Elevated temperature attenuates ovarian functions and induces apoptosis and oxidative stress in the American oyster, *Crassostrea virginica*: potential mechanisms and signaling pathways

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Abstract

Global climate change is predicted to intensify thermal stress in marine and coastal organisms, affecting their development, growth, and reproductive functions. In this study, we performed histological observations on ovarian development, immunohistochemical analyses of ovarian heat shock protein-70 (HSP70), nitrotyrosine protein (NTP, an indicator of reactive nitrogen species (RNS)), and dinitrophenyl protein (DNP, an indicator of protein oxidation) expressions, in situ TUNEL assay for cellular apoptosis, biochemical analyses of ovarian caspase-3/7 activity and protein carbonyl (PC, a measure of reactive oxygen species (ROS)) contents, nitrate/nitrite (NOx) levels, and extrapallial fluid (EPF, an important body fluid) pH in the American oyster, *Crassostrea virginica*. Oysters were exposed to medium (28 °C) and high (32 °C) temperatures under controlled laboratory conditions for 1 week. Oysters exposed to higher temperatures significantly decreased the number and diameter of eggs, and EPF protein concentrations compared with controls (24 °C). In contrast, EPF pH, ovarian HSP70 mRNA levels, and protein expression were increased after heat exposure, consistent with increased ovarian apoptosis. The enhanced apoptosis in ovaries was associated with increased ovarian caspase-3/7 activity, PC contents, NOx levels, and NTP and DNP expressions in heat-exposed oysters. Collectively, these results suggest that higher temperatures drastically increase RNS and ROS levels, increasing incidence of apoptosis and subsequently reducing ovarian functions in oysters.

Keywords Global warming · Oyster · Ovary · Heat shock protein · Apoptosis · Oxidative stress

Introduction

Global temperature increases, due primarily to anthropogenic emissions of greenhouse gases, impact marine and coastal environments and their inhabitants (Root et al. 2003). Average global temperature is increasing at the fastest documented rate and has reached a new record high (Morris et al. 2018). Additionally, the probability and severity of heatwaves

also increase under this changing climate as demonstrated in the European heatwave of 2003 (Stott et al. 2004). Impacts of intensified ultraviolet radiation penetration into the ocean present a major concern due to multitudes of environmental stressors already interacting in marine ecosystems; marine organisms including sea urchins, mussels, and oysters are directly influenced by such stressors (Bonaventura et al. 2011).

Free radicals such as reactive oxygen species (ROS, e.g., superoxide radical, hydrogen peroxide, hydroxyl radicals) cause severe oxidative damage and increase apoptosis in cells and tissues during environmental stress (Silmen et al. 2014, Di Meo et al. 2016). Even though ROS are necessary for normal cellular function, overproduction of ROS alters homeostasis and induces cellular stress (Di Meo et al. 2016). A previous study revealed heat shock increases the production of ROS, inducing oxidative damage (Dröge 2002, Bruskov et al. 2002). Heat stress also increases other reactive radicals such as reactive nitrogen species (RNS, e.g., nitric oxide, peroxynitrite) which leads to increased cellular apoptosis and oxidative stress (Silmen et al. 2014).

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Heat shock proteins (HSPs) are key chaperone proteins that protect animal cells and tissues under thermal stress (Feder and Hofmann 1999, Creagh et al. 2000). Multiple subunits of HSPs, such as HSP70, HSP90, and HSP100, bind together with the heat shock factor and then enter into the nucleus of a cell where they bind with other proteins (Giudice et al. 1999, Mayer and Bukau 2005, Javid et al. 2007). These key proteins also serve as regulators of normal cellular functions, assisting in proper folding, assembly, and transport of other proteins (Clark and Peck 2009). Changes in the HSPs expression are linked to major developmental changes such as gametogenesis, embryogenesis, and metamorphosis in marine shellfish such as clams, mussels, and oysters (Li et al. 2007, Ueda and Boettcher 2009, Ueda et al. 2009).

