50 gallons of water daily (Riisgard, 1988). Oyster gill comprises numerous demi branches of tissue in the mantle cavity and each demi branch divided into arms or lamellae which again divided into an outer ascending lamella and an inner descending lamella. Each gill is composed of two joined demi branch and each demi branch is composed of two marginally joined lamellae. Both lamellae are attached to each other by means of interlamellar junctions which is made of tissue with hemolymph vessels (Eble and Scro, 1996). Each lamella is made of several vertical filaments those arranged in a regular fashion of vertical folds called plicae and each filament is composed of simple epithelial tissue with cilia in frontal and lateral cells containing cilia (Eble and Scro, 1996). Each cell of epithelial tissue composed of microvilli containing mucous droplets and a minute filamentous glycocalyx or cell coat which is attached with the external surface of microvilli covering microvilli (Eble and Scro, 1996). Each filament also contains a pair of skeletal rods of fibrous protein and usually found beneath the epithelial tissue in frontal and lateral region (Brown 1952;

Le-Pennec et al., 1988). Each gill is attached with the open end of the oyster body called gill base and pointed end of each V is called gill margin (Eble and Scro, 1996; Galtsoff, 1964). Oyster gills along with the mantle play vital role in filtration for food (Galtsoff, 1964).

## Study Objectives

The aim of this study is to test the hypothesis that extreme seawater temperature causes oxidative damage in gills of American oyster. In addition, high temperature may induce the overproduction of reactive free radicals (i.e., ROS, RNS) that causes DNA stand breaks, consequently, increase the expression γH2AX, BAX, and CASP3, which may promote 8-OHdG regulation in gills of American oyster. The main goals of our research are:

(i) to determine the effects of elevated temperature on HSP70 expression in gills of American oyster,

(ii) to determine the effects of elevated temperature on 8-OHdG expression, DNA strand breaks and γH2AX expression in gills of oysters,

(iii) to determine the effects of elevated temperature on cellular apoptosis, BAX and CASP3 expressions in gills of oyster, and,

(iv) to determine the effects of elevated temperature on extrapallial fluid compositions in oysters.

# CHAPTER II

# MATERIALS AND METHODS

## Collection of Oysters

American oysters (*Crassostrea virginica*, average age: ~2-4 years) were randomly collected from oyster bed in the bay site of South Padre Island (geographical location: 26º04'30"N, 97º09'59"W) in Brownsville, Texas. Various physio-chemical parameters such as water temperature, pH, dissolved oxygen levels were measured using a YSI probe (YSI Professional Plus Water Quality Meters, Harrisburg, PA, USA) during the collection of oysters. Oysters were then quickly transported with areated seawater to laboratory in the University of Texas at Rio Grande Valley in Brownsville campus. The University of Texas at Rio Grande Valley Institutional Animal Care and Use Committee does not require any animal care and handling protocol for aquatic and/or terrestrial invertebrates. All oysters, however, were cared according to the Guide for Care and Use Animals for research in the National Research Council Committee of United States (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf).

## Laboratory Heat Exposure Experiment

For laboratory heat exposure experiment, oysters were randomly selected and placed in six glass aquaria (10 oysters/aquarium; capacity: 20 gallons; Tetra, Blacksburg, VI, USA) with recirculating seawater. Oysters were then acclimatized for 5 days under control laboratory conditions. A total of six portable water heaters (Top Fin, Franklin, WI, USA) were placed in the aquaria to maintain in control temperature condition beginning with room temperature (20-22$^{o}$C) around 4-5 days before entering actual experimental phase. Temperatures were then gradually increased (~1$^{o}$C/day) from 22 to 24$^{o}$C as control (2 aquaria), 28$^{o}$C as medium temperature (2 aquaria) and 32$^{o}$C as high temperature (2 aquaria). Physio-chemical parameters such as temperature, pH, and dissolved oxygen (DO) level (pH: ~6.62, average DO: ~5.46) were recorded with a YSI probe three times daily (morning, afternoon, and evening) during the experimental period. Oysters were fed frozen marine cuisine (San Francisco Bay Brand, San Francisco, CA, USA) once every alternative day during the experimental period. After a week of heat exposure, 60 oysters were collected (10 oysters/aquarium) for analysis. The experimental temperatures and exposure period of oysters used in this study were based on mortality, survivability and previous publication (Lowe et al., 2017; Casas et al., 2018; Jones et al., 2019; Nash and Rahman, 2019; Nash et al., 2019).

## Sample Collection and Fixation

Oysters were cracked open by using protective gloves and oyster knife (Academy, Brownsville, TX, USA). The body length and width were measured by using a centimeter scale and recorded as a unit of gram (average shell size:12 ~9 cm length, ~4 cm width, and ~5-6 g

meat weight) by using a digital balance (Carolina Biological Supply Company, Burlington, NC, USA). Extrapallial fluid was collected and pipetted in 1.5 ml Eppendorf tube. A portable pH meter (Oakton, California, USA) was used to obtain highly accurate pH data of extrapallial fluid. Extrapallial fluid glucose level was measured using a glucometer (Angel Holm, Sweden). For histological and immunohistochemical analyses, gill samples were collected and placed on embedding plastic cassette (Fisher Scientific, Hampton, NH, USA), and kept in a polyethylene plastic container with 4% paraformaldehyde solution (Acros Organics, Morris, NJ, USA) for 6-7 days at 4°C. For molecular and/or biochemical analysis, gill samples were collected and placed in 1.5 ml RNase-DNase free tube and stored in -80$^{o}$C for analysis.

## Embedding and Dehydration of Tissue Samples

After a week of fixation in paraformaldehyde solution, gill samples were taken out from the refrigerator and dehydrated with series of ethyl dilutions (50, 75, 95, and 100% 2X) at least 30 min for each. Tissue samples were immediately cleared by common clearing agent, xylene (Fisher Scientific) for 30 min each (3 times). Samples were then incubated with a mixture of xylene and melted paraffin (1:1) overnight. Following this, samples were infiltrated with paraffin (1 h each for 3 times) and finally embedded in liquid paraffin (Paraplast Plus, Fisher Scientific) using embedding cassette. The paraffin embedded tissue blocks were sectioned at 7 µm using a rotary microtome machine (Leica, Wetzlar, Germany) and transferred into warm water bath. Tissue sections were then scooped up on a positively charged glass slide (Superfrost Plus, Thermo Fisher, Waltham, MA, USA). Slides were then properly labelled and incubated on a slide warmer (35-40°C, at 24 h) to dry and remove extra water.

## Histological Staining of Tissue Samples

For histological analysis, paraffin embedded tissue slides were deparaffinized in xylene (3 times, 4 min each). Slides were then rehydrated with series of ethanol dilutions (100% 2X, 95, 75, and 50%) and incubated with hematoxylin stain (Sigma-Aldrich, St. Louis, MO, USA) for 3-5 min. Following this, the slides were washed with deionized (DI) water until their deep blue color disappeared. The hematoxylin is a basic stain that bind with the tissue nuclei and gives blue coloration (Chan, 2014). Slides were stained with eosin solution (Sigma-Aldrich) for 30 min. Eosin is an acidic and orange/pink colored dye that binds with proteins in the cytoplasm and in connective tissue (Chan, 2014). Slides were then washed and dehydrated through an increasing concentration of alcohol baths (50, 75, 95, and 100% 2X). Slides were cleared in xylene and mounted by using mounting medium (Cytoseal XYL, Richard-Allan Scientific, MI, USA) and cover slip (Fisher Scientific). Slides were then kept dry at room temperature for 30 min prior to microscopic observation. Histological pictures were taken by a photometrics Cool-SNAP camera (Nikon Eclipse E600, Nikon, Japan) using a light microscope (Photometrics, Tucson, AZ, USA).

## Immunohistochemical Analysis

For immunohistochemical analysis, gill tissue sections from the same blocks were deparaffinized in xylene (3X, 4 min each). The slides were dehydrated with series of ethanol dilutions (100, 95, 75, and 50%), and washed with 1X phosphate buffer saline (PBS) solution (Fisher Scientific) for 3 times (10 min each). Slides were then incubated with 1% bovine serum albumin (BSA, Fisher Scientific) blocking solution for 1 h at room temperature to avoid non-specific binding. Following this, slides were washed again with 1X PBS solution and incubated

with different primary antibodies (diluted 1:100 with 1X PBS solution); mouse anti-HSP70 (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-8OHdG (Japan Institute for the Control of Aging, Shizuoka, Japan), mouse anti-ssDNA (Millipore Sigma, St. Louise, MO, USA), mouse anti-dsDNA (Millipore Sigma, St. Louise, MO, USA), rabbit anti-caspase-3 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-BAX (Cell Signaling Technology, Danvers, MA, USA) or rabbit anti-γH2AX (Novus Biologicals, Centennial, CO, USA) at 4$^{o}$C for 48 h. The negative tissue slides were incubated with 1X PBS instead of primary antibody. Slides (including negative control) were then washed with 1X PBS solution (3X, 10 min each). After washing, tissue slides were incubated with either anti-mouse (Cell signaling, Boston, MA, USA) or anti-rabbit secondary antibodies (Southern Biotech, Birmingham, AL, USA) (diluted 1:100 with 1X PBS solution) for 1 h at room temperature. Slides were washed again with 1X PBS solution for 3 times (10 minutes each). Then, 3,3,'-diaminobenzidine (DAB) peroxidase substrate (diluted 1 drop substrate with 1000 ml with DAB solution) (Vector Laboratories Inc., Burlingame, CA, USA) was added according to manufacturer's guidelines and incubated for appropriate time until the color development to detect the immunoreactivity of HSP70, 8-OHdG, ssDNA, dsDNA, caspase-3, BAX and γH2AX proteins in tissue sections. Afterwards, slides were washed with DI water for 5 min to prevent the formation of deep background. Slides were then dehydrated in another series of ethanol dilutions, cleared in xylene and mounted with Cytoseal. The immunoreactive signals of HSP-70, 8-OHdG, ssDNA, dsDNA, γH2AX, BAX, and Caspase-3 were captured by a Cool-SNAP camera (Photometrics, Tucson, AZ, USA) using a compound light microscope (Nikon Eclipse E600, Nikon, Japan). The integrated density of protein expression was measured using ImageJ software described by Schneider et al. (2012).

## *In-situ* TUNEL Assay

Apoptotic cells in gills were detected using terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End labeling (TUNEL) detection kit (Dead End Colorimetric TUNEL system, catalog no. G7361, Promega) according to manufacturer's protocol. Briefly, paraffin-embedded gills were sectioned, deparaffinized with xylene, rehydrated with a series of ethanol dilutions, washed with 0.85% NaCl at room temperature for 5 min, and then rinsed with PBS. Slides were then incubated with protease K (20 mg/ml) for 15 min at room temperature and then rinsed with PBS. 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 mix (equilibrium buffer: biotinylated nucleotide mix: rTdT enzyme, 98:1:1) in a humidified chamber at 37$^{o}$C for 1 h. Slides were then rinsed in PBS and incubated with SSC buffer for 15 min at room temperature, rinsed in PBS, incubated with streptavidin HRP solution (1:500 in PBS), and stained with DAB substrate for 5 min. Sections were then rinsed in DI water, incubated with 1% methylene green for 5 min, dehydrated with series of ethanol dilutions, sequenced with xylene, and mounted with Cytoseal under glass coverslip.

## Extrapallial Fluid Glucose Level

Extrapallial fluid (body fluid) glucose level was measured using HemoCue Glucose 201 analyzer (Angel Holm, Sweden) and glucose microcuvette according to the manufacturer's protocol. Briefly, a drop of extrapallial fluid from the experimental sample was taken and put-on aluminum foil paper. A drop of extrapallial fluid was then loaded into microcuvette. The microcuvette were placed in glucose meter and the data was recorded.

## Extrapallial Fluid Protein Concentration

Extrapallial fluid protein concentration was measured using the technique developed by Bradford (1976). Briefly, 5 µl of extrapallial fluid was pipetted into a 5 ml protein assay solution (Bio-Rad, Hercules, CA, USA) and incubated at room temperature for 5 min. A Nanodrop (Thermo Scientific, Waltham, MA, USA) was used for protein measurement. A standard curve was likewise made of BSA solution (0, 62.5, 125, 250, 500 and 1,000 µg/ml) and absorbance was read at 595 nm. The absorbances (standard vs. samples) were plotted and the protein concentration was determined and expressed as µg/ml.

