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RESEARCH ARTICLE

Triploid Pacific oysters exhibit stress response dysregulation and elevated mortality following heatwaves

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Abstract

Polyploidy has been suggested to negatively impact environmental stress tolerance, resulting in increased susceptibility to extreme climate events. In this study, we compared the genomic and physiological response of diploid (2*n*) and triploid (3*n*) Pacific oysters (*Crassostrea gigas*) to conditions present during an atmospheric heatwave that impacted the Pacific Northwestern region of the United States in the summer of 2021. Climate stressors were applied either singly (single stressor; elevated seawater temperature, 30°C) or in succession (multiple stressor; elevated seawater temperature followed by aerial emersion at 44°C), replicating conditions present within the intertidal over a tidal cycle during the event. Oyster mortality rate was elevated within stress treatments with respect to the control and was significantly higher in triploids than diploids following multiple stress exposure (36.4% vs. 14.8%). Triploids within the multiple stressor treatment exhibited signs of energetic limitation, including metabolic depression, a significant reduction in ctenidium Na^+/K^+ ATPase activity, and the dysregulated expression of genes associated with stress response, innate immunity, glucose metabolism, and mitochondrial function. Functional enrichment analysis of ploidy-specific gene sets identified that biological processes associated with metabolism, stress tolerance, and immune function were overrepresented within triploids across stress treatments. Our results suggest that triploidy impacts the transcriptional regulation of key processes that underly the stress response of Pacific oysters, resulting in downstream shifts in physiological tolerance limits that may increase susceptibility to extreme climate events that present multiple environmental stressors. The impact of chromosome set manipulation on the climate resilience of marine organisms has important implications for domestic food security within future climate scenarios, especially as triploidy induction becomes an increasingly popular tool to elicit reproductive control across a wide range of species used within marine aquaculture.

KEYWORDS
climate change, *Crassostrea gigas*, functional genomics, multiple stressors, physiological tolerance, triploid mortality

1 | INTRODUCTION

Chromosome set duplication (polyploidy) occurs when there is total nondisjunction of chromosomes during mitosis or meiosis, resulting in cells or organisms with one or more additional homologous chromosome sets. Polyploid animals generally have larger cells, lower cell membrane surface area to volume ratios, slower cell division rates, and achieve larger sizes as adults ("polyploid gigantism") than diploid (2*n*) conspecifics (Gregory & Mable, 2005). Cell size differences have meaningful consequences for metabolism; smaller cells require more energy to maintain ionic gradients between the cytoplasm and their surroundings (Rolfe & Brown, 1997; Szarski, 1983) and have faster oxygen and nutrient diffusion rates than larger cells (Subczynski et al., 1989). As a result, polyploids have been reported to have lower mass-specific metabolic rates than diploids (Kozłowski et al., 2003; Starostová et al., 2009), are more susceptible to oxygen limitation under elevated temperature (Hermaniuk et al., 2021), and perform better within cold environments (Atkins & Benfey, 2008; Sambraus et al., 2017).

While phenotypic differences between polyploids and their diploid progenitors have been described within a variety of taxa, a mechanistic understanding of the relationship between polyploidy and stress tolerance remains elusive due to potential performance benefits (i.e., heterosis) and consequences (i.e., energetic costs associated with gene silencing; Doyle & Coate, 2019; Mittelsten Scheid et al., 1996; Van de Peer et al., 2021). A useful system to investigate this dynamic is oyster aquaculture. Techniques to induce triploidy (3*n*) in oysters were first developed in the 1980s and are now widely adopted by industry (Downing & Allen, 1987; Guo et al., 2009; Nell, 2002; Yamamoto et al., 1988). Oyster aquaculture leverages triploidy to induce reproductive impairment in an attempt to avoid the seasonal, energy-intensive cycle of gametogenesis that can result in enhanced mortality within the summer months (a phenomenon known as "summer mortality"; Perdue et al., 1981; Samain & McCombie, 2008). By partitioning energy toward somatic growth, triploid oysters display enhanced growth and meat weight, decreased time to market, and greater marketability year round (Allen & Downing, 1986, 1991; Dégremont et al., 2012; Normand et al., 2008; Shpigel et al., 1992).

Early investigations of the ability of triploidy to compensate for summer mortality in France were promising, with triploid oysters exhibiting lower mortality rates (Gagnaire et al., 2006; Samain, 2011), presumably due to enhanced energetic investment in growth and maintenance processes (Jouaux et al., 2013). However, in practice, triploids can experience enhanced mortality when grown under the same field conditions (a phenomenon known as "triploid mortality"). Triploid mortalities have been observed in France (Houssin et al., 2019), as well as the Eastern (Guévélou et al., 2019; Matt, 2018; Matt et al., 2020), Southern (Wadsworth, 2018; Wadsworth et al., 2019), and Western (Tim Morris, Pacific Seafood; Paul Taylor, Taylor Shellfish Co.; Kurt Grinnell, Jamestown S'Klallam Tribe, personal communications) coasts of the United States, resulting in substantial economic losses. While the selective mortality of triploid oysters has been linked to environmental variability at specific sites (*Crassostrea virginica*: Bodenstein et al., 2021; Guévélou et al., 2019; *Crassostrea gigas*: B. Eudeline, Taylor Shellfish, personal communication), it remains unclear whether ploidy manipulation, and the physiological and transcriptomic perturbations that result, enhance or decrease the risk of summer mortality.

Summer mortality rates of marine organisms are expected to increase along with the likelihood and intensity of marine and atmospheric heatwaves. Marine heatwaves (MHWs) are prolonged extreme oceanic high-temperature events that have far reaching ecological (Whalen et al., 2023) and economic consequences, with individual events resulting in direct economic losses of $800 million and more than $3.1 billion in indirect losses for multiple consecutive years (Smith et al., 2021). Atmospheric heatwaves can have similar impacts on marine environments, particularly in the intertidal zone. In the nearshore, increases in air temperature can result in ocean warming and present multiple abiotic stressors in rapid succession over a tidal cycle act that synergistically on physiology (Breitburg & Riedel, 2005). A poignant demonstration of this phenomenon was observed when the Pacific Northwestern region of the United States experienced an atmospheric heatwave that coincided with some of the lowest daytime low tides of the year during the summer of 2021 (Philip et al., 2021; White et al., 2022). In this case, a gradual increase in seawater temperature in shallow bays (20–30°C) was followed by exposure to high air temperatures (>40°C) over three tidal cycles, resulting in the estimated death more than one billion shellfish within the coastal and inland waters of Washington State and British Columbia and substantial losses for commercial and tribal growers (Cecco, 2021; Raymond et al., 2022).

The impact of polyploidy on the susceptibility of marine ectotherms to extreme climate events like heatwaves remains unclear. Differences in energy storage could benefit polyploids within multiple stressor scenarios, while changes in the genetic architecture that underly important stress response pathways could alter physiological tolerance limits (Holbrook et al., 2020). Using heatwaves and oyster aquaculture as a model, we conducted a hatchery experiment to identify key ploidy-specific differences in the stress response of diploid and triploid *C. gigas* using an energy-limited tolerance to stress framework (Sokolova, 2013; Sokolova et al., 2012) by monitoring changes in standard metabolic rate (SMR), metabolic enzyme activity rates, and gene expression patterns.