Oysters are intertidal bivalves, constantly exposed to extreme environmental fluctuations from immersion and/or emersion of tidal waves, making them highly tolerant to varying environmental conditions. During summer spawning, when heat stress is intensified, oysters must compromise their thermal tolerance and immune status to cope with increased energy expenditure, making them subject to higher mortality rates (Li et al. 2007). Post-spawning oysters in water temperatures greater than 40 °C face greater mortality rates, thus exhibiting decreased tolerance of higher temperatures when compared with pre-spawning oysters (Li et al. 2007). The rate of change in temperature, however, has greater effects than the temperature level itself (Shumway 1996). For example, oysters can die not only from exposure to short-term high temperatures, but also exposure to long-term low temperatures (Shumway 1996).

Heat stress directly affects larval and early spat development in the American oysters by inhibiting their growth (Ueda and Boettcher 2009, Lowe et al. 2017), potentially leading to abnormal gonadal development and impaired reproductive functions. It also affects gametogenesis and sex determination in the European flat oysters after long-term exposure (4 weeks; Zapata-Restrepo et al. 2019). The duration of heat exposure can vary in geographically distant oyster populations (e.g., northern oyster populations growing faster than conspecifics from southern populations when raised under the same conditions, Casas et al. 2018), and some oyster populations more frequently face short-term (few days) thermal stress events; therefore, it is imperative to further investigate the reproductive impairment and signaling pathways in oysters exposed to short-term heat stress. Considering the significant effects on the gene expressions (e.g., HSP70, anti-apoptotic protein, cadherin, etc.) and physiological functions (e.g., respiration rate, clearance rate, etc.) reported in the American oyster (Jones et al. 2019) after long-term (4 weeks) exposure to high temperature, it is expected that time-dependent responses exist in oyster populations. The aim of this study builds on the possibility of time-dependent responses to further understand molluscan thermal stress, and its impacts on reproductive fitness after short-term exposure.

The main objectives of this research were to investigate the effects of short-term exposure to elevated temperature (1 week) on ovarian development, extrapallial fluid (EPF, an important body fluid in marine invertebrates; Robertson 1939, Mangum and Shick 1972) conditions, cellular apoptosis, and oxidative stress in the American oyster.

American oyster (*Crassostrea virginica*), an edible and important marine shellfish species in the USA, has been an extensive biological asset for multidisciplinary studies including aquaculture, and physiological, ecotoxicological, and pathogenic research over the past decade (Shumway 1996). American oysters are dioecious, oviparous species of substantial commercial value (Eckelbarger and Davis 1996). Their distribution occurs along the east and west coasts of the USA as well as the east coast of Central and Northern South America, and even some coasts of Europe (Grizzle 1990, Eckelbarger and Davis 1996; Grizzle et al., 2018).

Materials and methods

Laboratory experiment

Young adult wild-caught American oysters (~ 2 years old, shell size: length 7.2 cm, width 3.3 cm, weight 4.7 g) were collected off the southern coast of Texas in the Gulf of Mexico according to the approved wildlife species capture rules and regulations by the Texas Parks and Wildlife Department, transported to laboratory, and placed in 20-gallon (~ 75 L) glass aquaria with recirculating seawater under controlled laboratory conditions. The University of Texas Rio Grande Valley Institutional Animal Care and Use Committee (IACUC) does not require an animal use and/or care protocol for research conducted on invertebrates including oyster. Over a 5-day period, two aquaria per treatment group (24, 28, and 32 °C) were gradually heated to their respective temperature using 22–40-W aquarium heaters (Top Fin, Franklin, WI, USA). The duration and experimental temperatures used in this study were based on oysters mortality (54% mortality occurs after 5-day exposure to 35 °C; Quick 1971) and survivability (thermal death point 48.5 °C; Henderson, 1929), and also previous publications (Lowe et al. 2017, Casas et al. 2018, Jones et al. 2019). Each aquarium was equipped with a recirculating flow through biological filter (Top Fin) and advanced power motor multi-stage system (Tetra, Blacksburg, VA, USA). Temperature, pH, and dissolved oxygen levels in each aquarium were measured using a YSI Professional Plus multiprobe system (YSI Incorporated, Yellow Springs, OH, USA). A single parameter water quality kit (Mars Fishcare North America, Inc., Chalfont, CA, USA) was used to measure ammonia and nitrite once a day. There were no major changes in the physiochemical parameters in water (pH ~ 8.1, salinity ~ 32–33 ppt, dissolved oxygen ~