## Statistical Analysis

All of the statistical analyses were performed using GraphPad Prism software (GraphPad San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were initiated in all experimental data. Student's *t*-test was also performed to compare the unpaired means. The mean ± standard error of the mean (SEM) was incorporated to express in all data. A $P$ value $<0.05$ was considered statistically significant.

# CHAPTER III

# RESULTS

## Effects of Elevated Temperature on Gill Morphology

Oyster gill plays an important role in gaseous exchange (Khondee et al., 2016). The effects of rising temperature on histological architecture in gills of oyster was carefully examined. Histological observations showed that elevated temperature markedly modulated the gill structure (i.e., gill lamellae, hemocyte infiltration, gill epithelium, mucocyte, mucus formation etc.) in oysters (Fig. 3A-C). The number of branchial lesions increased in medium and high temperatures (28 and 32$^{o}$C) compared to controls (24$^{o}$C). Since mucus acts as a natural barrier against environmental stresses (Espionosa et al., 2016), the effects of high temperature on mucocyte number were also monitored in gills of oysters. The number of mucocyte increased in gill filaments at 28 and 32$^{o}$C treatments compared to controls (Fig. 3B, C), although mucocyte number reduced in gills at 32$^{o}$C treatment group compared to 28$^{o}$C. High temperature also caused extensive mucus formation in gills of treatment groups compared to controls. The hemocyte infiltration within the intracellular space of gill filament was not prominent in experimental samples (Fig. 3A-C).

## Effects of Elevated Temperature on Heat Shock Protein-70 Expression

A key tool in detecting stress tolerance is the monitoring and characterization of heat shock proteins (HSPs) (Clark et al., 2008). Under heat stress, HSP70 increases expression and binds with other proteins/heat shock factors, which is maintained to resume regular cellular processes/functions (Williams et al., 1993). Therefore, we examined the effects of elevated temperature on HSP70 expression in gills of oysters by immunohistochemical (IHC) technique using HSP70 antibody and quantified the expression levels by measuring immunoreactive (IR) activity using ImageJ software. Oyster exposed to high temperatures showed an increased amount of HSP70 expression in gills compared to controls (Fig. 4A-C). HSP70 expression appeared to be high in medium (28$^{o}$C) and high temperature (32$^{o}$C) groups compared to controls (Fig. 4B, C). No IR signal was detected in gill tissues of negative controls (Fig. 4D).

The measured integrated optical density (OD) using ImageJ software demonstrated that HSP70 expression increased IR activity markedly in both medium and high temperature groups (Fig. 5). HSP70 expression increased around 1.3-fold ($P < 0.05$, OD: 0.96 ± 0.006) in medium and ~1.4-fold ($P < 0.05$, OD: 1.04 ± 0.008) in high temperature group compared to controls (OD: 0.75 ± 0.007), which indicates the synthesis of a higher amount of HSP70 expression in response to rising seawater temperature.

## Effects of Elevated Temperature on 8-Hydroxy-2'-Deoxyguanosine Expression

8-hydroxy-2'-deoxyguanosine **(**8-OHdG) is a base modification and considered a biomarker of oxidative stress and DNA damage in vertebrates (Arima, 2006). An increased amount of 8-OHdG is a sign of aging, and exposure to environmental toxicants (Inaba, 2011).

The effects of heat stress on 8-OHdG expression in gills of oyster was measured by IHC technique using 8-OHdG antibody. IHC results showed there was an increased amount of 8-OHdG expression in gills from different experimental groups (Fig. 6A-C). 8-OHdG expression appeared to be denser in both medium (28$^{o}$C) and high temperature (32$^{o}$C) groups compared to controls (Fig. 6B, C). No IR signal of 8-OHdG was observed in negative controls (Fig. 6D).

The IR intensity of 8-OHdG expression was obtained through ImageJ software. There was a significant ($P$<0.05) increase of 8-OHdG expression in both medium and high temperature groups compared to controls (Fig. 7). The IR intensity of 8-OHdG increased~1.2 fold (OD: 1.11 ± 0.008) and ~1.13-fold (OD: 1.09 ± 0.008) in medium and high temperature exposure groups, respectively, compared to control group (OD: 0.96 ± 0.006) indicating more base modifications in response to heat stress.

**Effects of Elevated Temperature on Single-Stranded DNA (ssDNA) Expression**

Single-stranded DNA (ssDNA) is an early biomarker of non-apoptotic DNA damage or single strand breaks of DNA (SSBs) (Nguyen et al., 1992; Chen et al. 1997). The effects of high temperature on ssDNA expression were measured and detected in oyster gills using IHC technique and antibody against DNA lesions, anti-ssDNA antibody. Oyster exposed to high temperature showed increased the amount of ssDNA expression in gills from different experimental groups (Fig. 8A-C). A dense immunoreactive (IR) signal was found in both medium (28$^{o}$C) and high temperature (32$^{o}$C) groups compared to control (Fig. 8B, C). There was an absence of ssDNA IR signal in negative control slides (Fig. 8D).

The integrated OD of ssDNA was also incremented in both medium and high temperature groups compared to controls (Fig. 9). The IR intensity of ssDNA increased ~1.07-fold ($P < 0.05$, OD: 0.81 ± 0.008) in medium temperature, and slightly decreased ($P < 0.05$, OD: 0.75 ± 0.007) in high temperature compared to control group (OD: 0.76 ± 0. 005), indicating more DNA strand breaks in response to high temperature.

**Effects of Elevated Temperature on Double-Stranded DNA (dsDNA) Expression**

Double-stranded DNA (dsDNA) is a biomarker of apoptotic cell death and found to occur at a later stage of DNA damage; double strand breaks (DSBs) (Wylie, 1980). The effects of high temperature on DNA double-strand breaks were monitored in gills using IHC technique and antibody against DNA double-strand breaks, anti-dsDNA antibody. Oyster exposed to high temperature showed an increase of dsDNA expression in gills at different experimental groups. A dense IR signal of dsDNA was found in both medium (28$^{o}$C) and high temperature (32$^{o}$C) groups compared to control group (Fig. 10B, C). However, negative control slides demonstrated no immunoreactivity of dsDNA (Fig. 10D).

The measured integrated OD of dsDNA from ImageJ analysis showed there was a marked increase of dsDNA IR intensity in both medium and high temperature groups compared to controls (Fig. 11). The IR intensity of dsDNA increased ~1.32-fold ($P < 0.05$, OD: 1.14 ± 0.007) in medium temperature and ~1.26-fold ($P < 0.05$, OD: 1.09 ± 0.008) in high temperature compared to control group (OD: 1.09 ± 0.006).

## Effects of Elevated Temperature on γH2AX Expression

γH2AX is a DNA repair protein (Nikitaki et al., 2015), a biomarker of apoptosis and DNA damage (Rogakou et al., 1998). Thus, in order to quantify the density of γH2AX expression in gills, oyster exposed to high temperature were examined their gill tissues using IHC technique and antibody raised against the phosphorylated form of H2AX, γH2AX. This phosphorylated form of H2AX is also an indicator of DNA double-strand breaks (DSBs) during DNA damage. Oyster exposed to high temperature showed an increase in γH2AX expression in gills of experimental groups (Fig. 12A-C). γH2AX expression appeared to be stronger in both medium (28$^{o}$C) and high temperature (32$^{o}$C) groups (Fig. 12B, C) compared to controls. The absence of γH2AX IR was found in negative control slides (Fig. 12D).

The integrated OD of γH2AX increased ~1.2 fold ($P < 0.05$, OD: 0.71 ± 0.009) in medium temperature and ~1.32-fold ($P < 0.05$, OD: 0.8 ± 0.007) in high temperature compared to control group (OD: 0.8 ± 0.007) (Fig. 13).

## Effects of Elevated Temperature on BAX Expression

BAX is a biomarker of apoptosis and plays a major role in mitochondria-mediated apoptosis, thus, affecting the mitochondrial outer membrane (Wei et al., 2001). The effects of heat stress on BAX immunoreactivity in oyster gills were measured by IHC technique using anti-BAX antibody. Oyster exposed to high temperature showed an increase in BAX expression in gills from experimental group (Fig. 14A-C). BAX IR signal appeared to be stronger in both

medium (28$^{o}$C) and high temperature (32$^{o}$C) groups compared to controls. No IR signal of BAX was observed in negative control slides (Fig. 14D).

The measured integrated OD of BAX increased in medium and high temperature groups (Fig. 15). The IR intensity of BAX increased ~1.53-fold ($P < 0.05$, OD: 0.63 ± 0.008) in medium temperature, and ~1.7-fold ($P < 0.05$, OD: 0.7 ± 0.005) in high temperature groups compared to controls (OD: 0.41 ± 0.002), indicating an increased rate of apoptosis with high temperature exposure.

## Effects of Elevated Temperature on CASP3 Expression

CASP3 is a biomarker of DNA damage since it acts as a key mediator and master enzyme during apoptotic DNA fragmentation (Nicholson and Thornberry, 1997). Thus, the effects of high temperature on CASP3 expression were monitored in oyster gills by IHC technique using caspase-3 antibody. Oyster exposed to high temperature showed an increase in CASP3 expression in gills of experimental group (Fig. 16A-C). CASP3 IR signal appeared to be stronger in high temperature (32$^{o}$C) group (Fig. 16C). No IR signal of caspase-3 was found in negative control slides (Fig. 16D).

The integrated OD of CASP3 after ImageJ analysis showed there was a marked increase of IR activity in both medium and high temperature groups (Fig. 17). The CASP3 IR intensity increased ~1.22-fold ($P < 0.05$, OD: 0.85 ± 0.008) in medium temperature and increased ~1.03-fold ($P < 0.05$, OD: 0.72 ± 0.007) in high temperature compared to control group (OD: 0.7 ± 0.005), indicating a higher rate of apoptosis activity in gills exposed to high temperature.

## Effects of Elevated Temperature on Cellular Apoptosis

Apoptosis is a natural process of cell death for development and growth in aquatic organisms including shellfish species (Gervais et al., 2015; Nash et al., 2019). Apoptotic cells in oyster gills were measured by *in situ* TUNEL assay using colorimetric staining. Oyster experienced of elevated temperature (32$^{o}$C) showed increased number of apoptotic cells in gills compared to control (Fig. 18A-C).

ImageJ analysis showed that there was a significant increase in integrated density of apoptotic cell. The OD of apoptotic cell increased ~1.5-fold ($P < 0.05$, OD: 0.64 ± 0.006) in and ~1.6-fold ($P < 0.05$, OD: 0.7 ± 0.005) in medium and high temperature respectively compared to control (OD: 0.44 ± 0.004) groups (Fig. 19).

## Effects of Elevated Temperature on Extrapallial Fluid Compositions

Extrapallial fluid (EPF) is important as it maintains osmoregulatory processes, body temperature, and various physiological functions in oysters (Allam and Paillard, 1998; Nash et al., 2019). The effects of high temperature on the biochemical composition of oyster EPF like proteins, carbohydrates (glucose) were measured. Oyster exposed to high temperature significantly increased EPF glucose level compared to controls (CTL 15.2±1.08, MT 27.92 ± 0.88, and HT 28.92 ±1.63 mg/L, ANOVA, $P<0.05$; Fig. 20).

Contrarily, EPF protein tended to decrease in medium and high temperature treated oyster compared to control (CTL 1.93 ±0.27, MT 0.8 ± 0.13, and HT 1.4±0.11mg/ml, ANOVA, $P<0.05$; Fig. 21).

# CHAPTER IV

# DISCUSSION

Although heat stress is one of the most well-studied stress factors, very little is known about its effects on DNA strand breaks in aquatic invertebrates, such as shellfish species (Kantidze et al., 2016; Gleason et al., 2017). Gill is one of the most sensitive organs and thus is considered an ideal to study the environmental stress responses in bivalves (Meistertzheim et al., 2007). Gill has a larger surface area compare to other organs as it directly exposed to the surrounding environment (Menicke et al., 2014). In this study, we demonstrated that rising seawater temperatures (28 and 32$^{o}$C) affect the morphology of gills, increase the expression of heat-stress chaperon biomarker (i.e., HSP70) and oxidative DNA damage indicators (i.e., 8-OHdG, γH2AX, ssDNA and dsDNA) as well as increase cellular apoptosis in gills of oysters. Interestingly, the rate of BAX and CASP3 expressions also significantly increased and correlated with higher rate of apoptotic cells in gills, which distinctly indicate DNA damage in cells and/or tissues of oysters. We also demonstrated that elevated temperature changes the compositions of EPF in oysters. Together, our results imply that rising seawater temperature drastically induces oxidative DNA damage and cellular apoptosis, and body fluid conditions in oysters.