2 | METHODS

Five hundred diploid (2*n*) and triploid (3*n*) Pacific oysters (*C. gigas*) were obtained from our commercial partners. Triploidy was induced through the exposure of larvae to a thermal shock during meiosis I (Yamamoto et al., 1988). Diploids and triploids were made from wild stocks of unknown parentage and were reared in common conditions until adulthood. Adult oysters were received in March 2021 and transferred to the Jamestown S'Klallam Point Whitney Shellfish Hatchery located on Dabob Bay, Washington (47°45″ N 122°50′ W).

Upon receipt, the ploidy of 15 individuals from each group was determined using a CyFlow™ Ploidy Analyzer (Sysmex America Inc.); remaining oysters were individually labeled by affixing numbered wire tags to the right shell valve using cyanoacrylate. The maximum shell length, width, and height of each oyster was recorded using vernier calipers to the nearest mm; measurements were taken at the beginning and end of experiments. Shell volume was estimated from measurements using the equation of a generalized ellipsoid. To determine the baseline condition of a subset of oysters upon arrival at the hatchery, gonadal sections were sampled for histological examination and dry tissue weight was measured by drying excised whole-body tissue at 60°C for 72 h. Dry tissue weight and reproductive condition (see Section 2.2) were determined for remaining oysters following tissue sampling or after the conclusion of experiments. If dry weight could not be determined for an individual due to mortality or other constraints it estimated from shell volume (see Figure S1). All data created during this research are available at https://doi.org/10.5281/zenodo.7693092.

2.1 | Experimental design

All oysters were allowed to acclimate to hatchery conditions (*T* ~17°C, salinity ~27 ppt, pH ~8; see Table 1) for 20 days (day −30 to −10) within 100 L tanks with flowing seawater. Throughout the experiment, a peristaltic pump maintained an algal density of 3×10^5 cells mL^{-1} within tanks. Oysters were fed a mixed live diet *Tetraselmis* spp., *Pavlova* spp., and *Chaetoceros* spp. in equal proportions. Following acclimation, oysters were haphazardly split into either the control, single stressor, or multiple stressor treatment (see Figure 1a); treatment groups were labeled as follows: diploid control (2*n*-C), diploid single stressor (2*n*-SS), diploid multiple stressor (2*n*-MS), triploid control (3*n*-C), triploid single stressor (3*n*-SS), and triploid multiple stressor (3*n*-MS). The starting sample size within each treatment was 112 oysters. Oysters within each treatment were placed in triplicate within PVC silos (76 mm diameter) with netting (3.2 mm mesh) affixed to the bottom, which were themselves placed within individually capped PVC sleeves (101 mm diameter) to separate the effluent of each cluster. A pseudo-replicated design was used to prevent the mortality of an oyster within a given treatment from impacting others outside of its respective cohort. Each silo was supplied seawater at a constant rate of 100 mL min^{-1} and had a turnover rate of 78 min.

The temperature (±0.1°C), salinity (±0.1), and pH (±0.01) of each treatment tank were continuously monitored using an A3 Apex Controller System (Neptune Systems). The accuracy of each monitored parameter, in addition to the dissolved oxygen concentration (±0.1 mg L^{-1}) within each silo to check for hypoxic conditions, was manually measured every 3–5 days using a multimeter (Hanna Instruments). Seawater temperature within the control treatment was allowed to match that of the water entering the hatchery, controlled to a maximum setpoint of 17°C within the header tank. Starting on day −10, the temperature within the single and multiple stressor treatments was increased at a rate of 2°C day^{-1} until a setpoint of 30°C was reached (hereafter designated day 0, start of stress treatment); seawater was heated at this rate to approximate the conditions experienced during the lead up to a MHW (Philip et al., 2021). Experimental tanks were heated by passing seawater through two inline Aqualogic Optima Compact Plus Heaters; individual setpoints within each tank were maintained dynamically through the control of three 800-watt titanium rod heaters by a ramp/soak temperature controller utilizing PID control (Auber Instruments).

On day 2, oysters within the multiple stressor treatment were removed from seawater and placed within 40 L custom-built warming chambers for 6 h (see Figure S3). The air temperature within chambers was controlled to a setpoint of 44°C through the dynamic control of three ceramic heat lamps by a temperature controller utilizing PID control. The humidity of each chamber was allowed to fluctuate with temperature. The internal temperature of a subset of oysters was monitored during warming trials; oyster tissue temperature was measured in real time by drilling a 1.5 mm hole into the top shell valve of each oyster, inserting a K-type thermocouple into the stomach, and sealing the hole with putty (see Figure S3). While in warming chambers, air temperature was held at approximately 44°C for 4 h, with an hour before and after exposure of ramp. Following warming, oysters were returned to 30°C seawater, where they were held for 30 days. Throughout the experiment, the mortality and metabolic rate of individually labeled oysters within each treatment were repeatedly monitored. Tissue from the ctenidia was also sampled from a subset of oysters preceding and following the application of stress treatments and saved for later enzymatic and transcriptomic analysis. More information regarding the method and sampling regime employed for each metric can be found below.

2.2 | Reproductive condition

Oyster reproductive condition ($n=15$ per ploidy, per treatment, per time point) was determined for a subset of individuals within the control, single stressor, and multiple stressor treatments

TABLE 1 Seawater temperature (°C), salinity (ppt), and pH (NBS scale) within each treatment (±SD).

Treatment	Temperature	Salinity	pH
Acclimation	17.3 ± 0.8 (15.6–18.9)	27.5 ± 0.9 (26.0–29.6)	8.10 ± 0.08 (7.85–8.20)
Control	17.1 ± 0.8 (14.7–18.9)	28.2 ± 1.7 (25.0–31.3)	7.93 ± 0.05 (7.81–8.01)
Single stressor	30.2 ± 0.5 (27.4–30.9)	28.9 ± 1.3 (26.2–31.6)	7.97 ± 0.04 (7.90–8.05)
Multiple stressors	30.4 ± 0.4 (28.3–30.9)	28.8 ± 1.0 (26.5–31.3)	8.04 ± 0.11 (7.90–8.23)