5.75–6.63 mg/L, ammonia ~ 0.1–0.2 mg/L, nitrite ~ 0.1–0.2 mg/L) during the experimental period. Oysters were fed frozen marine cuisine (San Francisco Bay Brand, Inc., Newark, CA, USA) every other day. After 1-week heat exposure, sixty oysters (10/aquarium) were collected for histological, immunohistochemical, and biochemical analyses. Extrapallial fluid (~ 1 mL) pH was measured using a portable pH meter (Eutech, Waltham, MA, USA) and stored at – 80 °C for later analysis of protein concentration. The gonad was dissected from each oyster and fixed in 4% ice-cold paraformaldehyde for histological and immunohistochemical analyses. Ovarian tissue samples were also stored at – 80 °C for analysis of nitrate/nitrite (NOx) levels, protein carbonyl contents, and mRNA levels.

Histology

To execute histology, ovarian tissue samples were dehydrated with a series of ethanol dilutions (50, 75, 95, and 100%), embedded in paraffin, and sectioned at 7 µm on a rotary microtome (Leica, Wetzlar, Germany). The tissue sections were deparaffinized with xylene, rehydrated with a series of ethanol dilutions (100, 95, 75, and 50%), and counterstained with hematoxylin and eosin solutions (Sigma-Aldrich, St. Louis, MO, USA).

Percentage of ova and oocyte, and egg diameter

The percentages of ova (mature eggs) and oocytes (immature eggs) were calculated in ovarian sections for all treatment groups. Each section was marked into equally sized quadrants using a ruler and marker to efficiently count the number of mature and immature eggs per slide. Each slide was individually and carefully observed under a light microscope. The ova and oocytes were identified, counted with a tally counter, and recorded for every slide at each treatment group. The diameter of eggs (75–100 eggs/slide) was measured with a micrometer using ImageJ software (National Institutes of Health, Bethesda, MD, USA) according to Schneider et al. (2012).

Immunohistochemistry

The immunofluorescent signals of heat shock protein-70 (HSP70), nitrotyrosine protein (NTP), and dinitrophenyl protein (DNP) expressions in ovarian tissues were detected according to the immunohistochemistry method described by Rahman and Thomas (2015, 2019). Briefly, paraffin-embedded tissues were sectioned at 7 µm on the microtome, deparaffinized with xylene, rehydrated with a series of ethanol dilutions (100, 95, 75, and 50%), and rinsed in phosphate buffer saline (PBS, Fisher Scientific, Hampton, NH, USA) three times for 10 min each. Slides were then blocked with 1% bovine serum albumin (BSA, Fisher Scientific) 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) primary antibodies were added to each slide (antibody dilution 1:100 with 1X PBS) and incubated at 4 °C overnight. The negative control slides were incubated with PBS instead of HSP70, NTP, or DNP primary antibodies. After incubations, slides were rinsed with PBS thrice for 10 min each and incubated with Alexa Fluor 488 or Texas Red goat anti-mouse (Invitrogen, Carlsbad, 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 DNP expression was detected using 3,3'-diaminobenzidine peroxidase (DAB, Vector Laboratories, Burlingame, CA, USA) substrate in dark condition. Slides were then washed with deionized water for 5 min, dehydrated with a series of ethanol dilutions, sequenced with xylene, and mounted with Cytoseal. The immunoreactive (IR) signals of HSP70, NTP, and DNP were captured by a Cool-SNAP camera (Photometrics, Tucson, AZ, USA) using a light microscope and the IR signals were estimated using ImageJ software (Schneider et al. 2012).