## Effects of Elevated Temperature on Morphology of Gills

In mollusks, gill tissue plays an important role in gaseous exchange (Khondee et al., 2016) as well as in internal defense (Piano et al., 2005). In this study, we have shown that elevated temperatures (28 and 32$^{o}$C) increased the number of mucocyte and mucus concentration in gill filaments which may affect respiratory system in oysters. Kennedy and Mihursky (1972) showed that elevated temperature (30$^{o}$C) drastically decreased the respiratory metabolism in soft-shell clam, *Mya arenaria* and dwarf surf clam, *Mulinaia lateralis*. Mucus in gills plays a key role in defense against physical, chemical, and biological invasions from direct exposure to surrounding water in aquatic organisms (i.e., fish, shellfish etc.; Espionosa et al., 2016). Several bioactive molecules such as HSPs, CASPs, etc. that embedded in their matrix facilitate the process by counteracting diverse stressors (Espionosa et al., 2016). The increased mucus secretion in gills or other pallial organs (e.g., mantle, connective tissue, etc.) may offset the defense mechanism as well as reduce the respiratory metabolism during and after the stress which may compromise the gill function. In the present study, the histological assessment revealed increased branchial lesions in gills of oysters exposed to high temperatures. Similar branchial lesions were also observed in gills of flat oyster, *Ostrea edulis* after 25$^{o}$C heat stress (Da Silva et al., 2006). These findings together our results suggest that high temperature changes the structural integrity as well as physiological functions of gills and/or other organs in marine bivalve mollusks.

## Effects of Elevated Temperature on HSP70 Expression

HSP70 plays a central role in folding/unfolding peptides and protecting cells/tissues during environmental stress (Liu and Chen, 2013). The present study showed that short-term (1 week) heat exposure (28 and 32$^{o}$C) induced HSP70 protein expression in gills of oysters. Tomanek and Zuzow (2010) investigated HSP70 isoforms; Hsp70 (11), Hsp70 (14), Hsp70 (15), Hsp70 (16), and Hsp70 cognate Hsc70 (12) in gills of Mediterranean blue mussel, *Mytilus galloprovincialis,* after 1 h of heat stress. They reported that Hsp70 (12) was higher at 24$^{o}$C, and Hsp70 (11) was higher at 28$^{o}$C, and Hsp70 (14), Hsp70 (15), and Hsp70 (16) isoforms were higher at 32$^{o}$C in gills of blue mussel. Ivanina et al. (2009) found that HSP69, an inducible isoform of the HSP70 family, was significantly elevated in gills of American oyuster in high temperatures (20, 24, 28, and 32$^{o}$C). Kefaloyianni et al. (2015) reported that hyperthermia (30$^{o}$C) induces HSP70 protein level (4.21 ± 0.50-fold) in gills of blue mussel, *M. galloprovincialis*. Zhu et al. (2016) demonstrated that HSP70 mRNA was dramatically increased in gills of pacific oyster, *C. gigas,* after 1 d of 35$^{o}$C heat stress. Similarly, acute heat stress (37$^{o}$C for 6 h exposure) induced increase HSP70 mRNA levels in gills of Hong Kong oyster, *C. hongkongensis* (Zhang and Zhang, 2012). In another study, HSP70 mRNA was markedly induced in gills of Hong Kong oyster after 1 h of 37$^{o}$C heat stress (Li et al., 2017). Additionally, Liu and Chen (2013) found that high temperature induced the expression of HSP69 isoform mRNA in gills of flat oyster. Li et al. (2019) also found HSP70 mRNA was markedly increased in liver of juvenile blunt snout bream fish, *Megalobrama amblycephala* after 3 d of 35$^{o}$C stress. Similarly, HSP70 mRNA was significantly increased in liver of juvenile turbot, *Scophthalmus maximus* after 6 h of 27$^{o}$C (Jia et al., 2020). HSP70 mRNA was significantly increased in different tissues of Ya-fish, *Schizothorax prenanti* after 25$^{o}$C heat stress (Peng et al., 2018). Artigaud et al., (2015) reported

HSP70 significantly increased in tissues of king scallop, *Pecten maximus* after 56 d of 25$^{o}$C heat stress. HSP70 mRNA was upregulated in gills of manila clam, *Ruditapes philippinarum* after 6 h of 30$^{o}$C heat stress (Menike et al., 2014). These results suggest that high temperature increases in HSP70 protein and mRNA expressions in different tissues/organs in fish and shellfish species. Since HSP70 is a bioactive molecule (Liu and Chen, 2013), increased HSP70 may provide extra support to organs/tissues in order to prevent greater stress.

## Effects of Elevated Temperature on 8-OHdG Expression

8-OHdG is one of the most prevalent oxidative DNA damages that occur in a wide range of organisms, including aquatic invertebrates and mammals (Li et al., 2005). The hydroxyl radical ($HO^{\bullet}$), is one of the most important oxygen-free radical that attacks and damages DNA strands (Valavanidis et al., 2009). The interaction of this hydroxyl radical with the nucleobase-guanine results in the formation of C8-hydroxyguanine or 8-OHdG (Valavanidis et al., 2009). In this study, we have shown that elevated temperature (28 and 32$^{o}$C for 1week exposure) induced increase expression of 8-OHdG in the gills of oysters. Similarly, high temperature (35.75$^{o}$C) for ~3 weeks caused an increase in 8-OHdG expression in gills of California mussel, *Mytilus. californianus* (Gleason et al., 2017). In addition to heat stress, the expression of 8-OHdG was reported in marine mollusks after exposure to environmental toxicants (Steinert, 1999). Canova et al. (1998) observed a significant increase of 8-OHdG in gills of Mediterranean blue mussel, *M. galloprovincialis* after expose to benzo[a] pyrene. In addition, Torres et al. (2002) detected higher levels of 8-OHdG in digestive glands of mangrove mussel, *Mytella guyanensis,* from a polluted mangrove area contaminated with trace metals (i.e., Hg, Pb, Cr, and Cd). However,

Akcha et al. (2000) observed contamination does not make any difference in the expression levels of 8-OHdG in gills of blue mussel, *M. galloprovincialis.* In contrary, Mallins and Haimanot (1991) found a significant increase in 8-OHdG levels in liver of English sole fish, *Parophrys vetulus*, collected from polluted area. Collectively, these results in conjunction with the present study suggest that elevated temperature as well as environmental pollutant induces oxidative DNA damage in fish and shellfish species. It is also envisaged that DNA damages in organism may not occur solely from a single stressor and could be linked to other factors.

## Effects of Elevated Temperature on DNA Strand Breaks

Heat shock acts as an agent for DNA damage and inhibits the DNA excision repair system; base excision repair and nucleotide excision repair (Kantidze et al., 2016). DNA single-strand breaks (SSBs) occur through the inhibition of DNA topoisomerase I (top1), which introduces temporary SSBs into DNA (Velichko et al., 2015). In this study we have shown that ssDNA protein expression increased in medium temperature ~1.2 fold, and ~1.9 fold in the high temperature group indicating faster rate of DNA single-strand damage with the increasing temperarture. Similarly, high temperature (32$^{o}$C for 30 min exposure) induced significant increase of ssDNA in hemocytes of California mussel, *M. californianus* (Yao and Somero, 2012). High temperatures (28 and 32$^{o}$C for 30 min to 2 h or 8 h) also caused a marked increase of ssDNA in the Mediterranean blue mussel (Yao and Somero, 2012). SSBs are the most frequent type of DNA damage and are often repaired without any mistake (Wallace, 1994).

Double strand breakage of DNA (DSBs) form through the inhibition of DNA topoisomerase II (top2), introducing temporary DSB fragments into DNA (Nitiss, 2009). Present

study showed that dsDNA increased ~1.7- fold at 28$^{o}$C and ~1.4- fold at 32$^{o}$C after 1-week exposure in gills of oysters. Similarly, 32$^{o}$C heat stress for 8 h induced a significant increase in DSBs in hemocytes of California mussel (Yao and Somero, 2012). High temperature (30$^{o}$C) also induced (2.18 ± 0.02-fold increase) p38 MAPK phosphorylation (a biomarker of DNA damage) within 15 min of heat stress in the mantle tissues of Mediterranean blue mussel (Kefaloyianni et al., 2005). DNA double strand damages correlated with DNA inhibition of both replication and transcription processes (Velichko et al., 2015). Together, our data suggest it is highly plausible that DSBs are less frequent, and if they occur, are non-repairable, may induce cellular apoptosis and thus pose a greater threat to cellular integrity during heat stress (Ori et al., 2004).

## Effects of Elevated Temperature on γ-H2AX Expression

γ-H2AX is a biomarker of DSBs which is induced by UV-radiation, drug, and chemical exposure, and endogenous DNA processes (Mah et al., 2010). DSBs lead to phosphorylation of histone, H2AX, a variant of the H2A protein family (a component of the histone octamer in nucleosomes) (Kuo and Yang, 2008). The newly phosphorylated proteins, γ-H2AX foci, subsequently recruit and localize DNA repair proteins, some of which are directly attached to γ-H2AX, while others are associated with the binding of proteins (Kuo and Yang, 2008). Current study showed a significant increase in γ-H2AX protein expression, concomitant with elevated temperature (28 and 32$^{o}$C), indicating more double strand breaks in oyster gills due to heat stress. Gonzalez-Romero et al. (2017) demonstrated that γ-H2AX protein expressed in gills of American oyster after exposure to red tides on the Florida coast. The higher expression of γ-H2AX in oyster gills in the present study, in accordance with previous other studies suggest that

high temperature as well as other environmental stressors act as precursor of DNA damages which subsequently localize and recruit other proteins.

## Effects of Elevated Temperature on Cellular Apoptosis

Apoptotic cell death process is a way of defense and adapted by organisms against any environmental stresses as they eliminate dead or infected cells (Ophelie et al., 2015). In this study, we have shown that high temperature (28 and 32$^{o}$C) induced apoptotic cells in gills of oysters. Recently, Johnstone et al. (2019) found that high temperature (32$^{o}$C) increased the apoptotic cells by ~3-fold in testicular tissues and by ~1.4-fold in ova of Atlantic sea urchin, *Arabacia punctulate*. Cherkasov et al. (2007) reported that elevated temperature (28$^{o}$C) significantly increases the apoptosis in hemocytes of American oyster. Similarly, Yang et al. (2017) noticed that apoptosis rate was markedly increased in hemocytes of pacific oyster, *C. gigas* after 24 h of 25$^{o}$C heat stress. Luo et al. (2019) showed that in rainbow trout, *Oncorhynchus mykiss*, apoptosis rate was markedly increased at 25$^{o}$C heat stress. Apoptosis may be considered as an integral aspect in the development and homeostasis process in organisms (Ophelie et al., 2015). The increased apoptotic cell in the current study together with previous studies demonstrate that heat stress leads to increased apoptotic activity which may induce DNA damages in oyster and other aquatic organisms.

## Effects of Elevated Temperature on BAX Expression

BAX is a proapoptotic protein that is positioned in the mitochondrial outer membrane and induces to release cyt *c* into the cytosol (Jia et al., 2020). BAX is also widely considered as

an apoptosis regulator protein (Zhao et al., 2012). In this study, we have shown that 1 week of heat stress (28 and 32$^{o}$C) resulted an increase in BAX protein expression in oyster. Similarly, Jia et al. (2020) reported that BAX mRNA was significantly increased in liver of juvenile turbot, *Scophthalmus maximus* after 6 h of 27$^{o}$C stress. Also, Cheng et al., (2015) reported that BAX mRNA was markedly increased in blood cell of pufferfish, *Takifugu obscurus* after 3 h of 34$^{o}$C stress. It has been reported that rising temperature temperature (from 20$^{o}$C to 37$^{o}$C) induces the activation of BAX in BL21 (DE3) RIPL cells (Bleicken et al., 2010). Luo et al., (2016) observed that higher temperature (40-43$^{o}$C) increases BAX mRNA levels in cultivated granulosa cells of mice. It has also been shown that heat stress (43$^{o}$C) induces cytochrome *c* release in mouse liver from mitochondrial outer membrane, thus indicating the role of BAX in cellular apoptosis (Pagliari et al., 2005). Recently, Gu et al. (2015) demonstrated that heat stress (43$^{o}$C for 2 h) increases the amount of BAX accumulation in HUVEC cell *in vitro*. During cellular apoptosis, BAX transfers to mitochondrial membrane and accelerates its ion voltage channel to improve the membrane permeability (Luo et al., 2016). Thus, cytochrome *c* released from the mitochondria through ion channels combines with apoptotic protease-activating factor, leading to active CASP9 and CASP3 (Luo et al., 2016). These results suggest that high temperature acts as an environmental stressor that induces BAX expression and cause apoptosis in cells/tissues.