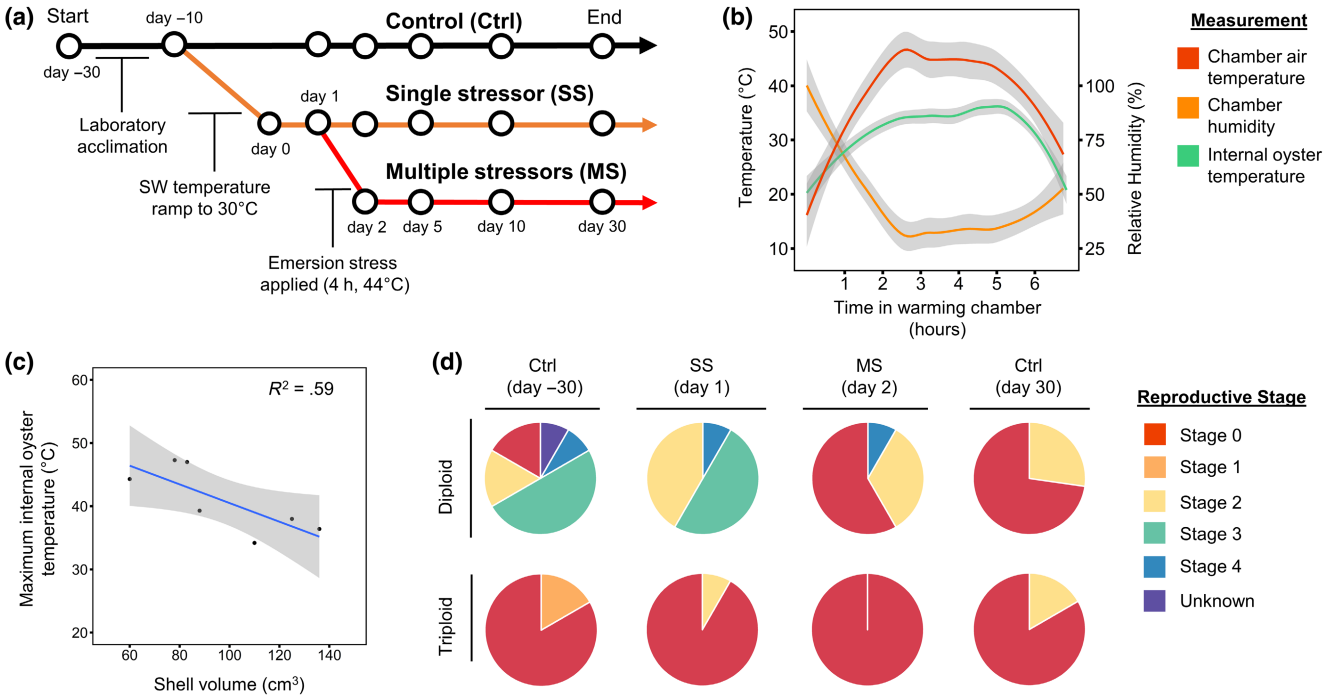

FIGURE 1 (a) Experimental design including a timeline of when stressors were applied. (b) Warming chamber air temperature, humidity, and internal oyster temperature during multiple stressor treatment on day 2. (c) Relationship between shell volume and the maximum internal (meat) temperature experienced by oysters within warming chambers. (d) Proportion of oysters within each ploidy at each reproductive stage upon receipt at the hatchery (before experiment control), after single and multiple stress exposure, and within the control treatment after 30 days (after experiment control); 25 oysters per ploidy were sampled for reproductive condition per time point.

through sampling and histological analysis of gonad tissue sections. Tissue was sampled from oysters within the control treatment upon receipt at the Point Whitney Hatchery (day −30) and after the conclusion of the experimental trials (day 30). Tissue was sampled from oysters within stressor treatments the day after conditions were applied (single stressor: day 1; multiple stressor: day 2). Sampled gonad tissue was placed within a histology cassette and fixed using the PAXgene tissue fixative and Stabilizer system (Qiagen). Samples were embedded in paraffin, stained with hematoxylin and eosin, sectioned, and imaged at 4×, 10×, and 40× magnification. Images of gonad sections were assigned to reproductive stages in accordance with ploidy-specific metrics as outlined by Ezgeta Balic et al. (2020) and Matt & Allen (2021), respectively. Gonad was assessed as either resting (stage 0), within an early (stage 1) or late growth stage (stage 2), mature (stage 3), spawning (stage 4), or unknown.

2.3 | Survival analysis

Oysters within each treatment group ($n = 88$ per treatment) were checked for mortality every 1–3 days over the course of experiments. The survival R package was used to compute survival curves using the Kaplan–Meier estimator from mortality data Therneau et al. (2022). The impact of ploidy and stress treatment on survival curves was assessed with the log-rank test using a significance cutoff of .05.

2.4 | Standard metabolic rate

Whole oyster oxygen consumption rate was measured as a proxy for SMR, at either 17°C (control) or 30°C (treatment). The SMR of 40 labeled oysters within the control, single stressor, and multiple stressor treatments was taken during the acclimation period (day −15 to −10), followed by repeated measurements of individuals on days 1, 2, 6, and 10 following initial stress exposure. The oxygen uptake rate of individual oysters was determined by closed-system respirometry wherein animals were placed in 1 L plastic chambers filled with air-saturated, UV-sterilized, and 0.2 um filtered seawater. Chambers were partially submerged within a recirculating water bath controlled to a temperature setpoint. For each measurement, a single oyster was placed within a chamber and allowed to recover from handling stress for at least 15 min; chambers were then closed, shielded from light, and the decline in O_2 concentration (mg O_2) was recorded for 1 h. Trials in which the oxygen saturation declined below 60% within a chamber were discarded. During each set of trials, the oxygen concentration of a blank chamber with an empty oyster shell was also recorded as an internal control. The mass-specific SMR (mg $O_2\,h^{-1}\,g^{-1}$) for each individual at a given time point and temperature was calculated using the following equation:

$$\mathrm{SMR} = \frac{[(m-b)\times V]\times 60}{w},$$

where m is the oxygen uptake rate of the linear portion of the oxygen concentration curve (mg min^{-1}), b is mean oxygen uptake rate of the

replicate blanks from that sampling day (mg min^{-1}), V is the volume of the chamber (L), and w is oyster dry tissue weight (g). The oxygen consumption rate of blank chambers as a function of oyster shell size is provided in Figure S2.

2.5 | Na^+/K^+ ATPase

Tissue was collected from oysters ($n = 12$ per ploidy, per treatment, per time point) within the control, single stressor, and multiple stressor treatments on days −10, 1, 2, 5, 10, and 30. Upon sampling, ctenidia were flash frozen in SEI buffer (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole; Zaugg, 1982) and stored at −80°C until analysis. The Na^+/K^+ ATPase activity of frozen samples was measured within 6 months according to the method of (McCormick, 1993) with modifications as described by (Bianchini & Wood, 2003) to improve assay performance for *C. gigas*. Briefly, two salt solutions (assay mix A and B) were used during analysis. The ATP concentration within assay mix A and B were decreased to 0.35 mM and the ouabain concentration in assay mix B was increased to 1 mM. Kinetic assays were run at 35°C for 10 min on a SpectraMax 190 plate reader (Molecular Devices).

2.6 | Citrate synthase

Ctenidium was collected from oysters ($n = 12$ per ploidy, per treatment, per time point) within the control and single stressor treatments on day 1 and the multiple stressor treatment on day 2, flash frozen, and stored at −80°C for later analysis. Citrate synthase enzyme activity was determined using the Abcam CS Assay Kit (ab239712; Abcam PLC). Briefly, 10–20 mg (wet weight) of ctenidium was homogenized in 350 µL of ice-cold CS Assay Buffer. The optical density of the supernatant in the presence of a developer and substrate (43 µL buffer, 5 µL developer, and 2 µL substrate) was measured in triplicate and averaged at 405 nm for 45–60 min at 25°C on a Wallac Victor 21420 Multilabel Counter Plate Reader (Perkin Elmer). Citrate synthase activity was standardized against the protein concentration of each sample using the Bovine Serum Albumin Assay (500-0201; Bio-Rad Laboratories, Inc.) and reported as nmol min^{-1} mg^{-1} protein after comparison with a glutathione (GSH) standard.

