Title: Impacts of Elevated Temperature, Decreased Salinity and Microfibers on the Bioenergetics and Oxidative Stress in Eastern Oyster, *Crassostrea virginica*

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Abstract

Projected increases in temperature and decreases in salinity associated with global climate change will likely have detrimental impacts on eastern oyster, *Crassostrea virginica*, as these variables can influence physiological processes in these keystone species. We set out to determine how the interactive effects of temperature (20°C or 27°C) and/or salinity (27‰ or 17‰) impacted the energetic reserves, aerobic and anaerobic metabolism, and changes to oxidative stress or total antioxidant potential as a consequence of an altered environment over a 21-day exposure. Gill and adductor muscle were used to quantify changes in total glycogen and lipid content, Electron Transport System and Citrate Synthase activities, Malate Dehydrogenase activity, Protein Carbonyl formation, lipid peroxidation, and total antioxidant potential. A second exposure was performed to determine if these environmental factors influenced the ingestion of microfibers, which are now one of the leading forms of marine debris. Elevated temperature and the combination of elevated temperature and decreased salinity led to an overall decline in oyster mass, which was exacerbated by the presence of microfibers. Changes in metabolism and oxidative stress were largely influenced by time, but exposure to elevated temperature, decreased salinity, the combination of these stressors or exposure to microfibers had small impacts on oyster physiology and survival. Overall these studies demonstrate that oyster are fairly resilient to changes in salinity in short-term exposures, and elevations in temperature or temperature combined with salinity result in changes to the oyster energetic response, which can be further impacted by the presence of microfibers.

1. Introduction

Eastern oyster, *Crassostrea virginica*, are widely distributed along the U.S. Atlantic coast and Gulf of Mexico, and are economically and ecologically important within estuarine environments. In 2020, 90 million pounds of marine aquaculture species were harvested from growers in the United States, netting $430 million. Approximately 42 million pounds of this yearly harvest came from oyster, with aquaculture facilities on the Atlantic coast earning over $134 million (NMFS, 2022). As a keystone species, oysters serve as "ecosystem engineers" within the estuary. Oyster reefs provide habitat for numerous species of shrimp, fish and crab, and also provide protection from coastal erosion. Additionally, these sessile bivalves rapidly filter their surrounding water for food, resulting in improved water quality and clarity (Kennedy, 1996). Oysters can filter particulates of 4-10µm out of the water column with nearly 100% efficiency (Ward and Kach, 2009; Ward et al., 2019), and have therefore been recognized as a valuable indicator species for aquatic pollution and environmental contamination.

Estuaries are one of the most dynamic environments on the planet, experiencing drastic changes in temperature, salinity, pH and dissolved oxygen (DO) on a daily basis. Oscillations in these environmental parameters are naturally driven by the processes of primary production (photosynthesis) and organismal respiration, coupled with tidal flow in and out of the estuary (Duarte et al., 2013; Baumann et al., 2015; Hendricks et al., 2015). Sessile bivalves that inhabit estuarine environments must therefore rely on physiological plasticity to cope with such changes (Pörtner, 2001). Eastern oyster have a reported temperature tolerance of -2°C to 36°C, and salinity tolerance of 5-40‰ (Galtsoff, 1964; Shumway, 1996). In western Long Island Sound, *C. virginica* exist sub-tidally, experiencing average summer water temperatures around 20°C, with average salinities around 27‰ (NOAA, 2023).

Burning of fossil fuels has led to an overall warming of the planet of about 1.0°C since the late 1800's. Much of this heat has been absorbed by the world's oceans, leading to an average increase in sea surface temperatures of about 0.6°C compared to pre-industrial values, heightening the thermal stress experienced by organisms that permanently reside in estuaries (IPCC, 2014). For *C. virginica*, increases in water temperature can have deleterious effects on physiological processes such as development, growth and reproduction (Shumway, 1996). In the northeast, climate change impacts extend to drastic changes in precipitation patterns; the frequency of heavy rain events has increased by over 55% in the last 100-years, decreasing the salinity of estuarine waters, and increasing pollution and contaminants from surface run-off (Easterling et al., 2017; Huang et al., 2017). As oyster are established osmoconformers (Prosser, 1973), any change in salinity necessitates regulating osmotic pressure of intracellular fluids, and has the potential to negatively impact oyster physiology to the same extent as changes in temperature (Prosser, 1973, Shumway, 1996). The combined effects of temperature and salinity changes can therefore affect almost every aspect of oyster physiology, predation rates and parasite-disease interactions (Shumway, 1996).

A frequent response in oyster under physiological stress is valve closure and a subsequent switch from aerobic to anaerobic metabolic pathways (Galtsoff, 1964; Loosanoff, 1966). Studies that have measured enzymatic activities relating to metabolism and oxidative stress in adult oyster have largely focused on the impacts of temperature or temperature combined with metrics such as ocean acidification or trace metal exposure (Dunphy et al., 2006; Lannig et al., 2006; Ivanina et al, 2013; Matoo et al., 2013; Li et al., 2017). Research exploring the impacts of osmotic stress or the interactive effects of temperature and salinity on oyster physiology have primarily focused on whole-organism metrics such as survival, growth, oxygen consumption,

and feeding and/or clearance rate (Heilmayer et al., 2008; La Peyre et al., 2013; Lowe et al., 2017; Casas et al. 2018; review in Pourmozaffar et al., 2020), impacts on hemolymph (Tirard et al., 1997), or the transcriptomic response of these stressors (Chapman et al., 2011; Jones et al; 2019). Tissue-level impacts of salinity changes have been documented (Wickes and Morgan, 1974; Cripps, 1977; Ballantyne and Berges, 1991), leaving the area of tissue-level bioenergetic response to the interactive effects of temperature and salinity relatively unexplored, despite these stressors changing concomitantly in Long Island Sound.

Adding complexity to the issues of how oyster may physiologically respond to projected environmental changes are the presence of microplastics (MP's); now considered to be the most prevalent form of debris in freshwater, oceanic and estuarine waters (Derraik, 2002; Andrady, 2011). Microplastic debris, defined as particles between 0.1µm-5mm, is comprised of spheres, pellets and synthetic fibers that remain suspended in the water column and are readily consumed by suspension filter feeders such as oyster (Arthur et al., 2009; Andrady, 2011; Wright et al., 2013; Cole, 2016; Waite et al., 2018). Microplastic contamination of estuaries is of particular concern as low tidal flushing rates maximize the residence time of particles. Several studies have confirmed estuarine bivalves to have greater amounts of MP's in their guts compared to their open-ocean counterparts (Bocchetti et al., 2008; Ward and Kach, 2009; Wegner et al., 2012), and much of this research has demonstrated microplastic spheres accumulate within the gills and gastrointestinal tracts of bivalves, negatively impacting their filtration efficiency and reproductive success (van Moos et al., 2012; Sussarellu et al., 2016; Smith et al., 2018; Carpenter et al., 2019). Interestingly, few experiments exploring the effects of microplastics in bivalves have included microfibers (MF's; microplastics released from synthetic clothing when washed, fragmented marine rope/net or fishing line), despite field data reporting microfibers are the most

prevalent form of microplastic debris ingested by bivalves (Mathalon, and Hill, 2014; Cole, 2016; Santillo et al., 2017; Waite et al., 2018).

While an established environmental stressor, studies that have addressed microplastic impacts through the lens of global climate change have largely explored how MP's contribute to oceanic warming, how changing environmental factors impact MP distribution and spreading, and overall impacts to ecosystems and biodiversity, focusing on bioaccumulation effects (See Sharma et al., 2023 and Tang, 2023 for reviews). Those studies that have aimed to discern the impacts of MP's on organismal physiology in tandem with climate change stressors, have focused on primary producers (Yang et al., 2020), freshwater ecosystems (Chang et al., 2022), or larval development (Bertucci and Bellas, 2021; Sui et al., 2022).

To this end, we investigated the interactive effects of elevated temperature, decreased salinity and microfiber exposure on the survival and physiology of eastern oyster, *Crassostrea virginica*. We specifically focused on measurements of energetic reserves (total glycogen and lipid content), and the enzymatic activity of aerobic (Citrate Synthase and Electron Transport System) and anaerobic (Malate Dehydrogenase) enzymes in gill and adductor muscles. Citrate Synthase (CS) is the first catalyzing step of the citric acid cycle, and has been established as a useful proxy of aerobic metabolism use in oyster of the genus *Crassostrea* (Ballantyne and Berges, 1991; Dunphy et al., 2006; Génard et al., 2011). Electron Transport System (ETS) activity is a useful estimate of energy consumption at the mitochondrial level, and has been used as a proxy for aerobic metabolic capacity (De Coen and Janssen, 1997; Andrade et al., 2018). Under anaerobic conditions, carbohydrates are utilized as the main source of energy in bivalves; ATP can still be generated using alternative metabolic pathways, resulting in the end products of succinate and alanine (de Zwaan, 1977). In bivalves under anaerobic conditions, glycolysis and

126 the citric acid cycle are connected by malate, which is then converted to succinate (de Zwaan,
127 1983). Therefore, Malate Dehydrogenase activity (MDH) was measured as a proxy for assessing
128 anaerobic metabolism use in oyster. It has been well established that increases in temperature are
129 linked to increased energy expenditure in ectotherms (Pörtner, 2001; Sokolova 2012). This
130 typically results in an increase in metabolic rate and subsequent generation of reactive oxygen
131 species; therefore, we quantified biomarkers for the oxidative stress response of oyster by
132 exploring changes in Protein Carbonyl (PC) formation, as well as malondialdehyde (MDA)
133 levels as an indicator of lipid peroxidation, and total antioxidant potential utilizing a ferric
134 reducing/antioxidant potential (FRAP) assay.