## Effects of Elevated Temperature on CASP3 Expression

CASP3 is a key enzyme in signaling pathways of apoptosis and executes cellular apoptosis (Zhao et al., 2012; Luo et al., 2016). In this study, we have shown a significant increment of CASP3 protein expression in gills of oysters after 28 and 32$^{o}$C stress (Fig. 15).

Similarly, CASP3 activity increased in gills of Mediterranean blue mussel, *M. galloprovincialis* after acute stress of 30ºC (Kefaloyianni et al., 2005). Li et al. (2016) demonstrated that CgCasp 3/7 (similar to CASP3) mRNA increased by 2.5- and 4.1-fold in gills of pacific oyster, *C. gigas* after 2 and 6 h of 27ºC stress, respectively. Yang et al. (2017) noticed CASP3 mRNA level was increased in hemocytes of pacific oyster, *C. gigas* after 25ºC stress in a time dependent manner. CASP3 was increased in gills of unionid mussel, *Unio tumidus* after 2 weeks of 25ºC stress (Falfushynska et al., 2018). Similarly, CASP3 was observed in gills of smooth clams, *Callista chione* and Antarctic scallop, *Adamussium colbecki* without refereeing temperature stress (Motta et al., 2013). However, Wang et al. (2018) found heat stress (25 and 35ºC) did not induced CASP3 mRNA in gills of green mussel, *Perna viridis.* It has been reported that CASP3 mRNA expression, CASP3 activity was significantly increased in hemocytes of white shrimp (*Litopenaeus vannamei*) after 22ºC for 2 d and 7 d stress. Jia et al. (2020) noticed that CASP3 mRNA level was significantly increased in liver of juvenile turbot, *Scophthalmus maximus* after 6 h of 27ºC stress. Cheng et al. (2015) reported that CASP3 mRNA was markedly increased in blood cell of pufferfish, *Takifugu obscurus* after 3 h of 34ºC stress. CASP3 mRNA levels was significantly increased in liver of pufferfish, *T. obscurus* after 1 h of 34 and 37ºC stress (Cheng et al., 2018). CASP3 activity was elevated in testis of sub-adult pejerrey, *Odontesthes bonariensis* after 12 h of 31ºC stress and 36 h of 29ºC stress (Ito et al., 2003). Li et al. (2019) found CASP3 mRNA levels was significantly increased in liver of juvenile blunt snout bream, *Megalobrama amblycephala* after 3 d of 35ºC stress. CASP3 is considered a major effector caspase when activated by initiator caspases (caspase-2,8,9 and 10), cleaves other protein substrates to generate apoptosis (Cohen, 1997; Fisher et al., 2003; Sakamaki and Satau, 2009). In addition, Iijima and Yokoyama (2007) reported that CASP3 dominated area was correlated to

higher rate of apoptosis. These results suggest that high temperature activates CASP3 in tissues/organs which triggers cellular injury or tissue damages in oysters as well as other aquatic organisms.

## Effects of Elevated Temperature on FPF Compositions

EPF is an important body fluid that is located in between the external epithelium of the mantle, so called pseudo-internal compartments, and the inner face of the bivalve shell (Allam and Paillard, 1998). EPF shows functional similarity to the hemolymphs of lower invertebrates, and vertebrate blood (Nash et al., 2019). EPF contains proteins, glycoproteins, amino acids, and carbohydrates, lipids, etc. (Yin et al., 2005). Since glucose is widely used as environmental stress indicator in fish (Gandar et al., 2017), we measured the glucose level in our study. We have shown that elevated temperature (28 and 32$^{o}$C) of 1 week increased the EPF glucose level in oysters. Similarly, glucose level in blood serum was significantly increased after 6 h of 27$^{o}$C stress of juvenile turbot, *Scophthalmus maximus* (Jia et al., 2020). Glucose is considered as vital fuel that fluctuates to meet the extra energy required in organism (Jia et al., 2020).

The present study also demonstrated that elevated temperature (28$^{o}$C) of 1 week markedly decrease EPF protein concentration in oysters. Recently, Nash et al. (2019) showed that EPF protein concentration significantly decreased in oyster exposed to heat stress. Similarly, EPF protein concentration was markedly decreased after 28 and 32$^{o}$C heat stress in Atlantic sea urchin (Johnstone et al., 2019). Since the EPF protein binds to $Ca^{2+}$ and may act as a precursor of the soluble organic matrix of the shell (Yin et al., 2005), a reduction of total protein concentration in body fluid, thus preventing accumulation, may inhibit the growth and development of shell in oysters and other aquatic organisms. Importantly, the biomolecular

components of oyster EPF are involved in shell formation (Misogianes and Chasten., 1979), defense, and regulatory processes in marine bivalves (Allam and Paillard, 1998). Therefore, it is envisaged to say that an increase or decrease in any of its components may shift major physiological functions in oysters and other closely related organism.

## Conclusion

Our finding shows that higher temperature causes detrimental changes in gill morphology, body fluid compositions, and increases cellular apoptosis and DNA-damage biomarkers. The present study provides, to the best of our knowledge, the first conclusive evidence and potential signaling pathways of oxidative DNA damage by rising seawater temperature in oysters. Since American oyster habitats show a wide fluctuation of temperature (from 4-5$^{o}$C during winter to 30-31$^{o}$C in summer) (Cherkasov et al., 2007), it represents an ideal model species to monitor thermal impacts in the Gulf of Mexico and the Atlantic coast. The effects of elevated temperature on oxidative DNA damages are limited and have only been studied in a few species, the rocky mussels (Gleason et al., 2017) and in the present study, the American oyster. Since, oyster is an important marine bivalve species, both ecologically and economically, it is vital to preserve this species in its proper habitat. The study of heat stress on American oyster contributes to the body of knowledge on the effects of global warming among multiple scales, ranging from the molecular level to tissue, and organ levels. Future studies may explore the DNA damages in other organs/tissues, and the prospects of DNA repairs in particular organs/tissues to identify potential genes which involve the potential repair mechanisms in oysters as well as other marine bivalve mollusks.

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# APPENDIX

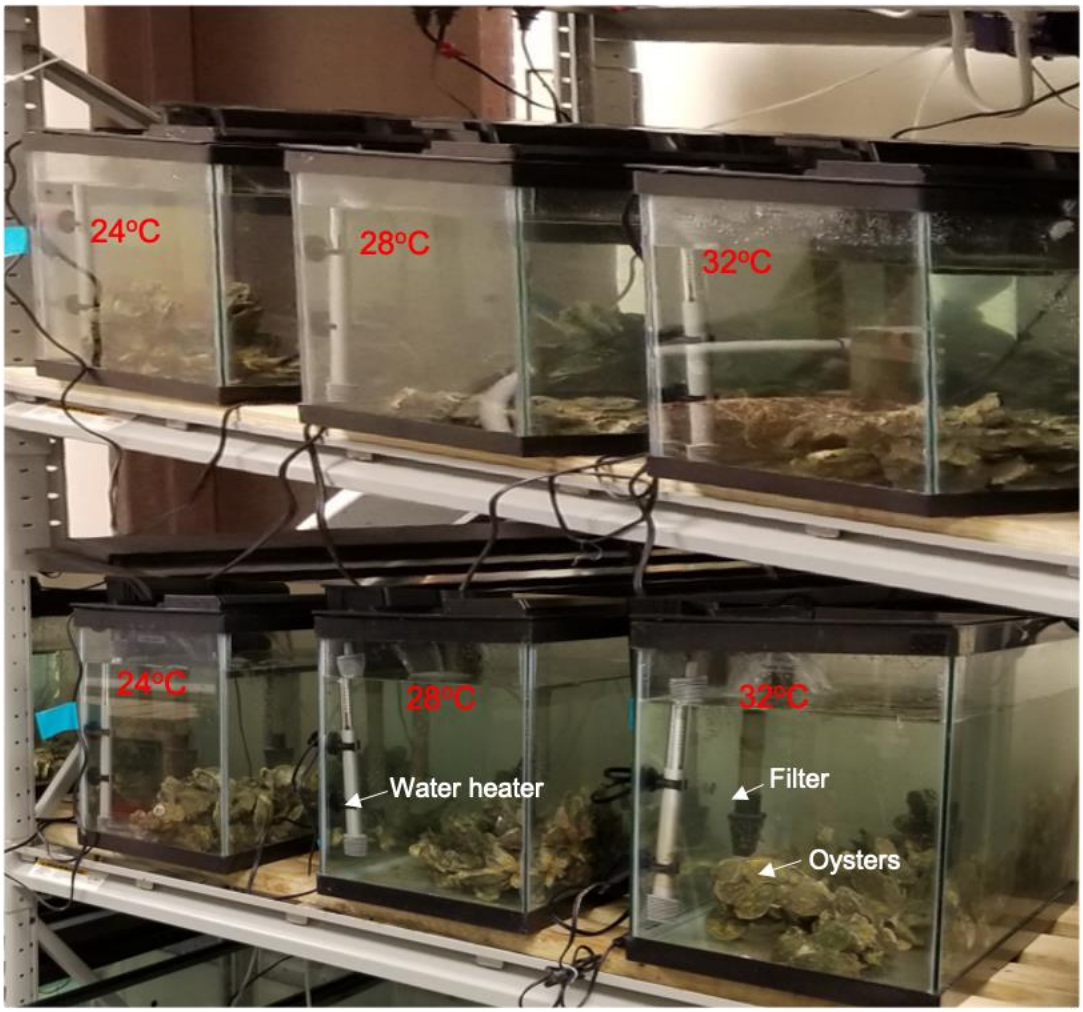


Figure 1. Laboratory experimental set up in six glass aquaria (capacity: 20 gallons) of three different temperature groups (24, 28, and 32°C) in recirculating seawater. Arrows indicate American oysters, filter and water heater.

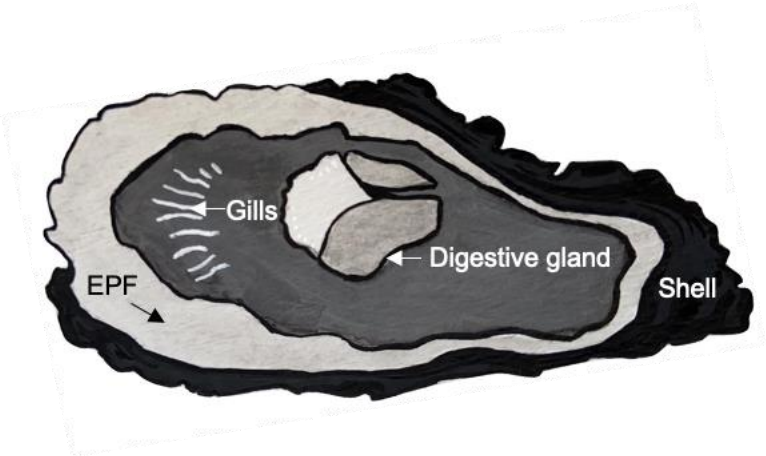


Figure 2. Anatomy of American oyster (*Crassostrea virginica*) showing internal organs (gills, digestive glands etc.) along with external shell. EPF, extrapallial fluid.

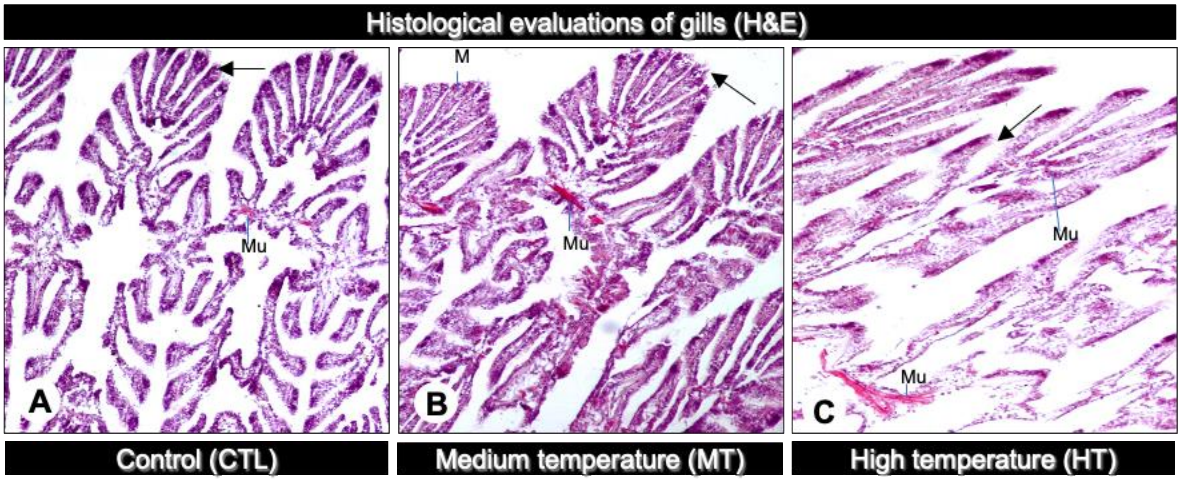


Figure 3. Oyster gill morphology after a week of exposure to 24, 28 and 32°C temperature. The gill tissue of control temperature (24°C) show less branchial lesions and mucus formation. Oyster exposed to elevated temperature (28 and 32°C) showed increasing mucocyte number and mucus formation in gill tissue in a temperature dependent manner. M, mucocyte; Mu, mucus; black arrow heads, branchial lesions. Sections were 7um thick and stained with H&E staining. (A) control temperature (24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). Magnification 10X.