Real-time quantitative PCR

HSP70 mRNA levels were quantified by qRT-PCR on total RNA using GoTag one-step qRT-PCR master mix (Promega, Madison, WI, USA) according to Rahman and Thomas (2017, 2018). Briefly, RNA was extracted from ovarian tissues using Tri reagent (Sigma-Aldrich) and treated with DNase to remove genomic DNA. qRT-PCR analyses were determined in a 25 µL RT-PCR mixture containing 100 ng RNA, 12.5 µL of 2× GoTag master mix, 1 µL of reverse transcriptase enzyme, and 125 nM gene-specific primers for oyster HSP70 (forward primer: 5'-CACATCTGGGAGGTGAGGA-3' and reverse: 5'-CTCAAATCTGGCTCGTGTGA-3', GenBank accession number: AJ271444). HSP70 transcript level was normalized for the quantification of oyster beta-actin (primers: forward 5'-CCCACACAGTCCCCATCTAC-3' and reverse: 5'-CTCC TGCTCGAAGTCCAAAG-3', X75894.1). Primer3 software (Untergasser et al. 2012) and the NCBI Primer BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) were used to evaluate the specificity of primers for qRT-PCR analysis. The relative HSP70 mRNA levels were analyzed using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Extrapallial fluid protein concentration

Extrapallial fluid (EPF) protein levels were analyzed using a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA, USA) according to the method described by Bradford (1976). Briefly, 5 µL of EPF was added to 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 1000 $\mu\text{g/mL}$) to measure EPF protein concentrations. Absorbance was read at 595 nm, and protein concentrations were expressed as microgram per milliliter.

Cellular apoptosis

Apoptosis in ovarian tissues was detected using in situ terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) detection kit (Promega) according to manufacturer's instructions and the method described by Ondricek and Thomas (2018). Briefly, paraffin-embedded ovarian tissues were sectioned at 7- μm sections, deparaffinized with xylene, and rehydrated with a series of ethanol dilutions. 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 °C for 1 h in a humidified chamber. After stopping TdT enzymatic reaction, sections were rinsed in PBS, incubated with anti-digoxigenin conjugate for 30 min, and stained with peroxidase substrate for 5 min. Sections were rinsed with deionized water, dehydrated with series of ethanol dilutions, sequenced with xylene, and mounted with Cytoseal. The integrated intensity of apoptotic cell bodies was measured using ImageJ software.

Measurement of caspase 3/7 activity

Caspase 3/7 activity in ovarian tissues was measured using Apo-ONE homogenous caspase 3/7 assay kit according to manufacturer's protocol (Promega).

Measurement of cellular NOx level

Concentrations of the stable endproducts of nitric oxide (NO) oxidation (nitrates/nitrites (NOx)) in ovarian tissues were measured using Griess reagent with a colorimetric assay kit according to the manufacturer's protocol (Enzy Life Sciences, Plymouth Meeting, PA, USA).

Endogenous protein carbonyl contents

Endogenous protein carbonyl (PC, a measure of ROS) contents were determined as an index of ROS production in oyster ovarian tissues during heat stress according to Weber et al. (2015). Briefly, ovarian tissue samples were homogenized in a glass homogenizer with ice-cold PBS buffer and centrifuged for 15 min at 4 °C. Two hundred microliters of the sample supernatant was added in 800 μL of DNPH to the sample and 800 μL of 2.5 N HCl to the control sample. Samples were then incubated for 1 h in dark condition. After adding 1 mL of 20% TCA, samples were centrifuged at 10,000 $\times g$ for 1 min at 4 °C. The pellet was washed three times with 1 mL of ethanol:ethyl acetate

mixture (1:1 ratio). The protein pellet was then dissolved in 500 μL of 6 M guanidine hydrochloric acid. PC contents were determined by the absorbance at the wavelength between 360 and 385 nm and expressed as nanomole per milligram protein.

Statistical analyses

All statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA, USA) and StatView (SAS Institute, NC, USA) computer software. All results are shown with the mean $\pm$ standard error of the mean (SEM). Analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) test was 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.