135 **2. Materials and Methods**

136 *2.1 Culture*

137 Adult *Crassostrea virginica* (mean weight of 102.7g ±17.2 and mean length 79.7mm ± 7.2) were
138 supplied from Copps Island Oyster Company in Norwalk, Connecticut. Once at the University of
139 Hartford, oyster were scrubbed of mud and epiphytes before being placed into one of two, 142-L
140 culture tubs, filled with artificial sea water (Instant Ocean, 27‰) and held at 20°C for at least
141 two weeks prior to experimentation. Temperature and salinity levels mimicked current summer
142 conditions in western Long Island Sound (NOAA, 2023). Water flow in culture tubs was
143 circulated at 12-L/min using a Penn-Plax 700 canister filter. Water quality (Ammonia, Nitrites,
144 Nitrates and pH) was measured daily for the first week in culture and once per week after (both
145 in culture and the exposure system). Water changes (10-20% of total water volume) were
146 performed if nitrogenous waste levels were elevated (above 0.25ppm). Temperatures were
147 checked daily with a hand-held K-type digital thermocouple (Gain Express, Hong Kong, China)
148 and refractometer (D-D True Seawater, Essex, UK) calibrated with distilled (DI) water once a

week. Oyster were fed LPB Shellfish Diet (Reed Mariculture, California, USA) diet three times a week at a density of 0.2-g/oyster both in culture and during experiments. All tanks were siphoned once a week to remove feces, pseudofeces, and maintain water quality.

2.2 Experimental Manipulations

Two separate experiments were performed, each lasting 21-days and using the same four treatments; a control, (C: 20°C and 27‰), a single stress treatment of decreased salinity (SS:DS: 20°C and 17‰), a single stress treatment of elevated temperature (SS:ET: 27°C, 27‰), and a multi-stress treatment (MS: 27°C and 17‰). Treatment levels were chosen based upon projected levels of Long Island Sound by the year 2300, and established physiological stress levels that are sub-lethal in *Crassostrea virginica* (Shumway, 1996; Sokolova, 2012; IPCC, 2014).

The recirculating exposure system consists of four mixing reservoirs for each treatment, which pumped water at a flow rate of approximately 1-L/min into four, 14-L experimental tanks randomly distributed on a shelving system. Tank effluent was pumped back into the mixing reservoir, where it was re-treated and aerated before circulating back to experimental tanks (See Supplemental Fig. 1). Treatment conditions were rotated between mixing reservoirs used in Experiment I and II to account for tank effects. Mixing reservoir conditions were monitored by Neptune APEX (Neptune Systems, California, USA) temperature and salinity probes and modules (PM2) calibrated with Neptune standards. Logging of these values was set for every 20-min over each experimental exposure. Elevated temperature treatments were generated and maintained using Eheim 150-watt thermostated heaters (Eheim, Germany) controlled by the Neptune APEX. Control temperatures were maintained using evaporative cooling fans (Petzilla, California, USA) in Experiment I. A small aquarium chiller (16-L, Poafamx, USA) as well as evaporative cooling fans were used to maintain a target of 20°C in the second experimental

exposure. Distilled water was added to each mixing reservoir when the salinity rose to 0.5ppt above target levels.

Oyster (n=18/treatment) were randomly selected from the culture tubs, weighed (Ohaus Navigator scale, New Jersey, USA), marked with colored lacquer, and placed into treatment tanks. Every 7 days, a sub-set of oyster (n=6/treatment), were weighed, and gill and adductor muscle collected and individually flash-frozen for later analysis. These tissues were selected for their aerobic (gill) and anaerobic (adductor muscle) functions in oyster (de Zwaan, 1983). All tissues were stored at -80°C until proteins were extracted.

2.3 Microfiber exposure

Microfibers were added to all treatments during Experiment II to explore impacts of MF exposure on oyster bioenergetics and oxidative stress. Blue MF's are found in high densities in oyster (Waite et al., 2018). We cut royal and navy-blue polyester and nylon threads (Coats and Clark, Connecticut, USA) to lengths of 1-3mm using a Bausch and Lomb StereoZoom 4 dissecting microscope (New Jersey, USA) at a density of 40 MF/oyster/treatment, which equates to 12 MF/L water in the exposure system (Waite et al, 2018). Microfibers were cured in 250-mL beakers for 5 days in culture water at 27‰ or culture water diluted with DI to 17‰ to allow a biofilm to form. An orbital shaker prevented the MF's from settling on the floor of the beakers, and maintained aeration of the water in the beaker. Once oyster were placed in experimental tanks, the entire volume of water and MF's was added to the sump. Aeration in the sump prevented MF's from settling, and tank inflow tubes were split to create circulation within each treatment tank that kept MF's suspended within the water column. This method of exposure allows oysters to filter MF's from the water column as in nature. Effluent water from tank cleaning was filtered and any MF's that were found in fecal matter were returned to the

appropriate treatment sump. Before tissues were collected each week, MF counts of gill and digestive tissues were performed under a dissecting microscope.

2.4 Biochemical Analyses

Proteins were extracted from oyster tissues by hand-homogenizing 50-70mg of frozen tissue on ice using a 10:1 ratio with buffers specific to each assay (described below). Homogenates were centrifuged, after which, cleared supernatant was immediately transferred to a fresh Eppendorf tube, aliquoted, and proteins quantified (Bradford, 1976). These supernatants were stored at -20°C until use.

2.5 Energy Reserves

Total glycogen content was quantified using the sulfuric acid method from Yoshikawa (1959). Samples were extracted using a 50mM potassium phosphate buffer containing protease inhibitor (pH=6.8), and were centrifuged for 10-min at 1,500g. Glycogen content was measured by incubating each sample (50µL) with 100µL of 5% phenol and 600µL of 60% H_2SO_4 at room temperature for 30-min and measuring absorbance of 250µL of sample in a polystyrene 96-well plate at 492nm (TECAN Infinite M Nano spectrophotometer with pathway correction; TECAN, North Carolina, USA). Total glycogen was determined comparing duplicate samples to a standard curve created from powdered oyster glycogen (Sigma Aldrich).

Total lipid content was measured using methods from Enzor et al. (2017). Samples were extracted using a 1x phosphate buffer solution with 1% Triton X-100 and centrifuged as described above. Duplicate samples (10µL) were combined with 290µL of Infinity™ Triglycerides Reagent and incubated at room temperature in the dark on an orbital shaker for 5-min, after which they were read at 500nm. Total triglycerides were calculated using a standard curve created from a Stan-Bio Triglyceride Standard (2g/L).

2.6 Metabolic Pathways

Aerobic metabolic pathways were investigated using spectrophotometric measurements of Citrate Synthase (CS) activity and Electron Transport System (ETS) activity. Levels of CS activity were quantified with tissue homogenates extracted using 50mM potassium phosphate buffer described above. Samples (10µL) were loaded in duplicate onto a 96-well plate; 200µL of CS buffer (50mM Imidazole pH=8.32, 1.5mM $MgCl_2$, 0.2mM Ellman's Reagent, 0.3mM Acetyl CoA, 1.0mM oxaloacetate) was added to initiate the reaction. A second set of duplicates was used as a blank and 200µL of CS buffer without substrate was added to measure background rates. The spectrophotometer was set to 412nm using a kinetic sweep of 20-min. Samples were run at their corresponding treatment temperature of 20°C or 27°C. The read was started immediately after oxaloacetate was added, and the plate shaken within the spectrophotometer (5-sec). Specific activity of CS was calculated by subtracting the mean background rate from the mean activity rate of each sample, and converting the difference into International Units (I.U.) using the extinction coefficient of 13.6mM/cm. Activities were standardized per g-protein in each sample.

Electron Transport System activity was measured using methods from King and Packard (1975), modified by De Coen and Janssen (1997). Gill and adductor muscle samples were extracted using a Tris-HCl buffer (0.1M Tris-HCl, pH=8.5, 15% [w/v] Poly vinyl pyrrolidone, 153µM $MgSO_4$, 0.2% [w/v] Triton X-100) and centrifuged at 3,000g for 10-min. Samples (50µL) were loaded in duplicate into a 96-well plate, after which 150µL of buffered solution (0.13M Tris HCl, 0.3% [w/v] Triton X-100, pH=8.5) and 50µL of NADPH solution (1.7mM NADH and 250µM NADPH) were added to each well. Reactions were initiated by the addition of 100 µL of 8mM p-Iodonitro Tetrazolium (INT). The spectrophotometer was set to 490nm

using a kinetic sweep of 10-min. Samples were run at their corresponding treatment temperature of 20°C or 27°C. The read was started immediately after INT was added, and the plate shaken within the spectrophotometer (5-sec). Specific activity of ETS was determined by the amount of formazan formed, using the extinction coefficient of 15,900M/cm. Activities were standardized per mg-protein in each sample.

Anaerobic metabolism use was quantified by measurements of cytosolic Malate Dehydrogenase (MDH) activity. Samples were extracted and measured as described under CS activity above, with a different assay buffer (200mM Imidazole pH=7.15, 0.2mM NADH, 0.2mM oxaloacetate), a wavelength of 340nm, and calculations used the extinction coefficient 6.22mM/cm.

2.7 Oxidative Stress and Antioxidant Potential

Levels of oxidative stress were investigated using measurements of Protein Carbonyl (PC) formation, and levels of malondialdehyde (MDA), which is a by-product of lipid peroxidation. Both measurements used samples that were extracted using a 50mM phosphate buffer solution, as described above. Protein Carbonyl formation was quantified using the alkaline method from Mesquita et al (2014), at a wavelength of 450nm. Samples were measured in duplicate and run at their corresponding treatment temperature. Activity was calculated using the average absorbances and the micromolar extinction coefficient of 2,4-dinitrophenylhydrazine reagent under alkaline conditions (.022308 µmol/cm at 450nm), and were standardized per g-protein in each sample.

Levels of MDA were determined using a thiobarbituric acid assay (Uchiyama and Mihara, 1978; Ringwood et al., 1999). Both standards of known MDA concentration and samples were mixed with 0.375% thiobarbituric acid and 2% butylated hydroxytoluene in a

1:14:0.14 ratio, heated for 5-min at 100°C, and centrifuged at 13,000g for 5-min. Duplicate 100µL samples were run at 532nm and MDA levels were expressed in µmol per g-protein in each sample.

Total Antioxidant Potential were measured using the ferric reducing/antioxidant potential (FRAP) analysis methods of Griffin and Bhagooli (2004). Samples (20µL) that were extracted using a 50mM potassium phosphate buffer were run in triplicate at 595nm at their corresponding treatment temperature, and a standard curve of concentrations of $FeSO_4*7H_2O$. Samples were standardized by g-protein.