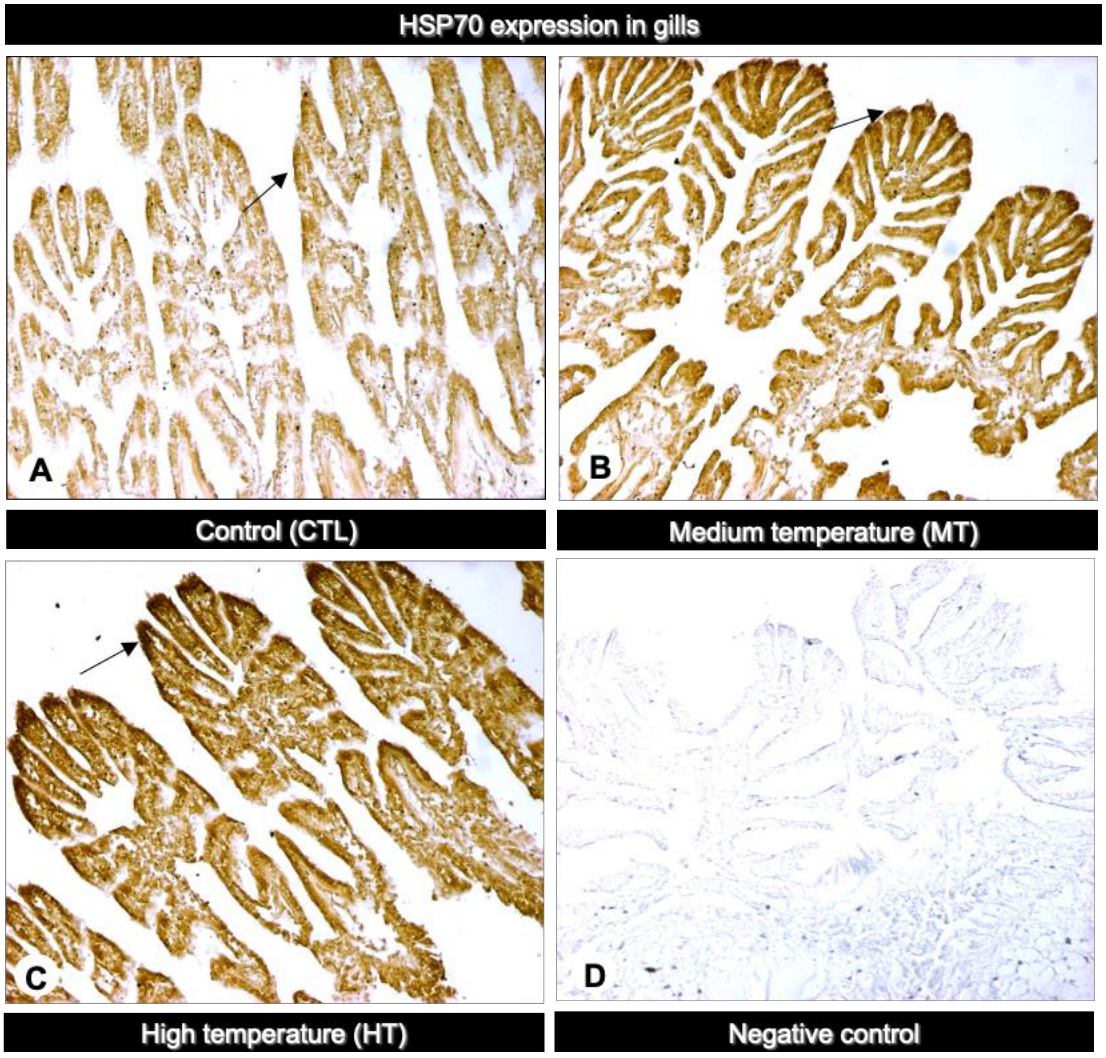


Figure 4. Effects of one-week heat exposure on heat shock protein-70 (HSP70) expression in gills of American oyster. (A-C) HSP70 expression in representative photographs of gills collected from oysters exposed to: (A) control temperature (24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). (D) Negative control. Arrows indicate HSP70 protein expression in gill. Darker brown color indicates higher HSP70 expression. Magnification 10X.

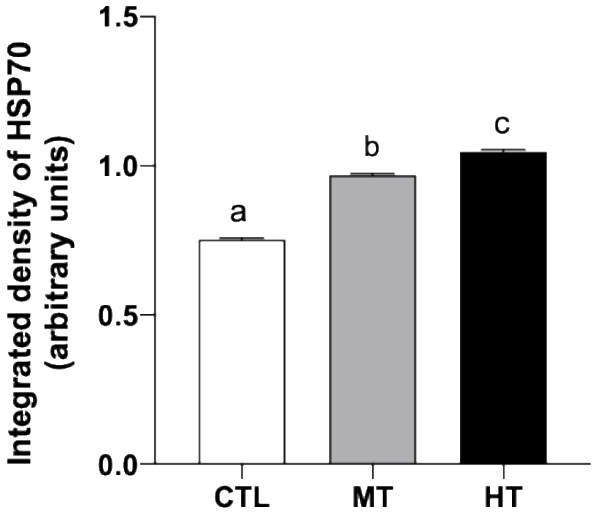


Figure 5. Effects of one-week heat exposure on integrated density of heat shock protein-70 (HSP70) in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

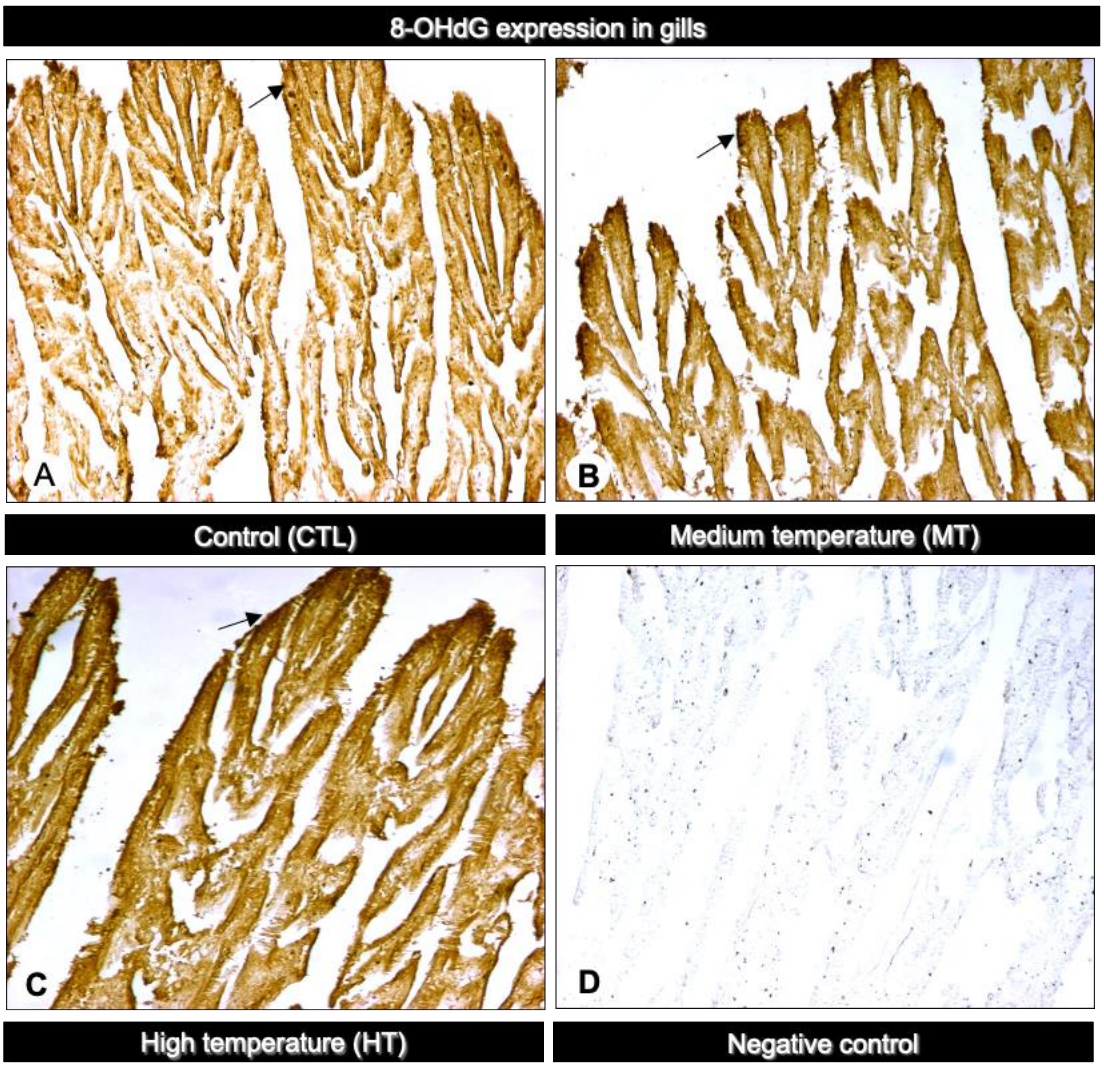


Figure 6. Effects of one-week heat exposure on 8-hydroxy-2'-deoxyguanosine (8-OHdG) expression in gills of American oyster. (A-C) 8-OHdG expression in representative photographs of gills collected from oysters exposed to: (A) control (temperature: 24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). Arrows indicate 8-OHdG expression in gill (D) Negative control. Darker brown color indicates higher 8-OHdG expression. Magnification 10X.

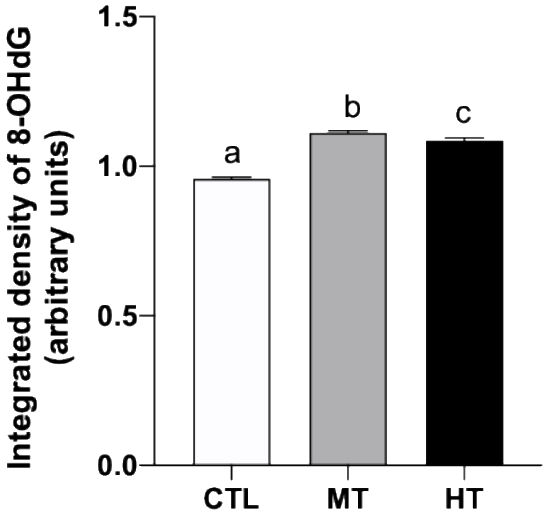


Figure 7. Effects of one-week heat exposure on integrated density of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