Results

Effects of heat stress on ovarian development

Ovarian functions were significantly attenuated in oysters exposed to high temperatures for 1 week compared with controls which was associated with a reduction in the percentage of fully grown eggs (mean ova 64.8% at 24 °C, 59.5% at 28 °C, and 43.3% at 32 °C, *P* < 0.05; Fig. 1a–d). Egg diameter in oysters was also significantly reduced after exposure to medium (MT 28 °C) and high (HT 32 °C) temperatures compared with controls (CTL 34.85 $\pm$ 0.82, MT 30.21 $\pm$ 0.53, and HT 28.03 $\pm$ 0.55 μm) (ANOVA, *P* < 0.01; Fig. 1e).

Effects of heat exposure on HSP70 expression and mRNA levels

Because HSP70 is known to play important physiological roles in the cells and tissues during heat stress (Mayer and Bukau 2005), we examined the expression of HSP70 in oyster ovaries by immunohistochemistry. Immunohistochemical detection of HSP70 in ovary sections showed that the immunoreactive expression of HSP70 appeared to be stronger in both the medium and high-temperature treatment groups compared with controls (Fig. 2a–c). The immunostaining intensity of HSP70 increased around 3-fold in medium and $\sim$ 7-fold in high temperatures (ANOVA, *P* < 0.01; Fig. 1d). Exposure to high temperatures caused a marked elevation ($\sim$ 5–33-fold) of ovarian HSP70 mRNA levels compared with controls (ANOVA, *P* < 0.05; Fig. 2e). No immunohistochemical signals were detected in the negative control sections of the ovary (Supporting Fig. 1).

Fig. 1 Effects of 1-week heat exposure on ovarian development in American oyster. Histological appearance of representative ovaries, **a** control (CTL 24 °C), **b** medium temperature (MT 28 °C), and **c** high temperature (HT 32 °C). **d** Ovarian development (oocyte and ova, $n = 8-12$), **e** egg diameter ($n = 75-100$). Arrowhead indicates eggs. Note: here and in subsequent figures exposure duration only refers to period oyster was exposed to target temperature; oysters were previously

exposed to an increasing temperature for the additional 5-day adjustment period. Each value represents the mean $\pm$ SEM. Whiskers indicate the minimum and maximum values for each data set. Center line inside the whisker box demonstrates mean value. Different letters indicate significant differences with a multiple range test, Fisher's PLSD (ANOVA, $P < 0.05$). An asterisk indicates a significant difference (Student's t test, $*P < 0.05$). Scale bar = 100 μ m

Fig. 2 Effects of 1-week heat exposure on heat shock protein-70 (HSP70) expression and relative mRNA levels in the ovary of American oyster determined by immunohistochemistry (**a-d**) and qRT-PCR (**e**), respectively. The relative HSP70 mRNA expression levels were normalized using beta-actin mRNA expression as the internal control. Each value represents the mean $\pm$ SEM, $n = 76-86$ (**d**), $n = 8$ (**e**). Arrowhead indicates

HSP70 expression in the egg. **d**, **e** Whiskers indicate the minimum and maximum values for each data set. Center line inside the whisker box demonstrates mean value. Different letters indicate significant differences in Fisher's PLSD test (ANOVA, $P < 0.05$). Control, CTL (24 °C); medium temperature, MT (28 °C); high temperature, HT (32 °C); IR, immunoreactive. Scale bar = 100 μ m

Effects of heat exposure on EPF conditions

Since EPF is an important body fluid that regulates many physiological functions in marine clams and oysters (Allam and Paillard 1998, Ma et al. 2007), we analyzed the biochemical compositions (total protein concentrations and pH) of EPF in oyster. Oysters exposed to higher temperatures significantly decreased EPF protein concentrations compared with controls (CTL 719.5 $\pm$ 106.4, MT 556.8 $\pm$ 65.8, and HT 452.7 $\pm$ 52.9 μ g/mL, ANOVA, $P < 0.05$; Fig. 3b). Contrarily, EPF pH tended to increase from control temperature to medium and high temperatures (CTL 6.56 $\pm$ 0.04, MT 6.7 $\pm$ 0.03, and HT 6.71 $\pm$ 0.06, ANOVA, $P < 0.05$; Fig. 3c).