2.8 Statistical Analysis

All data were analyzed using two-way Analysis of Variance (ANOVA) for the main effects of exposure time, treatment, and interaction between these main effects. Levene's test for Homogeneity of Variance was used, along with a Shapiro-Wilk test for normal distribution of data. A Tukey's HSD post-hoc test was performed to investigate pair-wise differences between treatments at each time-point of exposure. A second set of two-way ANOVA and Tukey's HSD post-hoc tests were performed to directly compare the measured responses of oyster from Experiment I and Experiment II. Findings were considered significant if $p<0.05$.

3. Results

Average temperature and salinity values over the course of both experiments are reported in Table 1. We were able to maintain elevated temperatures and/or decreased salinities at target levels in all treatments over the course of both 21-day exposures.

Mortality was low across both experiments, and was largely driven by increases in temperature. Neither experiment had any mortality during the first week of exposure. In Experiment I, only one oyster died (5% mortality in total), which was from the multi-stress

287 treatment during week 3. In Experiment II, a total of three oyster died during the second week of
288 experimentation. One oyster from the single stress of decreased salinity (5% mortality in total),
289 and two from the single stress treatment of elevated temperature (11% mortality in total, Table
290 2). No oyster died during the third week of experimental exposure.

291 Both navy and royal blue MF's were found in feces and pseudofeces during tank
292 cleanings; no clear trend of MF color or density across treatments was apparent (Table S1).
293 Microfibers were only found sequestered on gill tissues at the 7-day time-point in four different
294 oyster. All fibers were navy blue, and came from one oyster in the decreased salinity treatment
295 (one fiber counted), two oyster in the elevated temperature treatment (three fibers counted), and
296 one oyster in the control treatment (one fiber counted).

297 *3.1 Oyster Weight*

298 Experiment I: There was an overall trend of oyster losing mass across the 21-day exposure
299 period (Fig. 1, $p=0.028$, Table 3). Weight change at the 7-day timepoint was significantly
300 different than weight change at 14-days of exposure ($p=0.024$), most notably due to increases in
301 temperature. Oyster in the SS:ET treatment showed a larger change in weight from 7-days
302 (average gain 0.03g) to 14-days (loss of 0.43g, $p=0.019$). Oyster in the multi-stress treatment
303 continuously lost weight over the course of the exposure, with the largest decline occurring
304 between 7 and 14-days (average loss of 0.46g), with a significant decrease at 21-days of
305 exposure compared to control values (Fig. 1A, $p=0.037$).

306 Experiment II: Oyster in the control treatment maintained or gained weight at all three
307 timepoints in this exposure (Fig. 1B). There were significant main effects of treatment ($p<0.001$,
308 Table 4), yet no significant change in weight was noted between treatments at 7-days of
309 exposure. However, at 14-days, oyster in both the elevated temperature treatment and multi-

stress treatment lost significantly more weight compared to control oyster (Fig. 1B, $p=0.005$, $p<0.001$, respectively). This trend continued into 21-days in oysters exposed to both elevated temperature and decreased salinity in tandem (Fig, 1B, $p=0.005$).

When weight change in Experiments I and II were compared, a significant difference was found in control oyster at 7-days of exposure ($p=0.002$), and at all three time-points in oyster exposed to decreased salinity as a single-stressor (7-day: $p=0.002$, 14-day: $p=0.019$, 21-day: $p=0.028$). We recognize that Experiment I temperature levels (control and SS:DS) were higher than Experiment II, and could explain some of the difference in weight change seen between control oyster.

3.2 Energy Reserves

Gill Tissue: A significant decline in glycogen content was noted in gill tissues across treatments between 7 and 14-days of exposure in Experiment I oyster, and these levels remained low for the remainder of the exposure (Fig. 2A, $p<0.001$). A main effect of treatment was found in gill tissues ($p=0.033$, Table 3); total glycogen levels were below control values in all treatments across time, and were significantly different in multi-stress oyster at 7-days of exposure (Fig. 2A, $p=0.035$). The addition of MF's resulted in small changes to glycogen content in gill tissues, all below the significant level (Fig. 2C, Table 4). This resulted in glycogen content from Experiment I and Experiment II oyster being significantly different, most notably in the single stress treatment of decreased salinity ($p=0.013$) and the multi-stress treatment (Fig. 2A and C, $p=0.002$).

Similar results were seen when gill tissues were analyzed for total lipid content. Experiment I oyster showed a significant main effect of time ($p<0.001$, Table 3), with a notable lack of lipid use seen at 14-days of exposure when compared to 7 ($p<0.001$) and 21-days (Fig.

2E, p=0.016). No significant findings were found from Experiment II oyster, or when lipid levels of gill tissues between experiments were compared.

Adductor Muscle: A significant main effect of exposure time was seen in Experiment I oyster ($p<0.001$, Table 3), with a notable decline in glycogen content seen between 7 and 14-days of exposure (Fig. 2B, $p<0.001$). As with gill tissues, only small, non-significant changes in glycogen content were seen in adductor muscle samples in Experiment II oyster (Fig. 2D). When compared, the presence of MF's did have a notable effect on glycogen content as values from control oyster were significantly different at both 7 ($p=0.005$) and 21-days of exposure (Fig. 2B and D, $p=0.026$). Total lipid content from Experiment I oyster were similar between gill and adductor muscle tissues; a main effect of time was noted ($p<0.001$, Table 3), with a significant drop in 7 ($p<0.001$) and 21-day levels ($p=0.005$) when compared with 14-day samples (Fig. 2F). A significant main effect of exposure time was seen in adductor muscle in Experiment II oyster ($p=0.002$, Table 4). Lipid levels remained low at 7 and 14-days of exposure, and significantly increased at 21-days across treatments (Fig. 2H, $p=0.006$ and 0.016, respectively). The increase in total lipid content at 14-days (Experiment I) compared to 21-days (Experiment II) resulted in significant differences between exposures, specifically within control and SS:ET samples at 14-days ($p=0.038$, $p=0.021$, respectively), and only in the SS:ET oyster at 21-days (Fig. 2F and H, $p=0.004$).

3.3 Citrate Synthase Activity and Electron Transport System Activity

Gill Tissue: Two-way ANOVA did not find any main level effects of exposure time or treatment in gill tissue in Experiment I oyster CS activity, however, a Tukey's HSD post-hoc test found a significant decrease in the SS:DS treatment compared to control at 7-days (Fig. 3A, $p=0.033$, Table 3). Exposure to MF's did seem to affect CS activity in gill tissue; a main effect of time was

seen ($p=0.019$, Table 4), with CS levels steadily increasing across all treatments (Fig. 3C). Overall, CS levels were higher in Experiment II oyster in all treatments, however this was only found to be significant in the multi-stress oyster (Figs. 3A and C, $p=0.028$). Indeed, CS levels at 21-days in oyster exposed to increased temperature, decreased salinity and MF's showed the highest activity of 1.4 I.U/g-protein, compared to a value of 0.6 I.U./g-protein in oyster that were exposed to altered environmental conditions only.

We noted a distinct difference in the ETS activity between Experiment I and II oyster. Electron Transport System activity in Experiment I oyster displayed a main effect of time ($p<0.001$, Table 3). Activity levels remained similar between 7 and 14-days of exposure, with a marked increase in activity seen at 21-days (Fig. 3E, $p<0.001$). Gill tissues from Experiment II oyster also showed a main effect of time ($p=0.001$, Table 4), but the levels of ETS activity significantly rose at 14-days of exposure, a week sooner than noted in our first experiment (Fig. 3G, $p=0.001$). This difference in timing resulted in ETS values being significantly different between oyster across all treatments at 14-days of exposure between the two experiments (Fig. 3E and g, Control: $p=0.001$, SS:DS: $p=0.021$, SS:ET: $p=0.001$, MS: $p=0.038$).

Adductor Muscle: A significant effect of exposure time was found in both Experiment I and II oyster when analyzed for CS activity (Figs. 3B and D, $p=0.021$ and $p<0.001$, respectively, Table 3 and 4), however no specific treatment effects were noted. As with gill tissues, CS levels in oyster adductor muscle exposed to MF's were higher than those that were not. This elevation was found to be significantly different between the two experiments in multi-stress oyster at 14-days of exposure ($p=0.009$), and in all three treatments at 21-days (Fig. 3B and D, SS:DS: $p<0.001$, SS:ET: $p=0.028$; MS: $p=0.010$).

No significant main effects were noted in Experiment I or II when adductor muscle samples were analyzed for ETS activity. When directly comparing the two exposures, temperature had a significant effect, with the SS:ET samples being significant different at both 7-days ($p=0.045$) and 14-days ($p=0.016$) of exposure (Fig. 3F and H).

3.3 Malate Dehydrogenase Activity

Gill Tissue: No effects of treatment were noted in gill tissue from Experiment I oyster, but a main effect of time was seen (Fig. 4A, $p=0.001$, Table 3). An increase in MDH activity across treatments was noted, with single stress treatment values increasing to approximately 7 I.U./g-protein (Fig. 4A). Enzyme activities in the multi-stress oyster peaked at 14-days and remained at that level through 21-days of exposure. No main level effects were seen in Experiment II oyster (Table 4), yet MDH activity was decreased below control values at all time-points (Fig. 4C). This decrease was significant in both high temperature treatments at 7-days (SS:ET: $p=0.039$, MS: $p=0.002$), the single stress of elevated temperature alone at 14-days ($p=0.041$), and at both high temperature treatments at day 21 (Fig. 4C; SS:ET: $p=0.009$, MS: $p=0.033$).

Adductor Muscle: Overall, MDH levels were lower in adductor muscle compared to gill tissues (Figs. 4B and D). A main effect of time was noted in Experiment I ($p=0.006$, Table 3), largely driven by an increase in MDH activity in the low salinity treatment from 1 to 4.5 I.U/g-protein (Fig. 4B; $p=0.002$). Experiment II oyster samples showed a main effect of temperature (Table 4), with enzymatic activities of one or both of the two high temperature treatments significantly lower compared to control values at 7-days (Fig. 4D; MS: $p=0.049$), 14-days of exposure (SS:ET: $p=0.041$; MS: $p=.018$), and 21-days of exposure (SS:ET: $p=0.044$). No differences between MDH activity levels were noted between Experiment I or II oyster, regardless of tissue type.