2.7 | Differential gene expression analysis

Ctenidium was collected from oysters within the control, single stressor, and multiple stressor treatments on days 1 and 2, flash frozen, and stored at −80°C. Total RNA was extracted from 20 mg tissue samples homogenized in 1 mL of Trizol reagent (RNAzol® RT; Molecular Research Center Inc.). Homogenate was purified using the Direct-zol® RNA Purification Kit (Zymo Research) and genomic DNA was removed using the Turbo DNase Kit (Invitrogen). RNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies). RNA integrity was confirmed using the RNA 6000 pico assay on an Agilent 2100 Bioanalyzer. Twelve biological replicates from each treatment per ploidy ($N = 72$) were submitted for sequencing at the Genomic Sequencing and Analysis Facility at the University of Texas at Austin. Library preparation was performed using the QuantSeq 3′ mRNA-Seq protocol (v.015UG009V0251; Lexogen), also known as Tag-Seq. Tag-Seq generates cDNA from the 3′ end of mRNA strands with only one fragment per mRNA transcript; this approach avoids many of the computational issues commonly encountered during RNA-seq and the resulting sequence tags are considered direct proxies of gene expression levels. Additionally, tag counts are not influenced by gene length, making it ideal for differential gene expression (DGE) analysis (Asmann et al., 2009; Hong et al., 2011).

Bioinformatic workflows were performed using bash v.4.4.20 (www.gnu.org/software/bash/) and R v4.1.0 (www.r-project.org/) with the R Studio IDE v1.4.1717 (www.rstudio.com/). Sequencing reads were demultiplexed, filtered, and trimmed using Cutadapt v.4.1 (Martin, 2011); adapter sequences (e.g., AGATCGG), as well as poly-A and poly-G tails that exceeded eight base pairs (bp) were removed. The first 15 bp of the 3′ end of each fragment was trimmed to prevent the inclusion of low-quality reads (quality cutoff). After trimming, any fragment that was less than 20 base pairs were removed (minimum read length). Trimmed reads were aligned to the *C. gigas* genome assembly with mitochondrial genes added (Peñaloza et al., 2021) using Hisat2 v.2.2.1 (Kim et al., 2019), a splice aware aligner that has previously been used effectively with Tag-Seq data generated from bivalves (Gurr et al., 2022). Aligned reads were assembled against the mRNA genome feature track, merged using StringTie v2.2.0, and compiled using prepDE.py (Kovaka et al., 2019). Gene count matrices were analyzed with DESeq2 v.1.36.0 (Love et al., 2014) using the apeglm shrinkage estimator (Zhu et al., 2019). Genes with counts of more than 10 across at least one-third of samples were included in downstream analyses. Significant differentially expressed gene (DEG) lists were generated for each comparison using a Wald test p-value cutoff of .05 and the diploid control treatment as the baseline condition. Ploidy-specific DEG lists were generated with the same method using each ploidy's respective control as a baseline and removing transcripts expressed by both ploidies.

2.8 | Functional enrichment analysis

Functional enrichment analysis was performed to identify gene ontology (GO) terms significantly overrepresented within ploidy-specific DEG lists. Gene lists were analyzed for diploids and triploids within the single and multiple stressor treatments. To obtain GO terms associated with each DEG, uniprot Accession information for RNA nucleotide sequences (transcript IDs) was first obtained through a BLAST (Altschul et al., 1990) of the *C. gigas* genome (Peñaloza et al., 2021) against the Uniprot-SwissProt database (UniProt Consortium, 2019). A gene ID-to-GO term database was created by matching GO terms from the Uniprot-SwissProt database

to transcript IDs from the BLAST output using Uniprot Accession codes; GO terms associated with each DEG transcript ID were identified using this database.

Gene enrichment analysis was conducted using GOseq v.1.48.0 (Young et al., 2010). The Wallenius approximation was used to identify overrepresented GO terms within the Biological Processes GO branch, using all measured genes from individuals included in each analysis as a background. Enriched (overrepresented) GO terms were filtered using an adjusted *p*-value cutoff of .05, de-duplicated, and assigned a functional category. Functional category assignment was performed by categorizing identified GO terms into subgraphs (GOcats) based on a curated list of user-generated terms of interest (Hinderer 3rd & Moseley, 2020); Selected terms included "cellular processes" (including response to stimulus, transport), "regulation" (biological, epigenetic), "metabolism" (protein, RNA, other), "immune system processes," and "stress response" (including homeostasis, death). GO terms that failed to match to any defined GOcat were assigned to "other biological processes." The distribution of enriched GO terms within each functional category was compared with a Chi-squared test, using the results obtained for diploids within the single or multiple stressor treatment as the expected values. Identified GO terms for each analysis are available in Tables S12–S15.

2.9 | Statistical analyses

All statistical analyses were performed in R v.4.2.1 using the RStudio IDE v.2022.7.1.554. When applicable, analysis of variance was used to investigate differences in response variables (e.g., ATPase, citrate synthase) across stress treatments. For response variables with multiple observations from the same individual (e.g., metabolic rate), a repeated measures within-subject analysis of covariance was used to compare the impact of each factor (ploidy, stress treatment, duration of exposure), using oyster ID as a random effect. Assumptions of normality and homoscedasticity were assessed during model construction using the Shapiro test and a visual assessment of Q–Q and residual-fitted plots. The bestNormalize package was used to achieve normality when necessary (Peterson 2021) . For significant effects or their interactions ($\alpha = .05$), the agricolae package was used to perform pairwise comparisons of groups using the Tukey HSD post-hoc test (Felipe de Mendiburu & Yaseen, 2020).

3 | RESULTS

Ploidy analysis revealed that 100% of oysters tested ($n = 30$) corresponded to their presumed ploidy. The shell length, height, and width of oysters significantly varied across ploidy ($p < .001$), but not across treatment (Tables S1 and S2). Despite size differences, the dry tissue weight of oysters did not vary across ploidy or treatment (Table S2). Upon arrival at the hatchery, reproductive condition significantly varied across ploidy, with a larger proportion of diploids exhibiting later stage gonad development than triploids ($\chi^2_{df=1,N=24} = 78.1$, $p < .001$); this difference was maintained after single stress exposure ($\chi^2_{df=1,N=25} = 352$, $p < .001$). However, no difference across ploidy was observed following multiple stress exposure ($\chi^2_{df=1,N=25} = 9.57$, $p = .08$). On day 30 within the control treatment, there was also no significant difference in reproductive condition across ploidy ($\chi^2_{df=1,N=24} = 1.83$, $p = .87$). The proportion of oysters at each reproductive stage at each time point is presented in Figure 1d.

3.1 | Treatment conditions

Seawater temperature significantly varied across treatment ($p < .001$), with the single and multiple stressor treatments maintaining an elevated temperature with respect to the control for 30 days following the acclimation period (Figure S4). Seawater pH and salinity were not significantly different across treatments. The average (±SD) for each measured seawater parameter is presented in Table 1.

Diploid and triploid oysters within the multiple stressor treatment group were subjected to aerial emersion within one of four custom-built warming chambers on day 2 for 6 h. Oysters were afforded a 1 h ramp and cooling period preceding and following the maintenance of each chamber at a temperature setpoint of 44°C (Figure 1b). The mean air temperature within the maintenance period across chambers was 44.9 ± 1.6°C, corresponding to a mean relative humidity of 34.0 ± 4.0%. The mean internal temperature of oysters distributed across warming chambers was 33.9 ± 4.0°C while temperature was being maintained ($n = 7$; Figure 1b). The maximum internal temperature of oysters significantly varied with shell volume ($p = .04$, $r^2 = .59$, Figure 1b). The highest internal temperature recorded across all oysters monitored was 47.3°C.