Fig. 3 Effects of 1-week heat exposure on extrapallial fluid conditions in American oyster. **a** Anatomy (soft body parts: mantle cavity and internal organs) of American oyster. DG, digestive gland; EPF, extrapallial fluid. **b** EPF protein (prot.) concentrations (concentr.), **c** EPF fluid pH. Each value represents the mean $\pm$ SEM, $n = 13$ – 18 . Whiskers indicate the minimum and maximum values for each data set. Center line inside the whisker box demonstrates mean value. Different letters indicate significant differences in Fisher's PLSD test (ANOVA, $P < 0.05$). Asterisks indicate a significant difference (Student's t test, $**P < 0.01$). Control, CTL (24 °C); medium temperature, MT (28 °C); high temperature, HT (32 °C)

Effects of heat stress on apoptosis and caspase-3/7 activity

Apoptosis or programmed cell death is a natural process that occurs as part of an organism's development and growth (Gervais et al. 2015). Here, we observed how higher temperatures affect cellular apoptosis in ovarian follicles of oysters. Control temperature displayed few apoptotic cells while medium and high temperatures exhibited a large number of apoptotic bodies/cells in ovaries (Fig. 4a–c). Both TUNEL-positive follicles (CTL 6.66 $\pm$ 2.93% at 24 °C, MT 38.35 $\pm$ 1.93% at 28 °C, and HT 35.82 $\pm$ 3.51% at 32 °C) and intensity of apoptotic cells were significantly higher in medium and high temperatures compared with controls (ANOVA, $P < 0.05$; Fig. 4d, e).

Caspase-3/7 activity significantly escalated in ovaries of oysters exposed to high temperature, more than ~ 5 -fold higher than that in controls (ANOVA, $P < 0.05$; Fig. 3f).

Effects of heat exposure on nitrotyrosine protein expression

Nitrotyrosine protein (NTP, an indicator of reactive nitrogen species (RNS)) expression was detected in ovarian tissues of oysters in control, medium, and high temperatures through immunohistochemical analysis. NTP expression was significantly elevated in ovarian follicles of oysters exposed to medium and high temperatures compared with controls (Fig. 5a–c). The intensity of NTP was increased by ~ 2.7 -fold in medium temperature and ~ 3.5 -fold in high temperature compared with controls (ANOVA, $P < 0.05$; Fig. 5d). No immunohistochemical signals were detected in the negative control sections of the ovary (Supplementary Fig. 2).

Nitrate/nitrite (NO_x, an indicator of RNS) levels were significantly elevated around 1.6-fold in ovarian tissues of oyster exposed to medium and high temperatures, respectively (ANOVA, $P < 0.05$; Fig. 5e).

Effects of heat exposure on dinitrophenyl protein expression and protein carbonyl contents

To elucidate the possible relationship of protein oxidation and ROS production during heat stress, immunohistochemical analysis was conducted to examine the dinitrophenyl protein (DNP, a biomarker of protein oxidation; Simon 1953) expression in ovarian tissues. DNP expression significantly increased in both medium and high temperatures, displaying a very strong immunoreactive signal in oyster follicles at 32 °C compared with controls (Fig. 6a–c). Immunoreactivity intensity of DNP was also measured using ImageJ software. Results showed a much greater intensity in ovaries of oysters exposed to 28 °C and 32 °C compared with 24 °C (ANOVA, $P < 0.05$; Fig. 6d). No immunohistochemical signals were detected in the negative control sections of the ovary (Supplementary Fig. 3).