3.4 Protein Carbonyl Formation, Lipid Peroxidation and FRAP Analysis

Only small changes were noted when evaluating oyster tissues for PC formation. No significant effects of exposure time or treatment were found in gill tissues of either Experiment I or II oyster (Table 3 and 4), and no differences were found when comparing PC levels between experiments (Figs. 5A and C). When evaluating adductor muscle for PC formation, Experiment I oyster showed a significant effect of treatment (Fig.5B $p=0.015$, Table 3). Protein Carbonyl formation in the multi-stress treatment was significantly lower than control across time, ($p=0.011$) and at 21-days of exposure, the SS:ET treatment levels were significantly lower than control values (Fig. 5B5.4 vs. 13 mmol/g-protein; $p=0.025$). No discernable differences were seen in adductor muscle of oyster from Experiment II (Figs. 5D, Table 4).

A main effect of time was found when gill tissues from Experiment I oyster were evaluated for MDA levels (Fig. 5G $p=0.001$, Table 3). Levels of MDA increased over time with 7-day measurements significantly elevated above 21-day (Fig. 5G, $p<0.001$). While no main level effects were seen in gill tissue from Experiment II oyster (Table 4), MDA levels across all treatments were significantly higher than Experiment I oyster (Fig.5E and G, $p<0.001$ in all cases), indicating microfiber exposure had a pronounced effect on lipid peroxidation in gill tissues.

Adductor muscle samples from Experiment I showed a main effect of time ($p<0.001$), treatment ($p=0.037$) and a significant interaction ($p=0.006$) between these (Table 3). As with gill tissue, MDA levels appeared to significant increase over time (Fig. 5F, $p<0.001$). Experiment II oyster did not show any significant changes to MDA levels over time or in response to treatment (Fig. 5H, Table 4), but as with gill tissues, MDA levels in adductor muscle were significantly elevated in this exposure when compared to Experiment I (Fig. 5H, $p<0.001$ in all cases).

Gill tissues in both Experiment I and II oyster did not show any treatment effects when analyzed for total antioxidant potential, however an increase in antioxidant levels was seen in all treatments between 14 and 21-days of exposure in Experiment II oyster (Figs. 6A and C, $p=0.041$). Overall FRAP levels were similar in gill between the two experiments, and no differences between Experiments I and II were seen.

Total antioxidant potential of adductor muscle was significantly affected by treatment in Experiment I oyster (Fig. 6B, $p<0.001$, Table 3). An increase in FRAP level was found in response to temperature, most notably at 7 (SS:ET: $p=0.003$, MS: $p=0.006$) and 14-days (Fig. 6B; SS:ET: $p=0.047$, MS: $p=0.044$). By 21-days of exposure, levels in these treatments had dropped and were no longer different from control values of 0.95 mol/g-protein (Fig. 6B). Oyster exposed to MF's displayed a main effect of exposure time (Fig. 6D, $p=0.040$, Table 4), but no specific treatment effects were noted.

4. Discussion

In this set of experiments, we sought to discern the effects of exposure to elevated temperature, decreased salinity and MF's on the levels of energetic reserves, activity of aerobic and anaerobic enzymes, oxidative stress and subsequent antioxidant response in eastern oyster, *Crassostrea virginica*. To our knowledge, this is the first study to explore these stressors in tandem using this species, and the only exposure to allow oyster to passively filter microfiber from recirculating treatment waters, versus directly introducing microplastics at the shell aperture, or placing oyster in a container without water flow for a set period of time (i.e., a static system).

4.1 Effects of Temperature or Salinity

Survival, Energetic Stores and Metabolism: Oyster exposed to 27°C for 21-days in this study showed a significant drop in glycogen content in both gill and adductor muscle tissues between 7

447 and 14-days of exposure, indicating that energetic stores were being utilized. Lipid content did
448 change in these tissues as well, but not until later in the exposure period (between 14 and 21-
449 days), lending additional evidence that glycogen stores are the preferred first source of stored
450 energy in this species, particularly in response to elevated temperature (Galtsoff, 1964; de
451 Zwaan, 1983; Li et al., 2007; Yang et al., 2016). We also did not observe any mortality in our
452 oyster held at elevated temperatures during our first 21-day experiment, which could possibly be
453 explained by the use of glycogen stores we noted to off-set thermal stress. Previous studies on
454 bivalves have reported varying results in the metabolic response, use of energy stores, and
455 survival to elevations in temperature. Exposure to elevated temperature of 27°C from 22°C for
456 15-weeks resulted in marked decreases in tissue glycogen and lipid content in *C. virginica*,
457 indicating a shift from aerobic to anaerobic pathways (Ivanina et al., 2013). However,
458 measurements at both 2 and 8-weeks of this same exposure did not show any changes in
459 glycogen or lipid content as a result of elevated temperature, (Ivanina et al., 2013) nor were there
460 and significant changes to $\dot{M}O_2$ at these timepoints (Matoo et al., 2013). Oyster mortality of 20%
461 and 30% at 2 and 5 weeks, respectively, were noted in this study, despite any lack of energetic
462 store use (Ivanina et al., 2013).
463 Multiple studies have measured increases in ETS activity when bivalves are under physiological
464 stress, but this is not always coupled with other metrics associated with aerobic metabolism use
465 such as CS activity, or with changes of energetic reserve use or organism condition. We found a
466 notable increase in ETS activity in gill tissues from oyster in our study across treatments.
467 However, we did not find any change in CS activities, despite a notable decrease in energetic
468 reserves and oyster mass. A temperature increase of 8°C for 20-days more than doubled ETS
469 activity in *Crassostrea gigas*, with no changes seen in adenylate energy charge (Le Moullac et

al., 2007). Moreira and colleagues found a notable increase in ETS activity when *C. gigas* was exposed to elevated temperatures of 4°C for 28-days, but this was not coupled with any changes in glycogen content (2017). A significant increase in ETS activity was found after three hours of air exposure in *Mytilus galloprovincialis*, which also resulted in a decrease in lipid stores (Andrade et al., 2018). While ETS activity was not measured in a study by Dunphy and colleagues, a temperature increase of 5°C had no impact on the CS levels in adductor muscle of *C. gigas* at two or 16 weeks of acclimation (2006). A 30-day exposure to temperature increases of 8°C (20° to 28°C) doubled standard metabolic rates, decreased survival by 25%, but did not affect condition index in *C. virginica* (Lannig et al., 2006). In their study, Lanning et al (2006) postulated that 28°C is the suboptimal/pejus temperature for *C. virginica*; our data support this idea; approaching 28°C, we noted an increase in ETS activity and decrease in glycogen stores, despite no change in CS activity and decrease in oyster mass.

It is generally accepted that under hypo-osmotic stress, oyster will close their valves, and heavily rely on anaerobic sources of fuel for metabolic processes (See Pourmozaffar et al., 2020 for a review). While not directly measured in these experiments, we did note oyster in our low salinity treatments spent more time with their valves closed compared to control, elevated temperature, and multi-stress oyster. Despite the increase in ETS activity and decreases in energetic stores described above, exposure to decreased salinity significantly dropped CS activity in gill tissues at 7-days of exposure in our study. Changes in salinity seem to have less impact on energetic reserve and ETS activity in bivalves when compared to temperature effects. A decline of salinity of 5ppt had no impacts on glycogen stores or ETS activity levels over a 4-week exposure in *M. galloprovincialis* (Freitas et al., 2019). Glycogen stores were negatively impacted by both increases and decreases in salinity in clam *Venerupis philippinarum*, and mortality was

493 increased when salinities were dropped to 7 g/L compared to control values of 28 g/L (Carregosa
494 et al., 2014). However, only an increase in salinity was found to cause glycogen store use over a
495 48-hour exposure in *Crassostrea corteziensis* (Pérez-Velasco et al., 2022). A 4-6 week exposure
496 to hypo-osmotic stress showed a decrease in CS activity in both gill and adductor muscles but
497 these differences were not at a significant level (Ballantyne and Berges, 1991); our data follow
498 this trend; providing additional evidence that when presented as a single stressor, oyster can
499 compensate for hypo-osmotic stress in a relatively short time period. Indeed, MacFarland and
500 colleagues (2013) demonstrated complete osmotic equilibrium was achieved in *C. virginica* after
501 20 hours of exposure to hypo-osmotic stress in levels similar to this study (i.e., a change of
502 10‰).

503 Anaerobic Metabolism: Overall, MDH levels were decreased below control levels in
504 response to temperature in both gill and adductor muscle samples in this study. Interestingly, we
505 found MDH levels were higher in gill tissue compared to adductor muscle. Our findings concur
506 with other studies that have explored anaerobic metabolic activity in *C. virginica.* A decline in
507 MDH activity was noted when temperatures were increased from ~11°C to 23°C in *C. virginica*
508 adductor muscle (Ulrich and Marsh, 2006). This is further supported by work by Chambers and
509 colleagues (1975), who noted a significant seasonal effect on this enzyme; summer levels were
510 lower compared to winter values. This finding was also found in *C. virginica* when seasonal
511 effects were combined with lab-induced anoxia; MDH levels were naturally higher in colder
512 months (Greenway and Storey, 1999).

513 Cripps found a small decrease in MDH activity in gill tissues as a result of hypo-osmotic
514 stress (decrease from 20‰, to 8‰) after 20-days of acclimation, however MDH levels
515 significantly increased above control levels in adductor muscle (1977). Our data show a

consistent pattern of MDH levels at or near control-values across all three time points; the difference in our measurements are likely due to two possibilities. The first is population differences; oyster in Cripps study were collected from an intertidal population in Texas, versus our sub-tidal population from Long Island Sound. Previous research has shown variability in the stress response of intertidal versus sub-tidal species (Dunphy et al., 2006; Meng et al., 2018), which could explain the difference in MDH measurements between the two studies. While we do not know which growing beds the oyster in this experiment were collected from, we do know they were from the same general region of western Long Island Sound. Second is timing of collection; bioenergetics of oyster drastically change over seasons, largely due to reproductive events (Greenway and Storey, 1999). There is no mention of when oyster were collected in the Cripps study; we used oyster that were harvested in January and February to avoid any impacts of reproductive investments into tissues.