3.2 | Survival analysis

Mortality was observed within all treatment groups over the course of the experiment. The mortality rate for both diploids and triploids was elevated within the stress treatments with respect to the control and was significantly higher in triploids than in diploids following multiple stress exposure (Figure 2a,b). The cumulative mortality across treatments on day 30 was 5.7%, 9.1%, 14.8%, 22.7%, 9.1%, and 36.4% across the 2*n*-C, 2*n*-SS, 2*n*-MS, 3*n*-C, 3*n*-SS, and 3*n*-MS treatments, respectively. Survival probability was significantly impacted by ploidy ($p = .017$) and stress treatment ($p < .001$). Triploids within the multiple stressor treatment (3*n*-MS) had the lowest 1-month survival probability (0.7 ± 0.09), followed by the 2*n*-MS (0.86 ± 0.07), 3*n*-SS (0.88 ± 0.07), 3*n*-SS (0.93 ± 0.05), 3*n*-C (0.94 ± 0.05), and 2*n*-C (0.95 ± 0.05) treatments, respectively.

3.3 | Standard metabolic rate

The metabolic rate of oysters was significantly affected by the interaction of ploidy (diploid, triploid), stress treatment (control, single

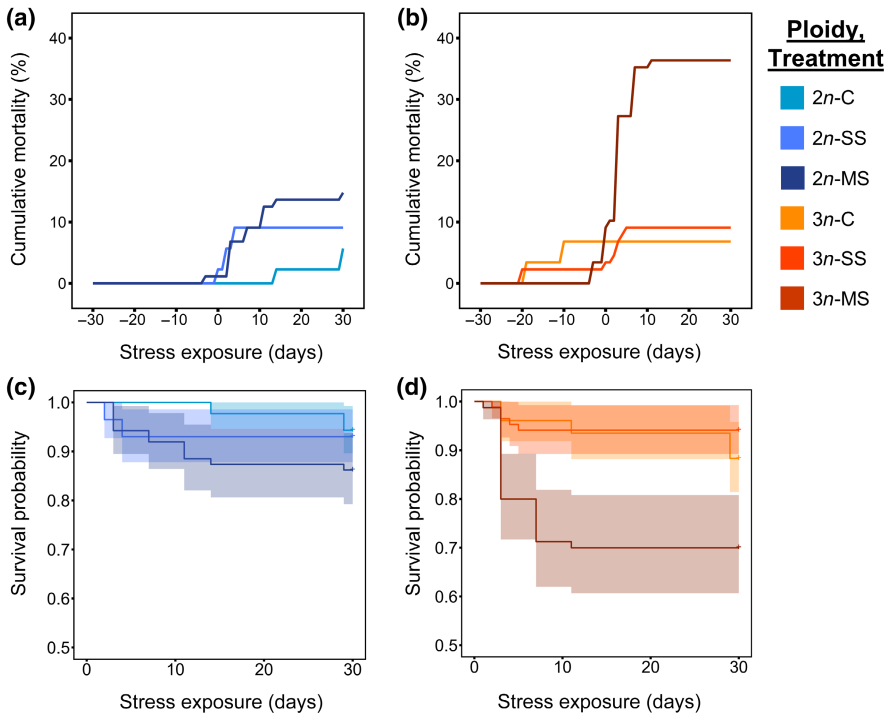

FIGURE 2 Cumulative mortality (a, b) and survival probability (c, d) for diploid (2*n*) and triploid (3*n*) adult Pacific oysters within hatchery stressor trials. All oysters were acclimated to hatchery conditions (day −30 to −10, 20°C) before inclusion in either the control (-C; 20°C), single stressor (-SS; SW temp = 30°C), or multiple stressor (-MS; SW temp = 30°C; aerial exposure = 44°C for 4 h on the second day of stress exposure) treatment.

stressor, multiple stressor), and the duration of stress exposure ($p < .001$; Table S3). Metabolic rate did not vary significantly across ploidy within the control treatment (20°C) and was similar during the acclimation period (day −30 to −10; "before experiment") and on day 10 ("after experiment"; Figure 3a). For diploids, metabolic rate was significantly elevated following multiple stressor exposure with respect to the single stressor treatment; this difference was maintained for up to 8 days after multiple stress exposure (Figure 3b,d). In contrast, no significant increase in metabolic rate was observed in triploids following multiple stressor exposure (Figure 3c,e). When compared with diploids, triploids experienced metabolic depression following multiple stress exposure (Figure 3f).

3.4 | Enzymatic assays

Na^+/K^+ ATPase activity was significantly impacted by the interaction of ploidy (diploid, triploid), stress treatment (control, single stressor, multiple stressor), and the duration of stress exposure ($p = .009$; Table S4). ATPase activity was significantly lower in triploids than diploids after multiple stressor exposure (Figure 4A; Figure S5). Citrate synthase was significantly impacted by stress treatment ($p < .001$; Table S4), with greater activity observed in the multiple stress treatment than the single stressor and control within both diploids and triploids (Figure 4B). Citrate synthase activity was not significantly impacted by ploidy ($p = .487$; Table S4).

3.5 | DEG analysis

Out of the 72 biological samples submitted for sequencing, eight were removed prior to analysis for quality concerns following the inspection of FastQC reports (Ewels et al., 2016). Sample sizes after quality control were 11, 11, 11, 10, 10, and 11 for the 2*n*-C, 3*n*-C, 2*n*-SS, 3*n*-SS, 2*n*-MS, and 3*n*-MS treatment, respectively. Remaining samples averaged 4.94 ± 1.1 million reads and achieved an average alignment rate of 87.8 ± 1.3% with the *C. gigas* genome (GenBank: GCA_902806645.1). *DESeq2* identified 470, 4267, 1809, 596, and 1579 significant DEGs associated with the 3*n*-C, 2*n*-SS, 3*n*-SS, 2*n*-MS, and 3*n*-MS treatment when the diploid control (2*n*-C) treatment was used as the baseline condition. To avoid the possible exclusion of triploid-specific transcripts, *DEGSeq2* was run a second time using each ploidy's respective control as a baseline; this analysis yielded 4258, 1300, 594, and 668 significant DEGs associated with the 2*n*-SS, 3*n*-SS, 2*n*-MS, and 3*n*-MS treatment and were used to generate ploidy-specific gene lists during later analyses. Identified gene lists for each comparison are provided in Tables S5–S11.

Principal component analysis demonstrated that the gene expression profiles of diploids within the single and multiple stressor treatments were more like each other than the control (Figure 5b). In contrast, the gene expression profiles of triploids within the single and multiple stressor treatments overlapped considerably with the control (Figure 5c). Across ploidy, triploids exhibited a greater degree of gene expression variation than diploids prior to being stressed (Figure 5d). The gene expression varied across ploidy considerably following stress exposure, with marginal overlap between individuals following exposure to elevated seawater temperature (Figure 5e) and no overlap following exposure to elevated seawater temperature and aerial emersion in combination (Figure 5f).