Fig. 4 Effects of 1-week heat exposure on ovarian apoptosis stained with in situ TUNEL assay in American oyster. **a–c** The presence of apoptotic nuclei shown as brown staining (arrowhead). **d** Percentage (%) of TUNEL-positive follicles ($n = 11$ – 14), **e** integrated intensity of apoptotic follicles ($n = 480$ – 481), and **f** caspase-3/7 activity in ovarian tissues ($n = 10$). Each value represents the mean $\pm$ SEM. Control, CTL (24 °C);

medium temperature, MT (28 °C); high temperature, HT (32 °C). Whiskers indicate the minimum and maximum values for each data set. Center line inside the whisker box demonstrates mean value. Different letters indicate significant differences in Fisher's PLSD test (ANOVA, $P < 0.05$). An asterisk indicates a significant difference (Student's t test, $*P < 0.05$). Scale bar = 100 μ m

Protein carbonyl (PC, an indicator of ROS; Dalle-Donne et al. 2003) contents were significantly elevated around 3.7-fold

and ~11.7-fold in ovaries of oysters exposed to medium and high temperatures, respectively (ANOVA, $P < 0.05$; Fig. 6e).

Fig. 5 Effects of 1-week heat exposure on nitrotyrosine protein (NTP) expression and nitrate/nitrite (NOx) levels in the ovary of American oyster determined by immunohistochemistry (**a–d**) and fluorometric assay (**e**), respectively. Each value represents the mean $\pm$ SEM, $n = 10$. Arrowhead indicates NTP expression in ovarian follicles. **d**, **e** Whiskers

indicate the minimum and maximum values for each data set. Center line inside the whisker box demonstrates mean value. Different letters indicate significant differences in Fisher's PLSD test (ANOVA, $P < 0.05$). Control, CTL (24 °C); medium temperature, MT (28 °C); high temperature, HT (32 °C); IR, immunoreactive. Scale bar = 100 μ m

Fig. 6 Effects of 1-week heat exposure on dinitrophenyl protein (DNP) expression and protein carbonyl (PC) contents in the ovary of American oyster determined by immunohistochemistry (**a–d**) and fluorometric assay (**e**), respectively. Each value represents the mean $\pm$ SEM, $n = 109$ – 123 (**d**), $n = 10$ (**e**). Arrowheads indicate DNP expression in ovarian follicles

(**a–c**). **d**, **e** Whiskers indicate the minimum and maximum values for each data set. Different letters indicate significant differences in Fisher's PLSD test (ANOVA, $P < 0.05$). Control, CTL (24 °C); medium temperature, MT (28 °C); high temperature, HT (32 °C); IR, immunoreactive. Scale bar = 100 μ m

Discussion

The influence of seasonal temperatures on gonadal development, photoperiod cycles, and perpetual winter conditions on gametogenesis including the initiation of gonadal development, maturation, and gamete resorption has previously been studied in oyster (Fabioix et al. 2005); however, many questions on the impacts of global warming in reproductive functions of oysters remained unanswered. In this report, we described the effects of high temperature on ovarian functions, cellular apoptosis, and oxidative stress in the American oyster. Although a large number of gonadal samples (60) were analyzed, a statistically insignificant number of males (5) were identified, disallowing us from further evaluating the effects on testicular functions. These findings indicate that high temperature attenuates ovarian functions, intensifies HSP70 expression, increases apoptosis, and, most importantly, increases NTP and DNP (biomarkers for RNS and ROS) expressions in ovarian tissues of American oyster.

The results of our study clearly demonstrate that exposure to medium and/or high temperatures (28 °C and/or 32 °C) for 1-week decreases the number of fully grown oocytes (ova) as well as reduces the diameter of ova in the American oyster. Effects of high temperature on gametogenesis have also been reported in other bivalves (Sastry and Blake 1971, Chavez-Villalba et al. 2002, Gosling 2015, Parker et al. 2018, Zapata-Restrepo et al. 2019). In the Pacific oyster, the speed of oocyte

development increases significantly with temperatures between 16 and 22 °C, but the growing speed of oocytes quickly decreases after exposure to 25 °C for 4 weeks, compared with 22 °C exposure (Chavez-Villalba et al. 2002). Similarly, in the European flat oyster, gametogenesis and sex determination are predominantly affected after exposure to 18 °C compared with 10 °C for 4 weeks (Zapata-Restrepo et al. 2019). In blue mussel, the lower and upper lethal temperatures are 0 and 23 °C, respectively, and the optimum temperature for gametogenesis is between 2 and 15 °C (Gosling 2015). Temperature also influences oocyte growth by regulating the transfer of nutrient reserves to the gonads in the Atlantic bay scallop (Sastry and Blake 1971). Collectively, our data suggest increasing temperature from global climate change imposes a great threat to the gametogenesis of marine mollusks.