4.2 Oxidative Stress and Antioxidant Response

Protein Carbonyl formation is an accepted measurement for levels of oxidative stress, which is typically accompanied by a change in antioxidants to off-set reactive oxygen species generated from increases in cellular respiration rates, most notably in response to increases in temperature (Lesser, 2006; Bal et al., 2021). As a second metric, we included measurements of lipid peroxidation to further elucidate if exposure to changes in temperature and /or salinity caused oxidative stress in oyster. Changes in free radical formation and antioxidant levels have also been measured as a response to disruptions of osmoregulation in both hypo and hypersaline environments in osmoconformers, but the response is highly variable (See Rivera-Ingraham and Lignot, 2017 for overview). There were no impacts of either increased temperature or decreased salinity on the PC formation of oyster in this study, but levels of MDA steadily increased over

our exposure period, indicating oxidative stress was present. Further evidence for this was the increase in total antioxidant potential in adductor muscle at both 7 and 14-days of exposure in the two high temperature treatments. Our data concur with findings from Matoo and colleagues, who found no increase in protein carbonyl formation at 2 or 8 weeks of exposure to 27°C; however, no difference in total antioxidant potential were found at 2 weeks of acclimation in their study, and levels dropped at 8-weeks (2013). Li and others found acute heat stress of 15°C resulted in accumulation of MDA in *C. gigas* species after 24-hours, but levels of superoxide dismutase declined during this same time-period (2017). A lack of change in lipid peroxidation, PC formation or antioxidant capacity was also found in *C. gigas* when exposed to multiple immersion-emersion cycles (Bruhns et al., 2023). Interestingly, the single stress of elevated temperature (8°C for 28-days) resulted in decreased levels of lipid peroxidation with no corresponding changes to superoxide dismutase or catalase in this same species (Moreira et al., 2017). The discrepancy in response requires further exploration, but the difference in findings could be due to methodology of measurement.

4.3 Combined Effects of Temperature and Salinity

The combination of elevated temperature and decreased salinity in this study significantly decreased oyster weight after 21-days of exposure. Electron Transport System activity levels did increase over time coupled with a decline in glycogen and lipid reserve, but there was no treatment effect noted in these metrics, and no changes in CS activity were found. Previous work has shown that when these environmental stressors are combined, bivalves will increase metabolic rates, but this is not always accompanied by an increase in feeding/clearance rate, leading to an overall decline in oyster condition, decrease in growth and even increased mortality (Heilmayer et al., 2008; Jones et al., 2009; Sokolova et al., 2012; La Peyre et al., 2013; Rybovich

et al., 2016; Casas et al., 2018; Bae et al., 2021). Studies have shown that the behavioral valve closure resulting from osmotic stress leads to decline in respiration rate, and increase in anaerobic metabolic pathways (See Pourmozaffar et al., 2020). In our study, we did not note any increase in MDH levels as a response to environmental perturbation. It is possible these findings would be different with a longer exposure period, or larger deviation from current environmental conditions. The low salinity level used in this study (17‰) is low for Long Island Sound, but is within the accepted salinity range of *C. virginica* (Loosanoff, 1966). Studies exposing oyster to hypo-osmotic stress noted a marked increase in mortality when high temperatures were combined with extremely low salinity values (1-5‰; La Peyre et al., 2013; Rybovich et al., 2016); we also observed mortality, but the 5% mortality of our multi-stress treatment (one oyster) is well below the reported mortality levels in other studies. Levels of protein carbonyl formation, MDA and total antioxidant potential responded very similarly to the combination of temperature and salinity to the effects of temperature alone; this lends further evidence to the supposition that increases in temperature largely drive the metabolic response of ectotherms.

4.4 Effect of Microfibers

While minimal, the presence of MF's did have a deleterious effect on oyster when compared to the response of environmental change alone. Oyster exposed to MF's lost more weight when compared with Experiment I oyster (no MF exposure) at 14-days of experimentation in both elevated temperature treatments, and at 21-days in the multi-stress treatment. We also found an overall increase in oyster mortality in this exposure compared to environmental perturbations alone. The presence of MF's has been shown to decrease feeding rates in bivalves (Shang et al., 2021), and result in a decrease in mass due to impacts on filtration efficiency (Carpenter et al., 2019). No change in weight between our Experiment I and Experiment II oyster in the hypo-

585 osmotic treatment, likely due to the behavioral valve closure described above. This was also
586 found by Du and colleagues; exposure to lowered salinities decreased clearance rates, resulting
587 in lower microplastic numbers in *C. gigas* (2023).

588 Microfiber exposure had no treatment effect on CS activity, but these levels were notably
589 higher when compared to oyster that were not exposed to MF's. This was also found when
590 comparing ETS activity between experiments; ETS levels in Experiment II oyster were
591 significantly higher at 14-days of exposure, indicating a need for energy at this time. A 14-day
592 exposure to microplastics at high concentrations (10^4 or 10^6 particles/L) drastically increased
593 energy demand and depleted energy stores in *Mytilus coruscus* (Du et al, 2023). While our
594 findings concur with the need for additional energy as a result of MF exposure, we did not
595 measure the expected decline in energy reserves, and found minimal changes to CS activity.
596 Levels of MDH were significantly decreased in our two elevated temperature treatments when
597 compared to control values. As oyster lost mass consistently, it is clear they were not able to
598 meet energetic demands of treatment exposure aerobically or anaerobically, and this lack of
599 stored energy use is an interesting finding that requires further investigation.

600 Previous work has demonstrated that the presence of MF's initiates an inflammation
601 response in bivalves, causing an increase in antioxidant levels (Cole et al., 2020; Du et al., 2023).
602 Our findings do not concur with these studies, which may be due to type of
603 microplastic/microfiber used, or concentration of microplastic in each treatment. The
604 recirculation system used in this study allowed oyster to freely filter MF's from the water volume
605 of 60-L that was constantly moving, unlike previous studies that have used smaller tank volumes
606 in a static system (Cole et al., 2020; Carpenter et al., 2019; Shang et al., 2021; Du et al., 2023).
607 Additionally, the concentration of MF's used here was based upon counts in a naturally occurring

population of *C. virginica* (Waite et al., 2018). Therefore, while we can conclude that the presence of MF's in an ecologically relevant concentration in concert with changes in temperature and salinity does have some effect on the bioenergetic response of *C. virginica*, further investigation is needed to discern if the weight changes, energy store use, levels of enzymatic activity and oxidative stress response measured in this study are due to physiological adaptation, or energetic trade-offs.

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CRediT authorship contribution statement

Tyler M. Mendela: Investigation, Writing- review and editing. Sean R. Isaac: Investigation, Writing- review and editing. Laura A. Enzor: Conceptualization, Funding Acquisition, Methodology, Investigation, Formal Analysis, Writing- original draft

Declaration of competing interest

The authors have no conflicts of interest to declare

Data availability

The data are available on request to the corresponding author

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	Control Temperature (°C)	Control Salinity (‰)	SS:DS Temperature (°C)	SS:DS Salinity (‰)	SS:ET Temperature (°C)	SS:ET Salinity (‰)	MS Temperature (°C)	MS Salinity (‰)
Experiment I	20.9 ± 0.9	27.6 ± 1.9	21.2 ± 0.8	17.4 ± 1.1	26.5 ± 0.4	27.2 ± 1.0	26.5 ± 0.4	17.3 ± 1.1
Experiment II	19.7 ± 0.5	26.9 ±1.7	20.2 ± 0.8	17.9 ± 1.7	26.8 ± 0.2	26.8 ± 0.8	27.0 ± 0.3	17.3 ± 0.6

Table 1. Average temperature (°C) and salinity (‰) data (±SD) recorded over Experiment I (environmental stress) and Experiment II (environmental stress + microfibers), each lasting 21-days. Measurements were logged every 20 minutes over the course of both experiments. Treatments included a control (20°C + 27‰), a single stress of decreased salinity (SS:DS, 20°C + 17‰), a single stress of elevated temperature (SS:ET, 27°C + 27‰), and a multi-stress treatment (MS, 27°C + 17‰)

	Experiment I				Experiment II			
	Control	**SS:DS**	**SS:ET**	**MS**	**Control**	**SS:DS**	**SS:ET**	**MS**
Day 0-7	0	0	0	0	0	0	0	0
Day 8-14	0	0	0	0	0	1	2	0
Day 15-21	0	0	0	1	0	0	0	0

Table 2. Recorded mortality of oyster during Experiment I (environmental stress) and Experiment II (environmental stress + microfibers), each lasting 21-days. Treatments included a control (20°C + 27‰), a single stress of decreased salinity (SS:DS, 20°C + 17‰), a single stress of elevated temperature (SS:ET, 27°C + 27‰), and a multi-stress treatment (MS, 27°C + 17‰)