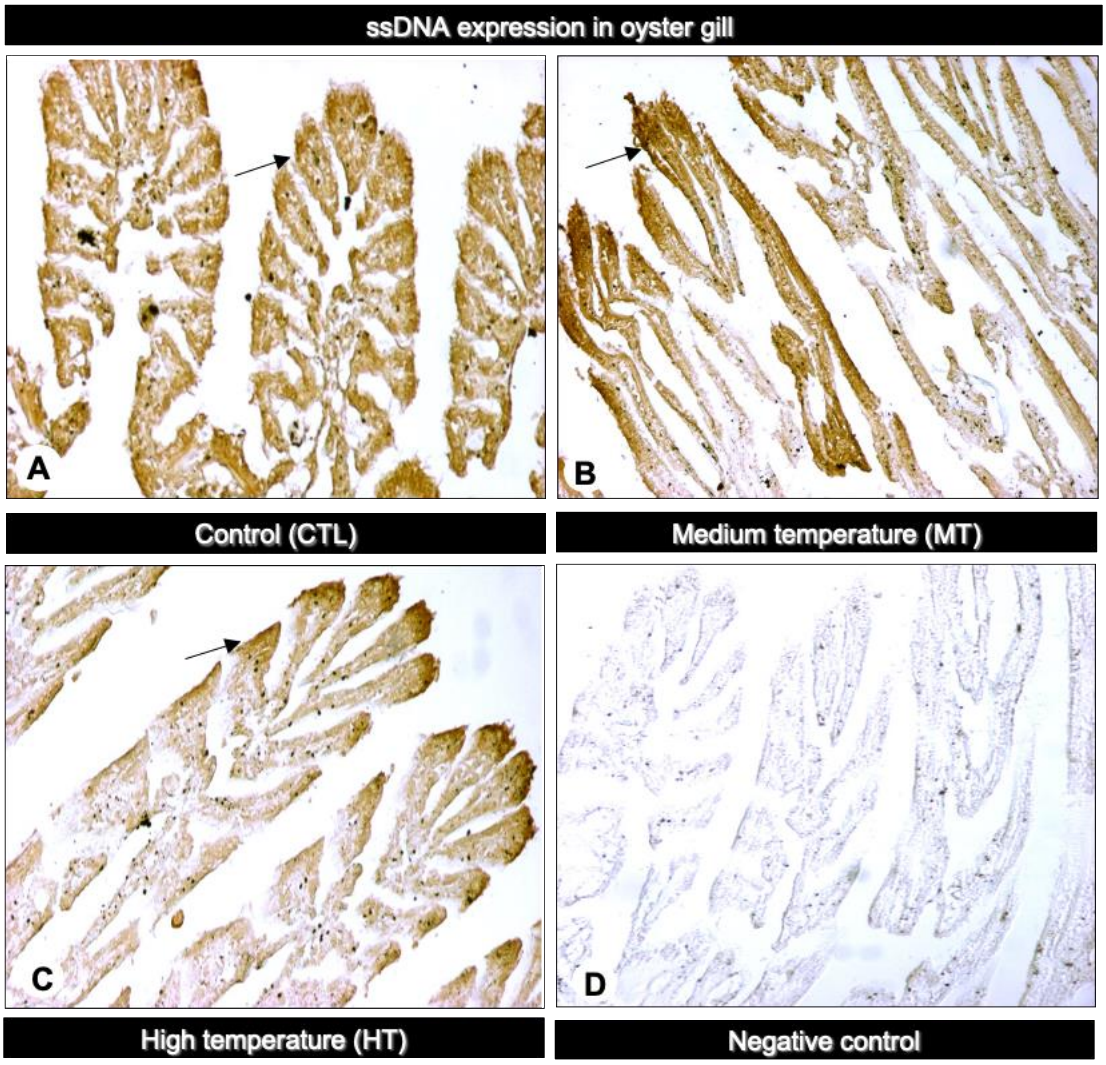


Figure 8. Effects of one-week heat exposure on single-stranded DNA (ssDNA) expression in gills of American oyster. (A-C) ssDNA expression in representative photographs of gills collected from oysters exposed to: (A) control (temperature: 24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). (D) Negative control. Arrows indicate ssDNA expression in gill. Darker brown color indicates higher ssDNA expression. Magnification 10X.

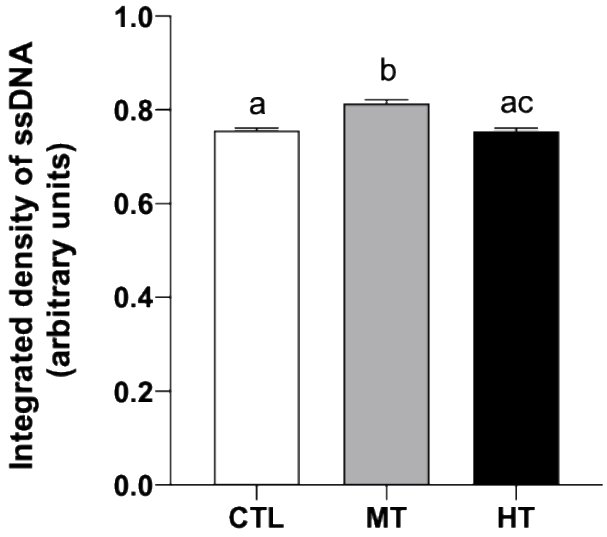


Figure 9. Effects of one-week heat exposure on integrated density of single-stranded DNA (ssDNA) in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Letter indicates no significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

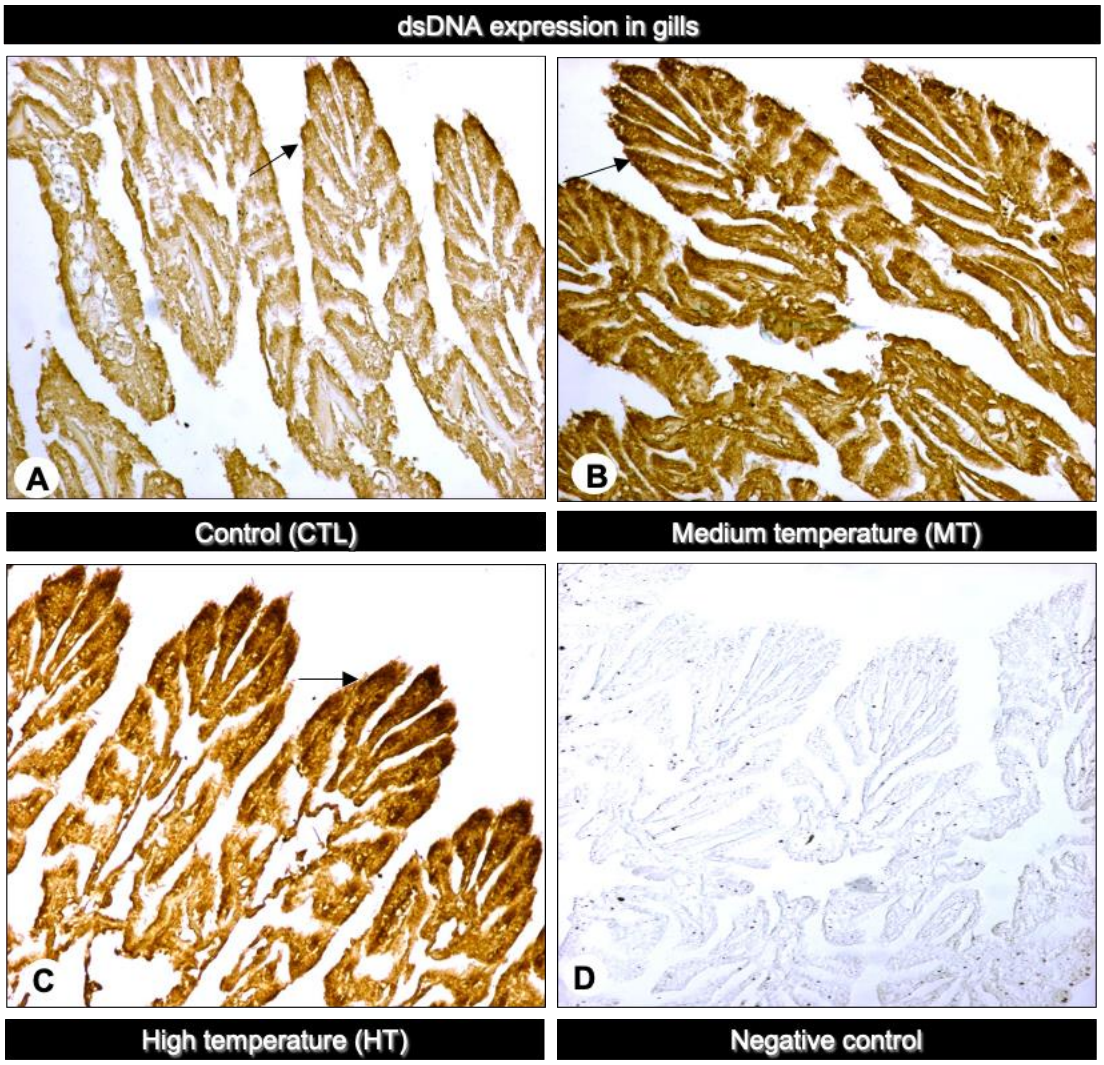


Figure 10. Effects of one-week heat exposure on double-stranded DNA (dsDNA) expression in gills of American oyster. (A-C) dsDNA expression in representative photographs of gills collected from oysters exposed to: (A) control temperature (24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). (D) Negative control. Arrows indicate dsDNA expression in gill. Darker brown color indicates higher dsDNA expression. Magnification 10X.

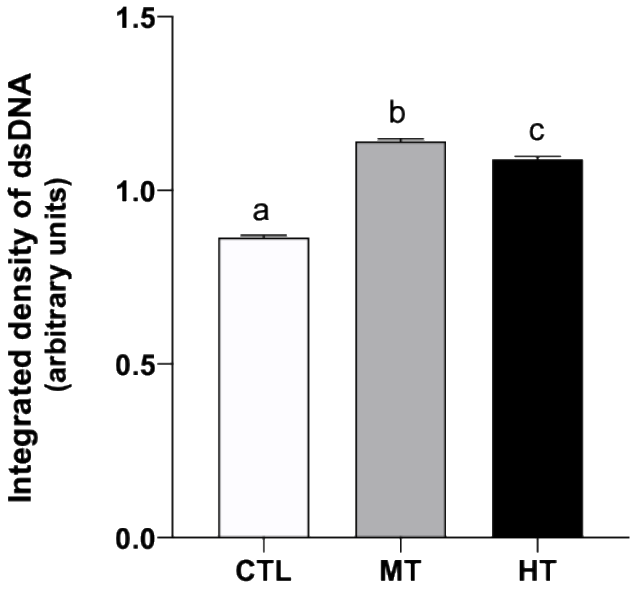


Figure 11. Effects of one-week heat exposure on integrated density of double-stranded DNA (dsDNA) in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

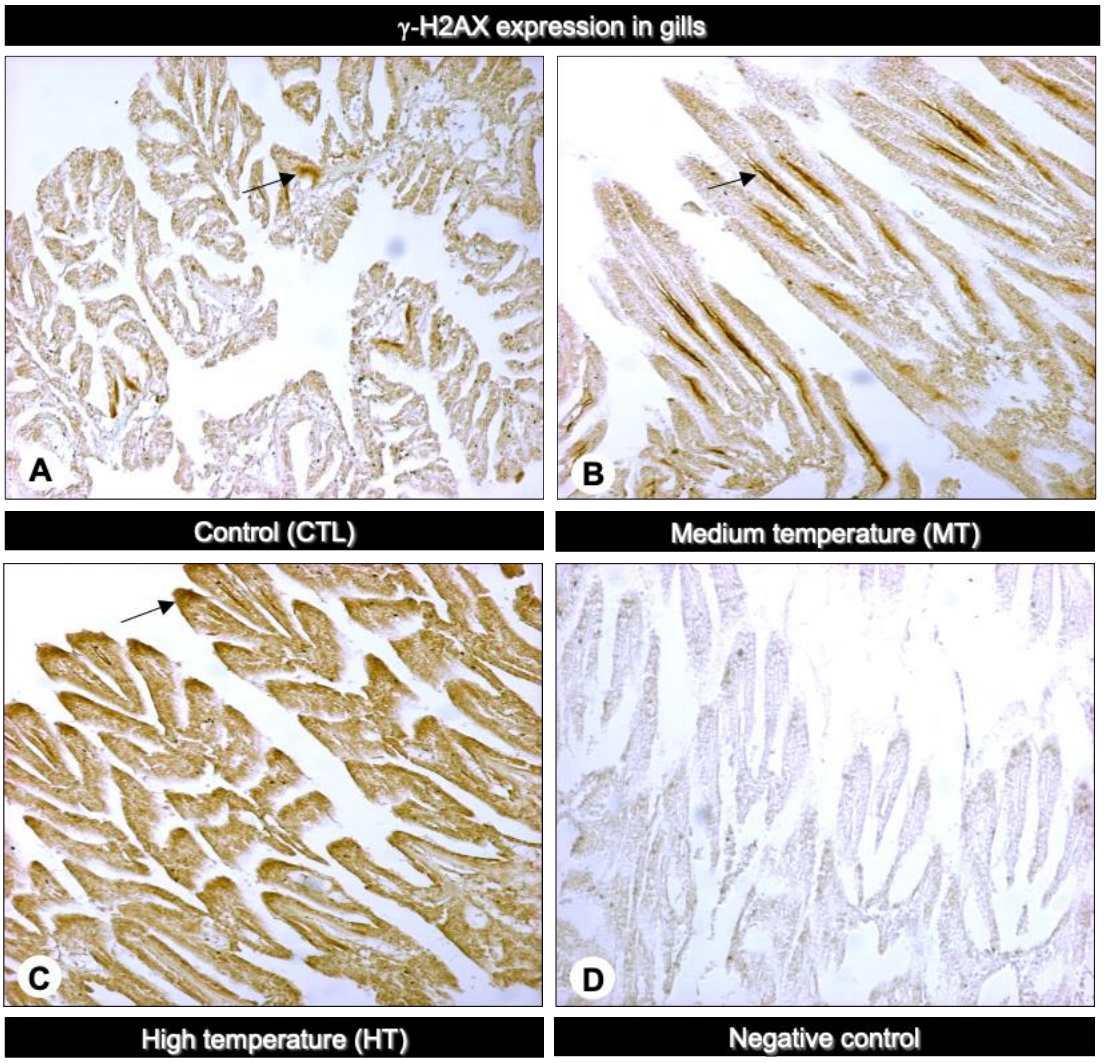


Figure 12. Effects of one-week heat exposure on γ-histone family member X (γ-H2AX) protein expression in gills of American oyster. (B-D) γ-H2AX expression in representative photographs of gills collected from oysters exposed to: (A) control (temperature: 24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). (D) Negative control. Arrows indicate γ-H2AX gill. Darker brown color indicates higher γ-H2AX expression. Magnification 10X.