The number of DEGs identified and their expression varied across ploidy and treatments. Four hundred and seventy genes were determined to be differentially expressed between diploids (2*n*-C) and triploids (3*n*-C) within the control treatment. After single stress

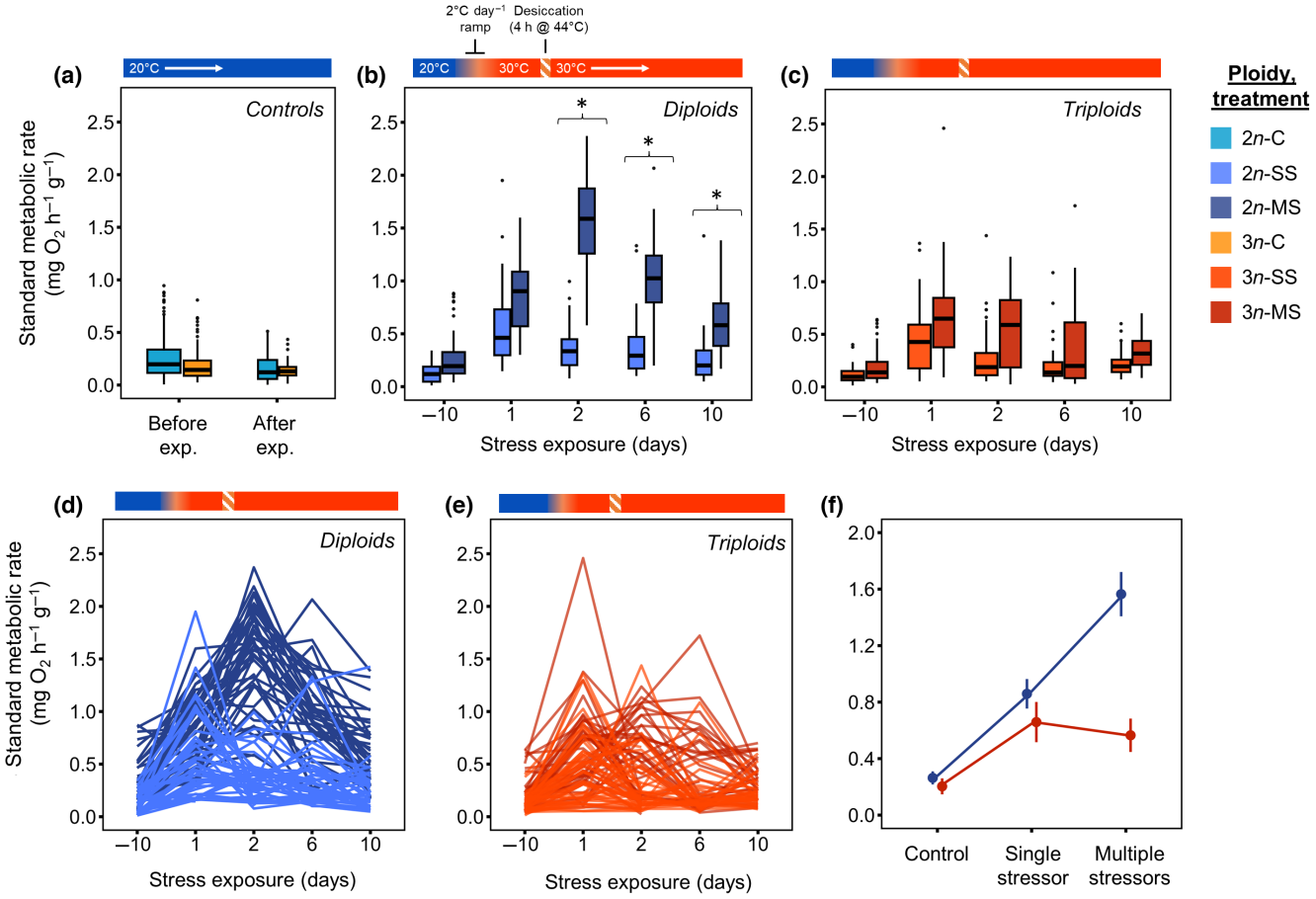

FIGURE 3 The impact of ploidy, stress type, and exposure duration on the standard metabolic rate of adult Pacific oysters (a-c). Diploid (2*n*) and triploid (3*n*) oysters were acclimated to hatchery conditions (day −30 to −10, 20°C) before inclusion in either the control (-C; 20°C), single stressor (-SS; SW temp = 30°C), or multiple stressor (-MS; SW temp = 30°C followed by aerial exposure = 44°C for 4 h) treatment. Oxygen consumption was repeatedly sampled from individually labeled oysters ($n = 30–45$ per treatment), each of which are represented as lines in panels (d) and (e). The mean metabolic rate (±95% CI) of oysters preceding and directly following multiple stress exposure are presented in panel (f). Asterisks indicate when metabolic rate varied significantly for a given ploidy across treatments (alpha = 0.05).

exposure, a 2.4-fold greater number of significant DEGs were observed in diploids (2*n*-SS; 4267 DEGs) than triploids (3*n*-SS; 1809), while the opposite was found after multiple stress exposure, where a 2.7-fold greater number of DEGs were identified in triploids (3*n*-MS; 1579) than diploids (2*n*-MS; 596). A summary of the number of DEGs identified and their commonality across ploidy and treatment groups is presented in Figure 6a. Under control conditions, a nearly equal proportion of DEGs exhibited either increased or decreased expression within triploids when compared with the diploid control (3*n*-C; 51.5% increased, 48.7% decreased). Similar parity was observed in triploids following single (3*n*-SS; 48.8% increased, 51.2% decreased) and multiple stress exposure (3*n*-MS; 46.2% increased, 53.8% decreased). However, the same was not found in diploids, which had a greater proportion of downregulated DEGs following single stress exposure (2*n*-SS; 42.5% increased, 57.5% decreased) and more upregulated DEGs following multiple stress exposure (2*n*-MS; 60.9% increased, 39.1% decreased). A summary of DEG expression patterns is presented in Figure S6 and Figure 6b,c.

The 50 DEGs with the largest increase or decrease in expression following stress treatment for each ploidy are presented in Figure 6d. Both ploidies increased the expression of transcripts associated with cellular stress following single stress treatment, including cyclic AMP-dependent transcription factor ATF-3 and HSP 83-like, while only triploids decreased APP1 and Rho1. Both ploidies increased the expression immune-related genes after single stress exposure, including complement C1q tumor necrosis factor-related protein 7 (C1QTNF7) and proline-rich transmembrane protein 1 (PRTP), while simultaneously decreasing the expression of ryncolin-1. Following multiple stress exposure, both ploidies drastically increased the expression of a suite of stress response proteins, including heat shock proteins (HSP68, HSP70-B2) and BAG family molecular chaperone regulator 3 (BAG3). Only triploids increased the expression of the immune-related genes metalloproteinase inhibitor 3 (TIMP3) and hemagglutinin/amebocyte aggregation factor isoform X1 (HAAF-X1).