	Measurement	Exposure Time	Treatment	Interaction
Experiment I	**Weight Change**	$F_{2,82}$=3.729 **p=0.028***	$F_{3,82}$=1.849 p=0.145	$F_{6,82}$=0.617 p=0.716
	GC	Gill: $F_{2,59}$=15.570 **p<0.001***	Gill: $F_{3,59}$=3.106 **p=0.033***	Gill: $F_{6,59}$=0.414 p=0.867
		AM: $F_{2,60}$=45.384 **p<0.001***	AM: $F_{3,60}$=0.729 p=0.539	AM: $F_{6,60}$=0.442 p=0.847
	LC	Gill: $F_{2,59}$=8.150 **p<0.001***	Gill: $F_{3,59}$=0.265 p=0.850	Gill: $F_{6,59}$=0.508 p=0.800
		AM: $F_{2,59}$=9.554 **p<0.001***	AM: $F_{3,59}$=0.128 p=0.943	AM: $F_{6,59}$=1.231p=0.303
	CS	Gill: $F_{2,35}$=0.107 p=0.899	Gill: $F_{3,35}$=0.939 p=0.432	Gill: $F_{6,35}$=1.518 p=0.201
		AM: $F_{2,36}$=4.326 **p=0.021***	AM: $F_{3,36}$=0.255 p=0.857	AM: $F_{6,36}$=0.677 p=0.669
	ETS	Gill: $F_{2,58}$=12.128 **p<0.001***	Gill: $F_{3,58}$=0.756 p=0.523	Gill: $F_{6,58}$=0.664 p=0.679
		AM: $F_{2,58}$=2.031 p=0.140	AM: $F_{3,58}$=0.015 p=0.998	AM: $F_{6,58}$=1.039 p=0.410
	MDH	Gill: $F_{2,35}$=8.035 **p=0.001***	Gill: $F_{3,35}$=1.572 p=0.213	Gill: $F_{6,35}$=1.473 p=0.216
		AM: $F_{2,34}$=6.006 **p=0.006***	AM: $F_{3,34}$=1.921 p=0.145	AM: $F_{6,34}$=1.895 p=0.110
	PC	Gill: $F_{2,31}$=0.875 p=0.427	Gill: $F_{3,31}$=1.484 p=0.238	Gill: $F_{6,31}$=1.008 p=0.438
		AM: $F_{2,34}$=2.860 p=0.071	AM: $F_{3,34}$=4.021 **p=0.015***	AM: $F_{6,34}$=1.262 p=0.301
	MDA	Gill: $F_{2,59}$=7.376 **p=0.001***	Gill: $F_{3,59}$=0.064 p=0.979	Gill: $F_{6,59}$=0.537 p=0.778
		AM: $F_{2,59}$=11.593 **p<0.001***	AM: $F_{3,59}$=3.008 **p=0.037***	AM: $F_{6,59}$=3.446 **p=0.006***
	FRAP	Gill: $F_{2,35}$=0.503 p=0.609	Gill: $F_{3,35}$=2.121 p=0.115	Gill: $F_{6,35}$=0.784 p=0.588
		AM: $F_{2,35}$=0.478 p=0.624	AM: $F_{3,35}$=12.329 **p<0.001***	AM: $F_{6,35}$=0.827 p=0.557

Table 3. Two-way ANOVA results for the main effects of exposure time, treatment, and interaction of exposure time and treatment on measurements from Experiment I (temperature and salinity exposure) gill and adductor muscle tissues. Measurements include: total glycogen content (GC), total lipid content (LC), Citrate Synthase activity (CS), Electron Transport System activity (ETS), Malate Dehydrogenase activity (MDH), Protein Carbonyl formation (PC), Lipid peroxidation, measured as levels of malondialdehyde (MDA), and total antioxidant potential, as determined by a ferric reducing/antioxidant potential assay (FRAP). Significant findings are marked by an asterisk and are in bold.

	Measurement	Exposure Time	Treatment	Interaction
Experiment II	**Weight Change**	$F_{2,55}$=1.199 p=0.309	$F_{3,55}$=9.551 **p<0.001***	$F_{6,55}$=1.625 p=0.158
	GC	Gill: $F_{2,55}$=2.627 p=0.081 AM: $F_{2,53}$=0.241 p=0.787	Gill: $F_{3,55}$=0.231 p=0.874 AM: $F_{3,53}$=1.607 p=0.199	Gill: $F_{6,55}$=0.683 p=0.664 AM: $F_{6,53}$=1.332 p=0.259
	LC	Gill: $F_{2,54}$=2.619 p=0.082 AM: $F_{2,55}$=6.774 **p=0.002***	Gill: $F_{3,54}$=0.64 p=0.978 AM: $F_{3,55}$=0.447 p=0.720	Gill: $F_{6,54}$=0.072 p=0.998 AM: $F_{6,55}$=0.716 p=0.638
	CS	Gill: $F_{2,34}$=4.433 **p=0.019*** AM: $F_{2,34}$=20.387 **p<0.001***	Gill: $F_{3,34}$=0.976 p=0.416 AM: $F_{3,34}$=0.478 p=0.700	Gill: $F_{6,34}$=0.106 p=0.995 AM: $F_{6,34}$=0.936 p=0.482
	ETS	Gill: $F_{2,53}$=7.540 **p=0.001*** AM: $F_{2,54}$=0.774 p=0.466	Gill: $F_{3,53}$=.175 p=0.913 AM: $F_{3,54}$=0.363 p=0.780	Gill: $F_{6,53}$=1.045 p=0.407 AM: $F_{6,54}$=0.969 p=0.455
	MDH	Gill: $F_{2,33}$=2.019 p=0.149 AM: $F_{2,31}$=0.574 p=0.569	Gill: $F_{3,33}$=10.396 **p<0.001*** AM: $F_{3,31}$=10.333 **p<0.001***	Gill: $F_{6,33}$=1.610 p=0.175 AM: $F_{6,31}$=0.750 p=0.614
	PC	Gill: $F_{2,35}$=0.388 p=0.351 AM: $F_{2,30}$=6.312 p=0.089	Gill: $F_{3,35}$=0.482 p=0.586 AM: $F_{3,30}$=5.568 p=0.088	Gill: $F_{6,35}$=1.152 p=0.257 AM: $F_{6,30}$=1.041 p=0.233
	MDA	Gill: $F_{2,53}$=0.353 p=0.704 AM: $F_{2,50}$=1.710 p=0.191	Gill: $F_{3,53}$=0.472 p=0.703 AM: $F_{3,50}$=0.399 p=0.755	Gill: $F_{6,53}$=0.283 p=0.943 AM: $F_{6,50}$=0.397 p=0.878
	FRAP	Gill: $F_{2,33}$=3.205 **p=0.049*** AM: $F_{2,32}$=3.554 **p=0.040***	Gill: $F_{3,33}$=0.856 p=0.459 AM: $F_{3,32}$=2.042 p=0.128	Gill: $F_{6,33}$=0.708 p=0.646 AM: $F_{6,32}$=0.413 p=0.865

Table 4. Two-way ANOVA results for the main effects of exposure time, treatment, and interaction of exposure time and treatment on measurements from Experiment II (temperature, salinity and microfiber exposure) gill and adductor muscle tissues. Measurements include: total glycogen content (GC), total lipid content (LC), Citrate Synthase activity (CS), Electron Transport System activity (ETS), Malate Dehydrogenase activity (MDH), Protein Carbonyl formation (PC), Lipid peroxidation, measured as levels of malondialdehyde (MDA), and total antioxidant potential, as determined by a ferric reducing/antioxidant potential assay (FRAP). Significant findings are marked by an asterisk and are in bold.

Fig. 1: Average weight change (g ±SE; n=6) for *Crassostrea virginica* exposed to environmental stressors (A) or environmental stressors and microfibers (B) for 7, 14 and 21 days to a control treatment (20°C + 27‰; black bars), a single stress of decreased salinity (SS:DS, 20°C + 17‰; dark grey bars), a single stress of elevated temperature (SS:ET, 27°C + 27‰; light grey bars), or a multi-stress treatment (MS, 27°C + 17‰; white bars with crosshatches). Asterisks indicate a significant difference from control oyster.

Fig. 2: Average glycogen content (mg glycogen/gram fresh tissue weight ±SE; n=6; A-D) and lipid content (mg total triglyceride/gram fresh tissue weight ±SE; n=6; E-H) for *Crassostrea virginica* tissues exposed to environmental stressors (Gill tissues: A and E, Adductor Muscle: B and F) or environmental stressors and microfibers (Gill tissues: C and G, Adductor Muscle: D and H) for 7, 14 and 21 days to a control treatment (20°C + 27‰; black bars), a single stress of decreased salinity (SS:DS, 20°C + 17‰; dark grey bars), a single stress of elevated temperature (SS:ET, 27°C + 27‰; light grey bars), or a multi-stress treatment (MS, 27°C + 17‰; white bars with crosshatches). Asterisks indicate a significant difference from control oyster.

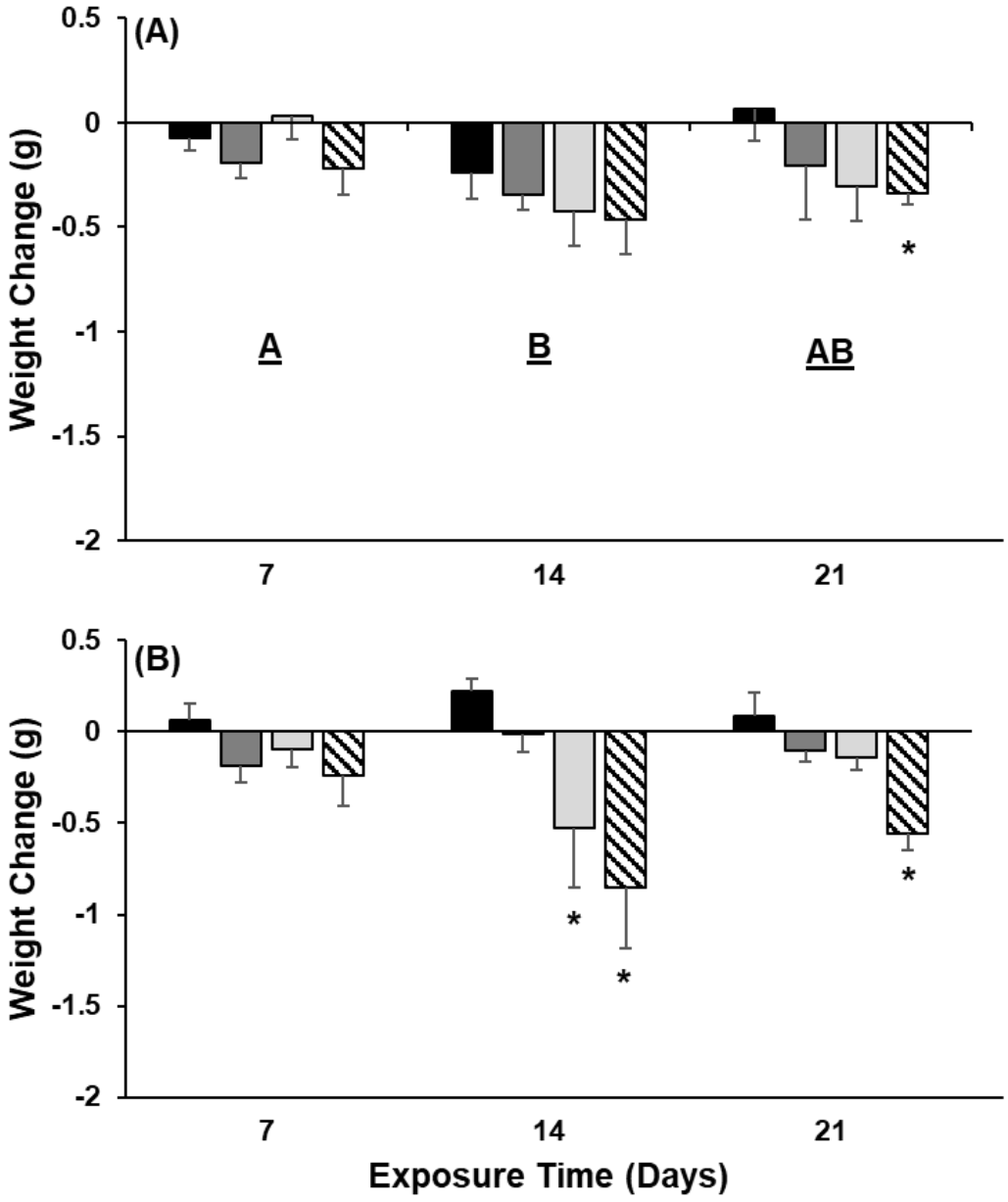
Fig. 3: Average Citrate Synthase enzyme activity (CS: I.U./g-protein ±SE; n=4; A-D) and Electron Transport System activity, (nmol formazan produced/minute/mg-protein ±SE; n=6; E-H) for *Crassostrea virginica* tissues in response to environmental stressors (Gill tissues: A and E, Adductor Muscle: B and F) or environmental stressors and microfibers (Gill tissues: C and G, Adductor Muscle: D and H) exposed for 7, 14 and 21 days to a control treatment (20°C + 27‰; black bars), a single stress of decreased salinity (SS:DS, 20°C + 17‰; dark grey bars), a single stress of elevated temperature (SS:ET, 27°C + 27‰; light grey bars), or a multi-stress treatment (MS, 27°C + 17‰; white bars with crosshatches). Asterisks indicate a significant difference from control oyster.