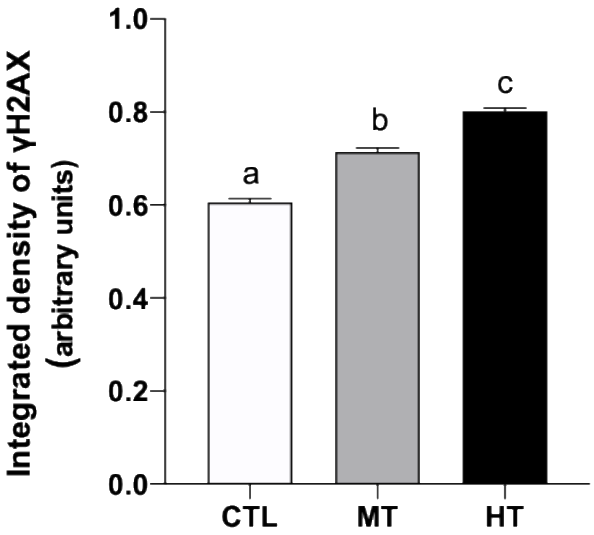


Figure 13. Effects of one-week heat exposure on integrated density of γH2AX in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

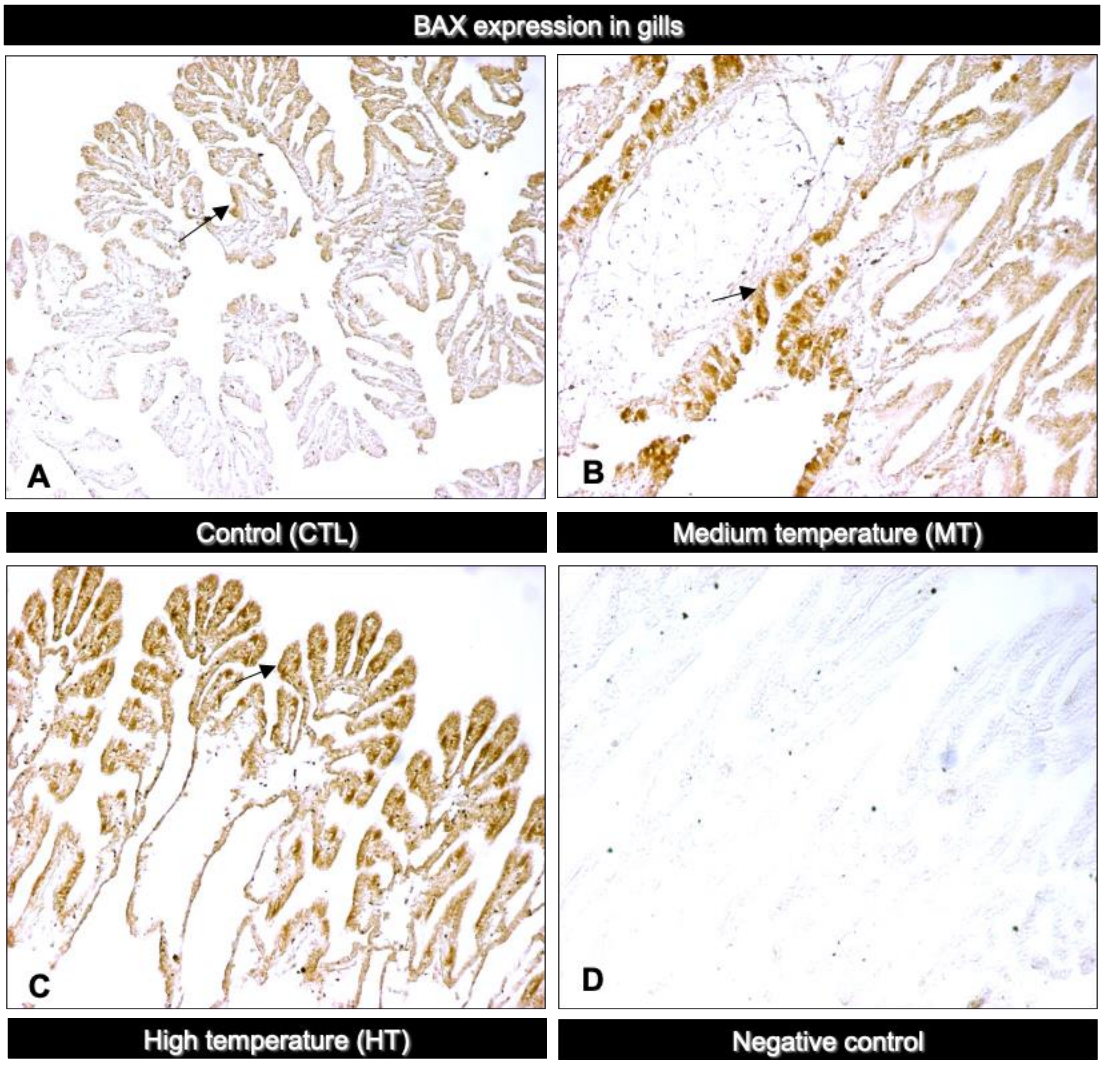


Figure 14. Effects of one-week heat exposure on Bcl-2-associated-X (BAX) protein expression in gills of American oyster. (A-C) BAX expression in representative photographs of gills collected from oysters exposed to: (A) control temperature (24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). (D) Negative control. Arrows indicate BAX protein expression in gill. Darker brown color indicates higher BAX expression. Magnification 10X.

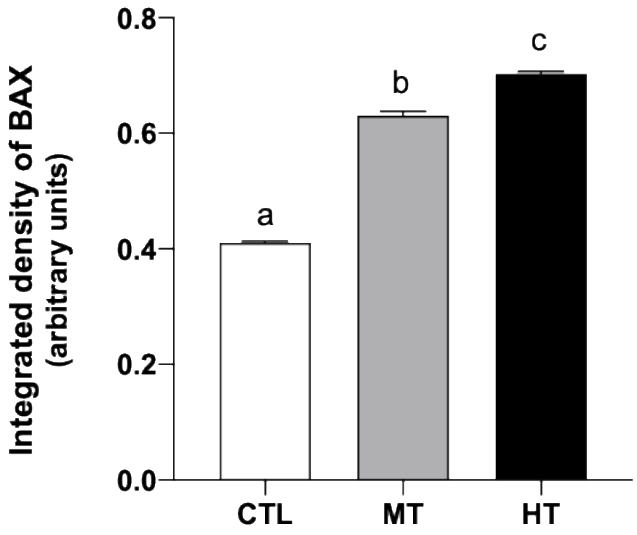


Figure 15. Effects of one-week heat exposure on integrated density of BAX in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT):28°C, and high temperature (HT): 32°C. Letter indicates no significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

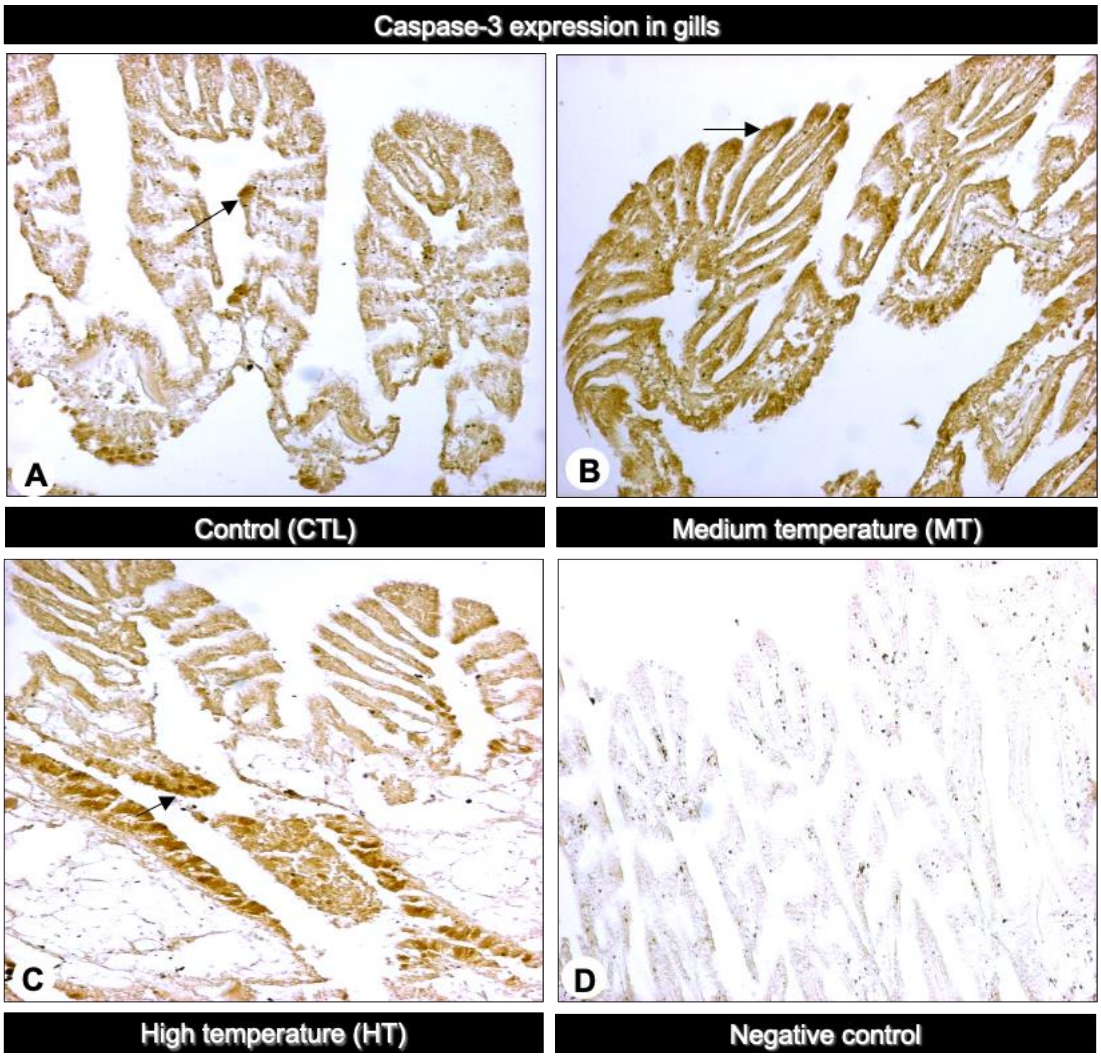


Figure 16. Effects of one-week heat exposure on caspase-3 expression in gills of American oyster. (A-C) Caspase-3 expression in representative photographs of gills collected from oysters exposed to: (A) control (temperature: 24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). (D) Negative control. Arrows indicate caspase-3 protein expression in gill. Darker brown color indicates higher caspase-3 expression. Magnification 10X.