The 50 DEGs with the greatest divergence in expression across ploidy following stress exposure are presented in Figure 7, categorized by their identified or putative role in the cellular stress response (a), immune response (b), or metabolism (c). Observed differences following single stress exposure were observed in heat shock proteins (HSP27, HSP70-12A), baculoviral IAP repeat-containing proteins

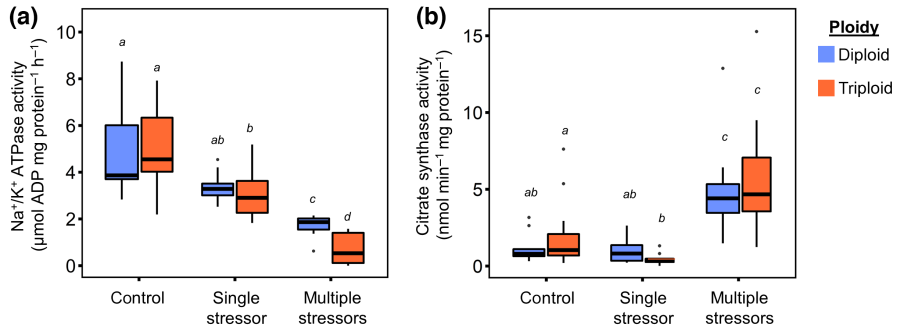

FIGURE 4 The impact of ploidy and stress exposure on Na^+/K^+ ATPase (a; $n = 10–13$ per treatment; 1 day after stress) and citrate synthase (b; $n = 9–12$ per treatment; 1 day after stress) enzyme activity of adult Pacific oysters. Diploid (2*n*) and triploid (3*n*) oysters were acclimated to hatchery conditions (20 days, 20°C) before inclusion in either the control (-C; 20°C), single stressor (-SS; SW temp = 30°C), or multiple stressor (-MS; SW temp = 30°C; aerial exposure = 44°C for 4 h) treatment. Italicized letters represent the result of post-hoc Tukey HSD comparisons across groups; boxplots that share a letter within panels are not significantly different. All enzyme activity was measured within the ctenidium.

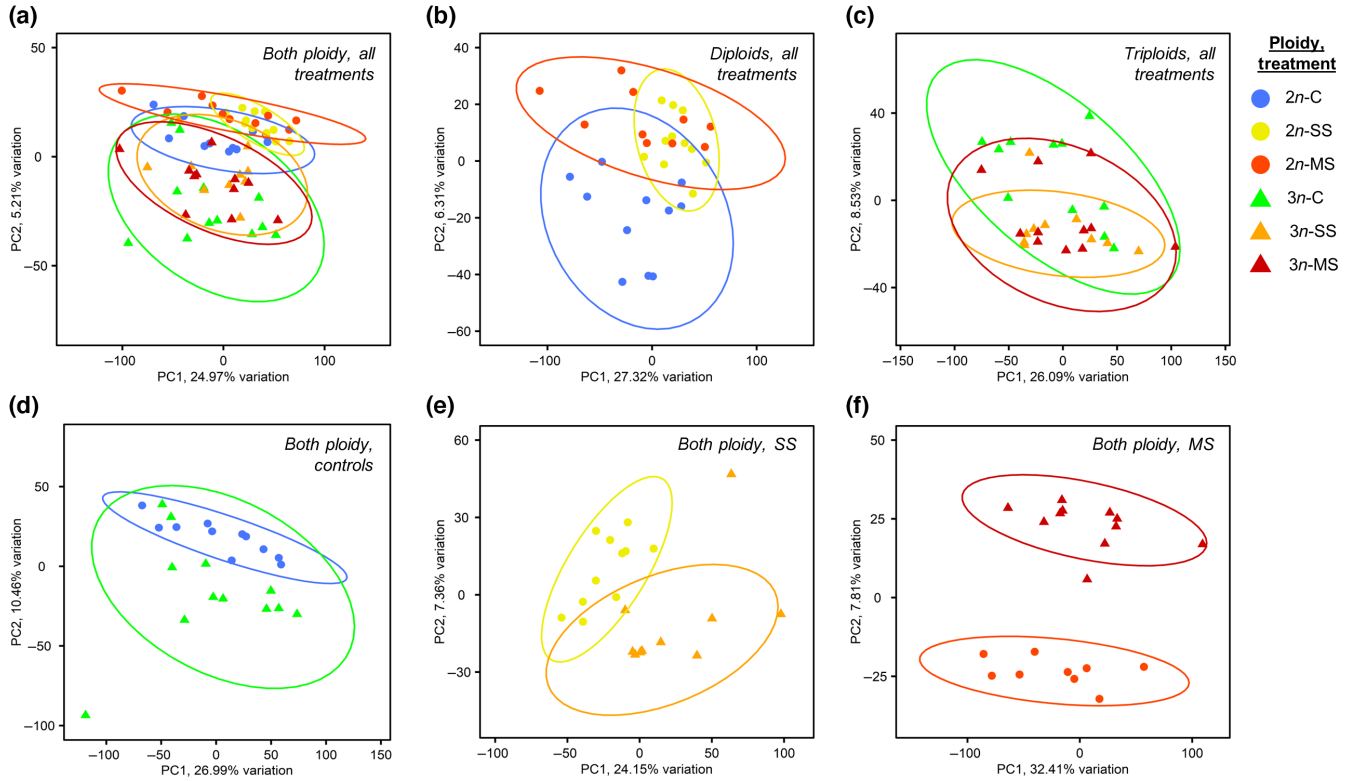

FIGURE 5 Principal component plots comparing gene expression across all treatments (a), within diploids (2*n*; b) or triploids (3*n*; c), or across ploidy within the control (-C; d), single stressor (-SS; e), or multiple stressor (-MS; f) treatments.

(BIRC2, BIRC7-A), caspases (CASP3, CASP7), and immune response genes, such as toll-like receptors (TRL4) and C-type lectin domain family member proteins (CLEC4M). Following multiple stress exposure, the expression of heat shock proteins (HSP70-B2, HSP83-like), E3 ubiquitin-protein ligases (RNF14, RNF34-like, TRIM13, TRIM36, TRIM71), transient receptor potential cation channel subfamily M members (TRPM1-like, TRPM2-like, and TRPM7–10), myeloid differentiation primary response protein MyD88, complement C1q-like protein 4 (C1QL4), and prostaglandin E2 receptor EP4 subtype (PTGER4) greatly differed between diploids and triploid.

3.6 | Functional enrichment analysis

Ploidy-specific DEGs from the single stressor and multiple stressor treatments were used in functional enrichment analysis. Following