Fig. 4: Average Malate Dehydrogenase activity (MDH: I.U./g-protein ±SE; n=4) for *Crassostrea virginica* tissues in response to environmental stressors (Gill tissues: A, Adductor Muscle: B) or environmental stressors and microfibers (Gill tissues: C, Adductor Muscle: D) exposed for 7, 14 and 21 days to a control treatment (20°C + 27‰; black bars), a single stress of decreased salinity (SS:DS, 20°C + 17‰; dark grey bars), a single stress of elevated temperature (SS:ET, 27°C + 27‰; light grey bars), or a multi-stress treatment (MS, 27°C + 17‰; white bars with crosshatches). Asterisks indicate a significant difference from control oyster.

Fig. 5: Average Protein Carbonyl formation (PC: mmol/g-protein ±SE; n=4; A-D) and lipid peroxidation, measured as malondialdehyde levels (MDA; µmol/mg-protein ±SE; n=6; E-H) for *Crassostrea virginica* tissues in response to environmental stressors (Gill tissues: A and E, Adductor Muscle: B and F) or environmental stressors and microfibers (Gill tissues: C and G, Adductor Muscle: D and H) exposed for 7, 14 and 21 days to a control treatment (20°C + 27‰; black bars), a single stress of decreased salinity (SS:DS, 20°C + 17‰; dark grey bars), a single stress of elevated temperature (SS:ET, 27°C + 27‰; light grey bars), or a multi-stress treatment (MS, 27°C + 17‰; white bars with crosshatches). Asterisks indicate a significant difference from control oyster.

Fig. 6: Average total antioxidant potential (FRAP: mol/g-protein ±SE; n=4) for *Crassostrea virginica* tissues in response to environmental stressors (Gill tissues: A, Adductor Muscle: B) or environmental stressors and microfibers (Gill tissues: C, Adductor Muscle: D) exposed for 7, 14 and 21 days to a control treatment (20°C + 27‰; black bars), a single stress of decreased salinity (SS:DS, 20°C + 17‰; dark grey bars), a single stress of elevated temperature (SS:ET, 27°C + 27‰; light grey bars), or a multi-stress treatment (MS, 27°C + 17‰; white bars with crosshatches). Asterisks indicate a significant difference from control oyster.

943 Fig. 1



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946 Fig. 2

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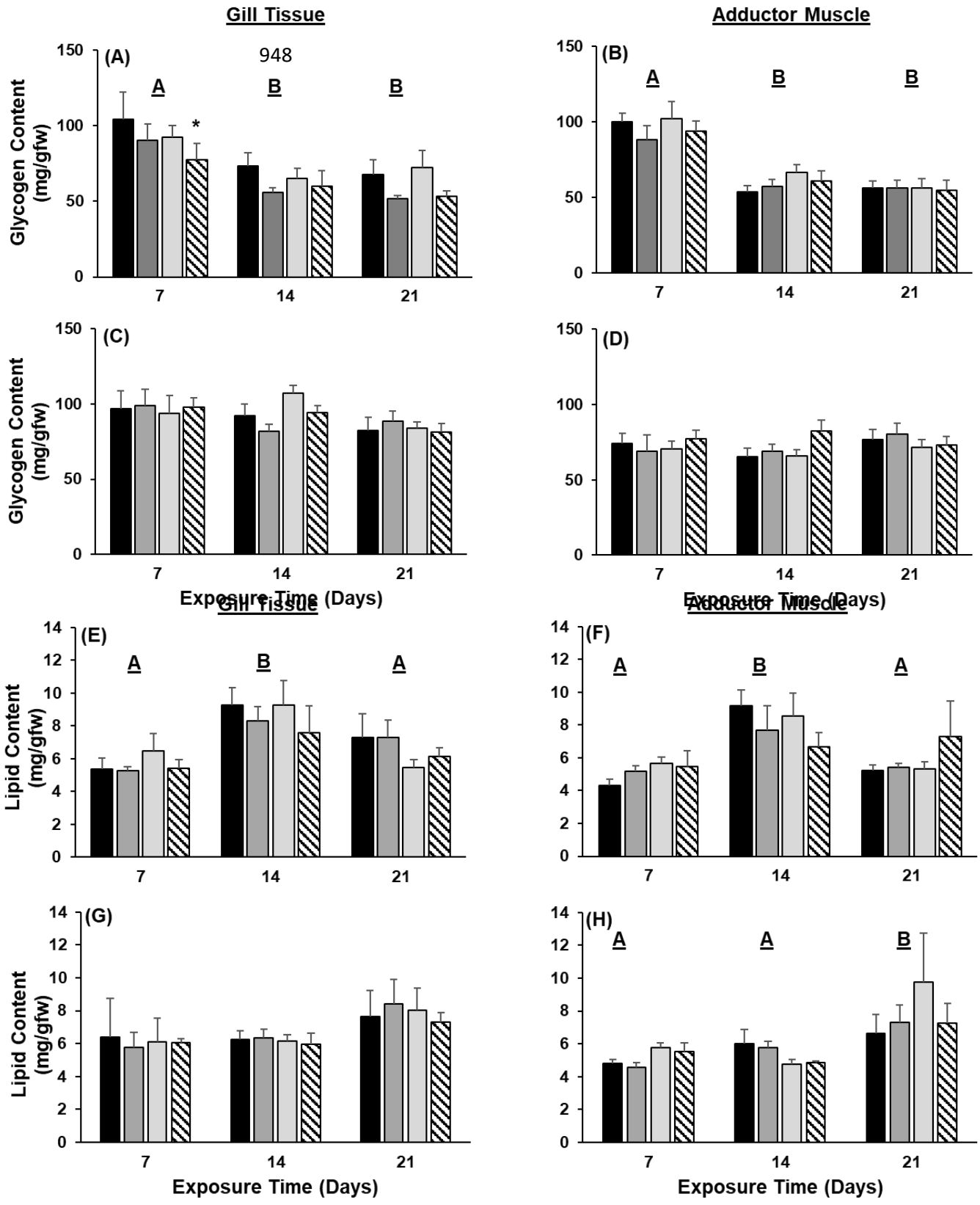

Gill Tissue
Adductor Muscle
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
A
B
*
Glycogen Content (mg/gfw)
Lipid Content (mg/gfw)
Exposure Time (Days)
7
14
21
0
50
100
150
2
4
6
8
10
12
14