An important finding of this study is that elevated water temperature increases extrapallial fluid (EPF, an important body fluid in marine bivalves; Allam and Paillard 1998, Ma et al. 2007) pH levels in oysters. In normal conditions, EPF pH is naturally acidic, but our results indicate that heat stress shifts EPF pH from acidic toward neutral. Oyster exposed to a high temperature significantly decreased EPF protein concentrations. It is therefore envisaged that a decrease in EPF protein concentrations correlates to a decrease in gametogenic production, suggesting that EPF is a leading determining factor in the decline of gonadal development. Results from the immunohistochemical analysis revealed an increase of HSP70

expression in the eggs of American oyster ovary, which could lead to extensive developmental changes in gametogenesis, embryogenesis, and metamorphosis from mutations in the DNA sequence (David 1997, Ueda et al. 2009).

An interesting result of this study is the increasing trend in the number of apoptotic eggs/cells and caspase-3/7, a master enzyme of apoptosis (Mosser et al. 1997), activity in oyster ovary exposed to high temperature. Caspase-3/7 serves as a critical heat-sensitive target leading to cell death as activated by a subunit of the heat shock protein, HSP70 (Mosser et al. 1997). It has also been shown that marine bivalves exposed to environmental stress protect themselves by increasing peroxynitrite, a RNS (Shumway 1996). Peroxynitrite is formed by the combustion of nitric oxide and a superoxide radical that reacts with tyrosine residues such as 3-nitrotyrosine protein (NTP, a RNS) (Torreilles and Romestand 2001). In this study, we have shown that increases in caspase-3/7 activity, PC contents, NOx levels, and NTP and DNP expressions from elevated temperature are the limiting factors responsible for apoptosis and indicators of oxidative stress. Since caspase activity is changing with increasing temperatures (Fig. 4f), this may cause DNA damage and/or DNA methylation in oyster gonads (Gavery and Roberts 2010).

Conclusions

This study, to the best of our knowledge, reports the first clear findings of the impacts of short-term high temperature on ovarian functions, extrapallial fluid conditions, cellular apoptosis, and oxidative stress in the American oysters that inhabit marine coastal environments along the entire Atlantic coasts to the Gulf of Mexico. Here, average water temperatures are not only rising in response to changing global climate, but are also regionally susceptible to increased instance of extreme heatwaves (Turner et al. 2017, Jones et al. 2019, Deng et al. 2018). This research not only progressed our knowledge on the effects of higher temperatures in oyster reproductive functions, but also the potential mechanisms and implications that are associated with damage from increasing temperatures such as increased HSP70 expression, apoptosis, and oxidative stress in marine mollusks. Collectively, our data propose that higher temperatures from global climate change will result in negative impacts of ovarian development and reproductive performances in the American oyster. Seawater conditions such as temperature, biotic factors, seasonality, and acidification (Parker et al. 2018), all play vital roles in influencing gonadal development in marine biota. The 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 its 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. Future research will be focused on epigenetic modifications such as global DNA methylation, a heritable epigenetic mark involving the covalent transfer of a methyl group to DNA-base cytosine (Riviere 2014, Suarez-Ulloa et al. 2015), in gonads of oyster exposed to high temperature, as well as a more in-depth analysis of the impacts of gonadal functions in male oyster exposed to high temperature.

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Author contributions S.N. and M.S.R. designed the experiment, S.N. ran the experiment, S.N. prepared histology and immunohistochemistry slides, S.N. and J.J. analyzed histology slides, S.N. and M.S.R. analyzed apoptosis, S.N. and M.S.R. analyzed the biological and biochemical indicators of body fluid and ovarian tissues, S.N. and M.S.R. led to writing the manuscript. S.N., J.J. and M.S.R. reviewed the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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