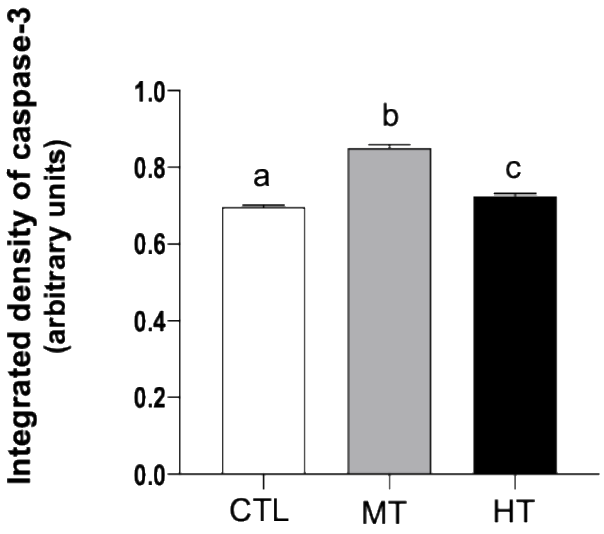


Figure 17. Effects of one-week heat exposure on integrated density of caspase-3 in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Letter indicates no significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

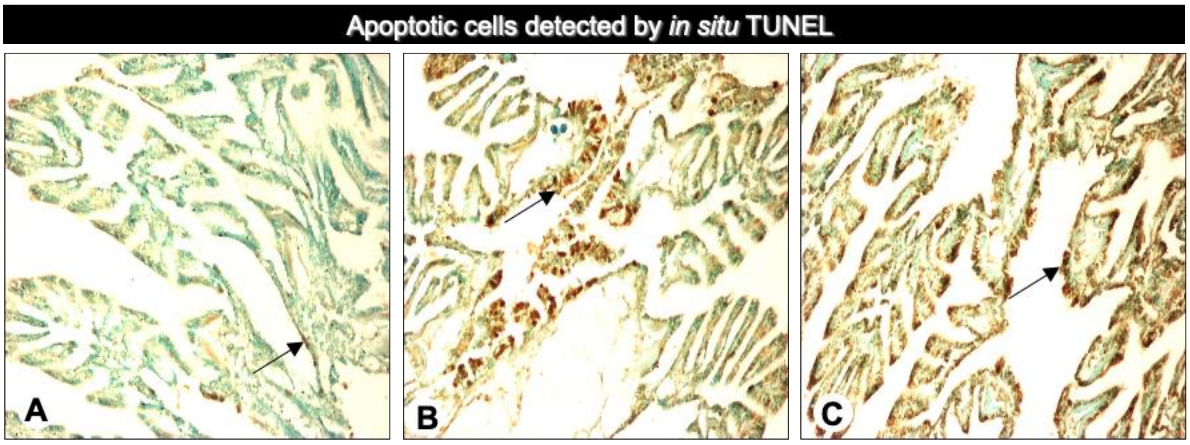


Figure 18. Effects of one-week heat exposure on gill apoptotic cell stained with *in situ* TUNEL assay in the American oyster. The presence of apoptotic nuclei shown as dark brown staining indicated by the arrows. (A) control temperature (24°C), (B) medium temperature (28°C), and (C) high temperature (32°C). Magnification 10X.

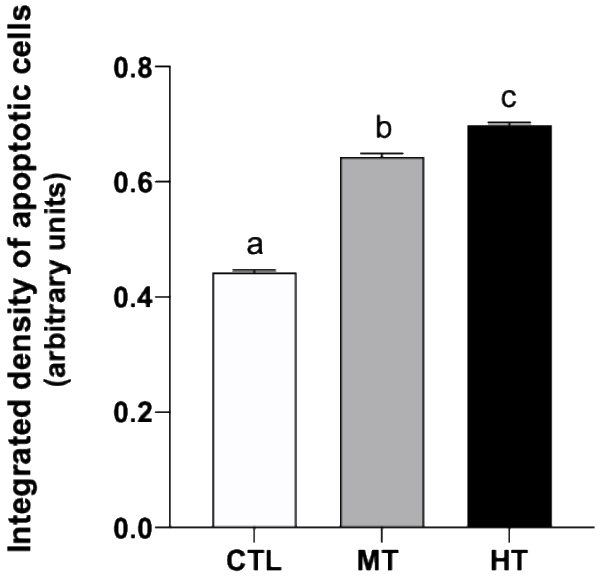


Figure 19. Effects of one-week heat exposure on integrated density of cells stained with *in situ* TUNEL assay in the American oyster gills. caspase-3 in gills of American oyster. The expressions were estimated by measuring the integrated density of staining using ImageJ software. Control (CTL) temperature: 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letter indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

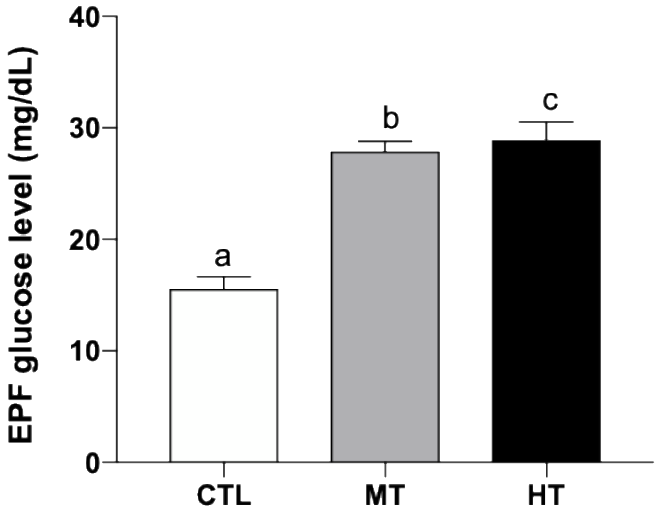


Figure 20. Effects of one-week heat exposure on extrapallial fluid (EPF) glucose level of American oyster. Control (CTL): 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

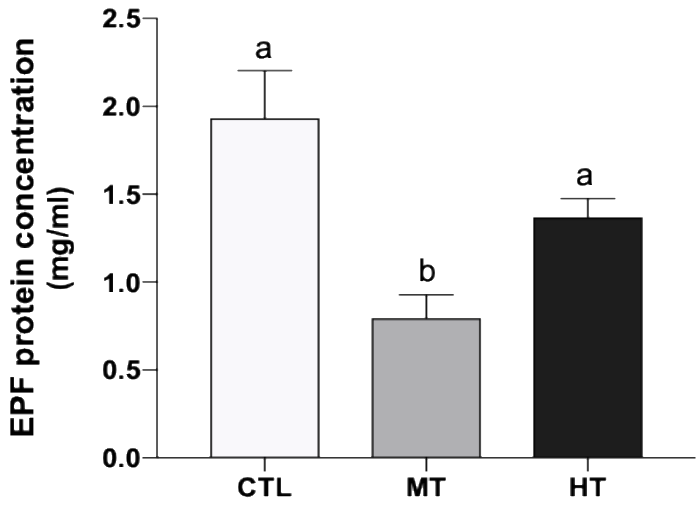


Figure 21. Effects of one-week heat exposure on extrapallial fluid (EPF) protein concentration of American oyster. Control (CTL): 24°C, medium temperature (MT): 28°C, and high temperature (HT): 32°C. Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Each value represents the mean ± SEM (N=9).

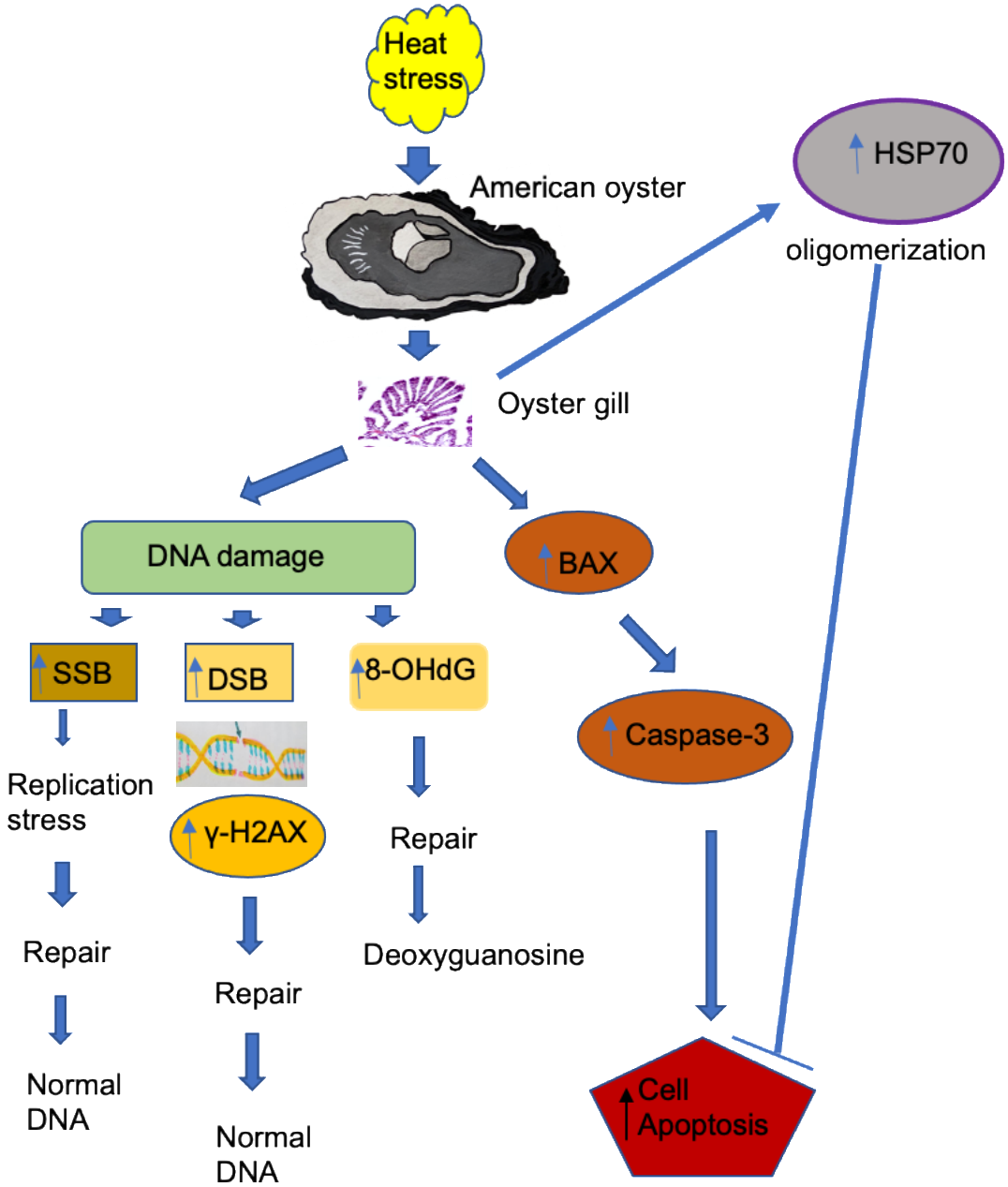


Figure 22. A representative summary of the results of current study. Schematic diagram of heat stress and oxidative DNA damage and apoptosis in American oyster gill. Synthesis of 8-hydroxy-2'-deoxyguanosine (8-OHdG), γH2AX, Single and Double-stranded DNA breaks. Oligomerization of Heat Shock Protein (HSP 70), Synthesis of apoptosis protein; BAX, and Caspase-3.

# BIOGRAPHICAL SKETCH

Md Faizur Rahman completed his Bachelor of Sciences and Master of Sciences in Zoology from the University of Dhaka. During his master's degree he conducted his thesis work on 'breeding ecology and conservation issue of threatened Lesser Adjutant'. He presented his thesis work on the '11$^{th}$ biennial international conference' hosted by the Zoological Society of Bangladesh. He worked as a science teacher in a middle school in Bangladesh. He attended as a trainee student in a certified chartered accountant firm, S.F. Ahmed & Co. Limited. He started his graduate career at the University of Texas Rio Grande Valley in Earth, Environmental and Marine Sciences in August 2018 for seeking his master's degree in Ocean, Coastal and Earth Sciences. He has been awarded university's prestigious college of Science's Dean's Graduate Research Assistantship Scholarship for his outstanding academic achievements. He began his research work under the supervision of Dr. Saydur Rahman, Assistant Professor, at the University of Texas Rio Grande Valley. During his academic year he maintained a good CGPA (3.84) while performing his intensive research work. He has submitted his abstract to the college of sciences annual meeting 2020. He is preparing to present his work in national as well as outside the United States. He has been selected as active judge for the Rio Grande Valley local fair 2019. He received his master's degree in December of 2020 in Ocean, Coastal, and Earth Sciences. He plans to extend his work in cancer research in world's famous school/institution.

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