Fig. 3

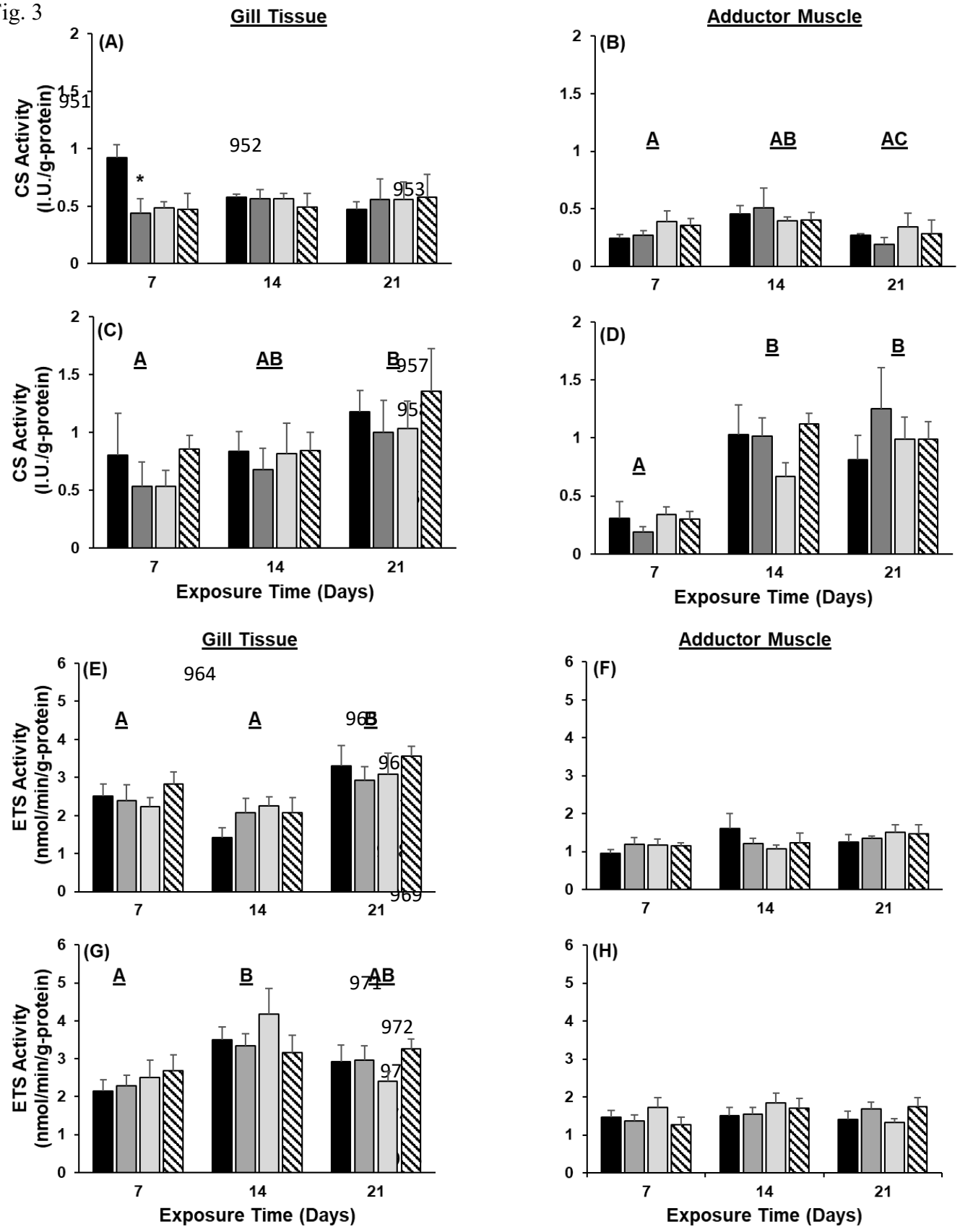

Gill Tissue
Adductor Muscle
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
CS Activity (I.U./g-protein)
ETS Activity (nmol/min/g-protein)
A
AB
AC
B
Exposure Time (Days)
7
14
21
*

Fig. 4

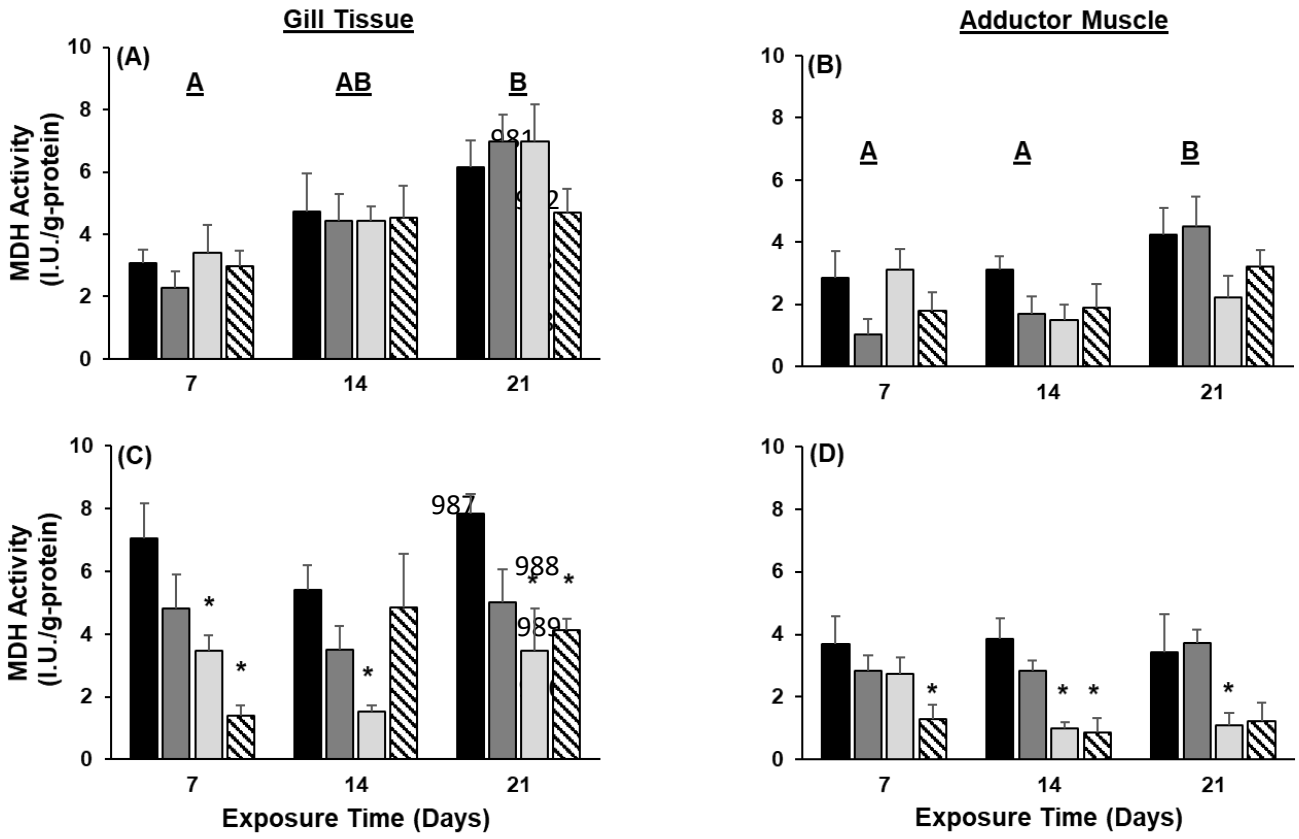
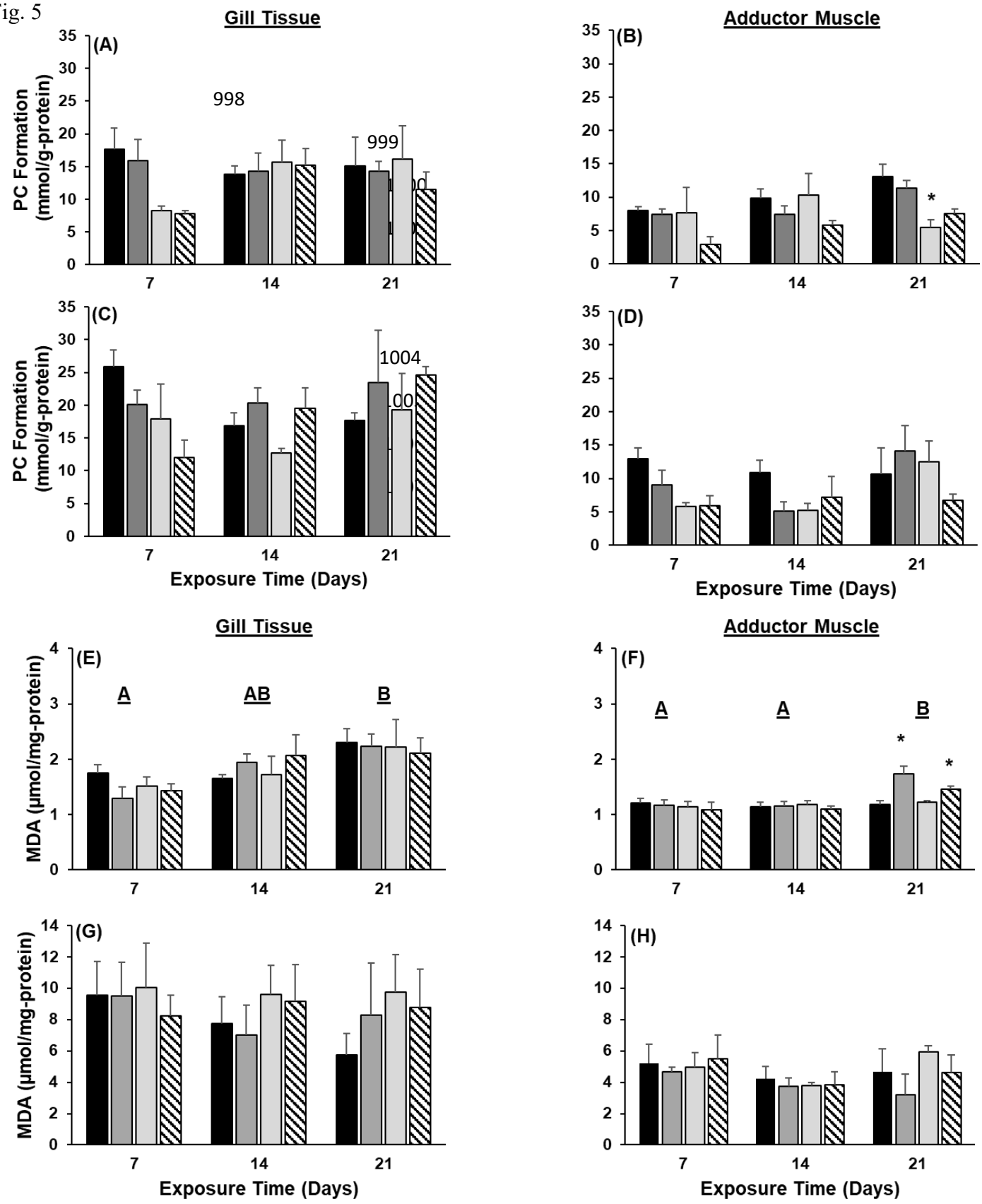

Gill Tissue
Adductor Muscle
(A)
(B)
(C)
(D)
A
AB
B
A
A
B
MDH Activity (I.U./g-protein)
Exposure Time (Days)
7
14
21
0
2
4
6
8
10
*

Fig. 5


Gill Tissue
Adductor Muscle
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
PC Formation (mmol/g-protein)
MDA (µmol/mg-protein)
Exposure Time (Days)
7
14
21
A
AB
B
*

1024 Fig. 6

1025