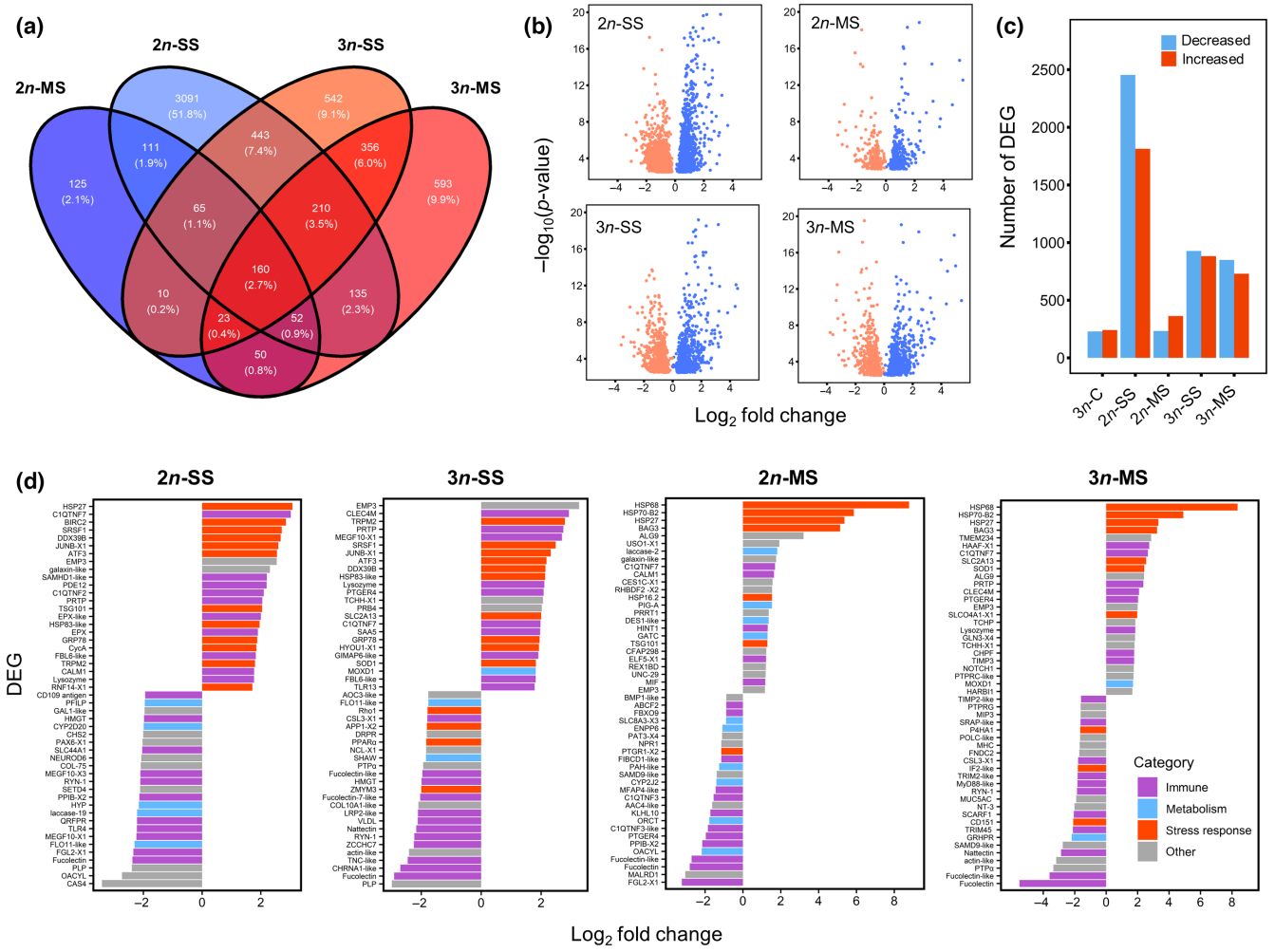

FIGURE 6 (a) Venn diagram of overlapping and contrasting differentially expressed genes (DEGs) identified within diploid (2*n*) and triploid (3*n*) oysters following each treatment (-SS: single stressor; -MS: multiple stressor). (b) Volcano plots displaying the significance (*p*-value) and expression (Log_2 fold change) of DEG within each ploidy following single and multiple stress treatment. (c) The number of significant DEG identified within triploids under control conditions (3*n*-C), within each ploidy following single (2*n*-SS, 3*n*-SS) and multiple (2*n*-MS, 3*n*-MS) stress exposure, whether their expression increased or decreased with respect to the diploid control. (d) The 25 DEGs with the greatest observed increase and decrease in expression within diploids and triploids following single or multiple stress exposure.

single stressor exposure, 3650 DEGs were uniquely associated with diploids, while 692 were associated with triploids (Figure 8a). Significant single stressor DEG mapped to 100 diploid-specific and 66 triploid-specific enriched GO terms associated with biological processes (Figure 8b); the distribution of GO terms within each functional category was significantly different across ploidy ($\chi^2_{df=6,N=65} = 37.41$, $p < .001$) following single stressor exposure, with 11.9% and 29.2% of terms associated with stress and immune processes within diploids and triploids, respectively. Following multiple stressor exposure, 418 DEGs were uniquely associated with diploids and 492 were associated with triploids (Figure 8c). Significant multiple stressor DEG mapped to 74 diploid-specific and 50 triploid-specific enriched GO terms associated with biological processes (Figure 8d); the distribution of GO terms associated with functional categories was also significantly different across ploidy ($\chi^2_{df=6,N=50} = 38.79$, $p < .001$), with 10.81% of diploid-specific and 26.0% of triploid-specific terms associated with stress and immune processes.

4 | DISCUSSION

The primary objective of this study was to determine whether polyploidy impacts the resilience of Pacific oysters to heatwaves. The mortality rate of diploid and triploid oysters was similar after a gradual elevation in seawater temperature to 30°C (single stressor) that replicated conditions present during a MHW. However, when followed by aerial emersion that replicated conditions present at low tide during an atmospheric heatwave, triploids exhibited a 2.5-fold higher mortality rate than diploids (36.4% vs. 14.8%). Elevated mortality in triploids was paired with metabolic rate depression, a measurable decrease in Na^+/K^+ ATPase within the ctenidium, and the dysregulated expression of genes the encode heat-responsive molecular chaperones, as well as antiapoptotic and immune-related proteins. These results suggest that polyploidy alters the genetic architecture that regulates the stress response in *C. gigas*, negatively impacting physiological tolerance limits and increasing susceptibility to extreme climate events.

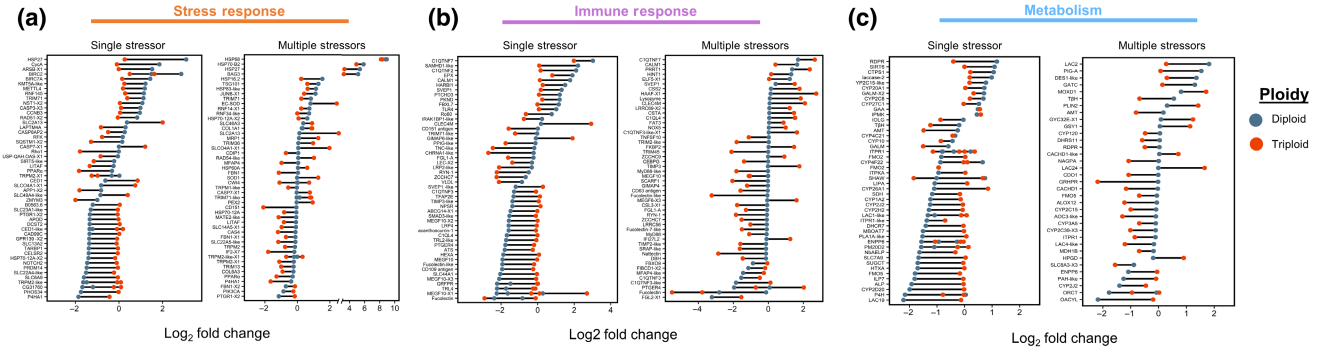

FIGURE 7 The 50 differentially expressed genes that displayed the greatest divergence in expression between diploid (2*n*) and triploid (3*n*) Pacific oysters following single or multiple stress exposure, categorized by their identified or putative role in (a) stress response, (b) immune response, or (c) metabolism.

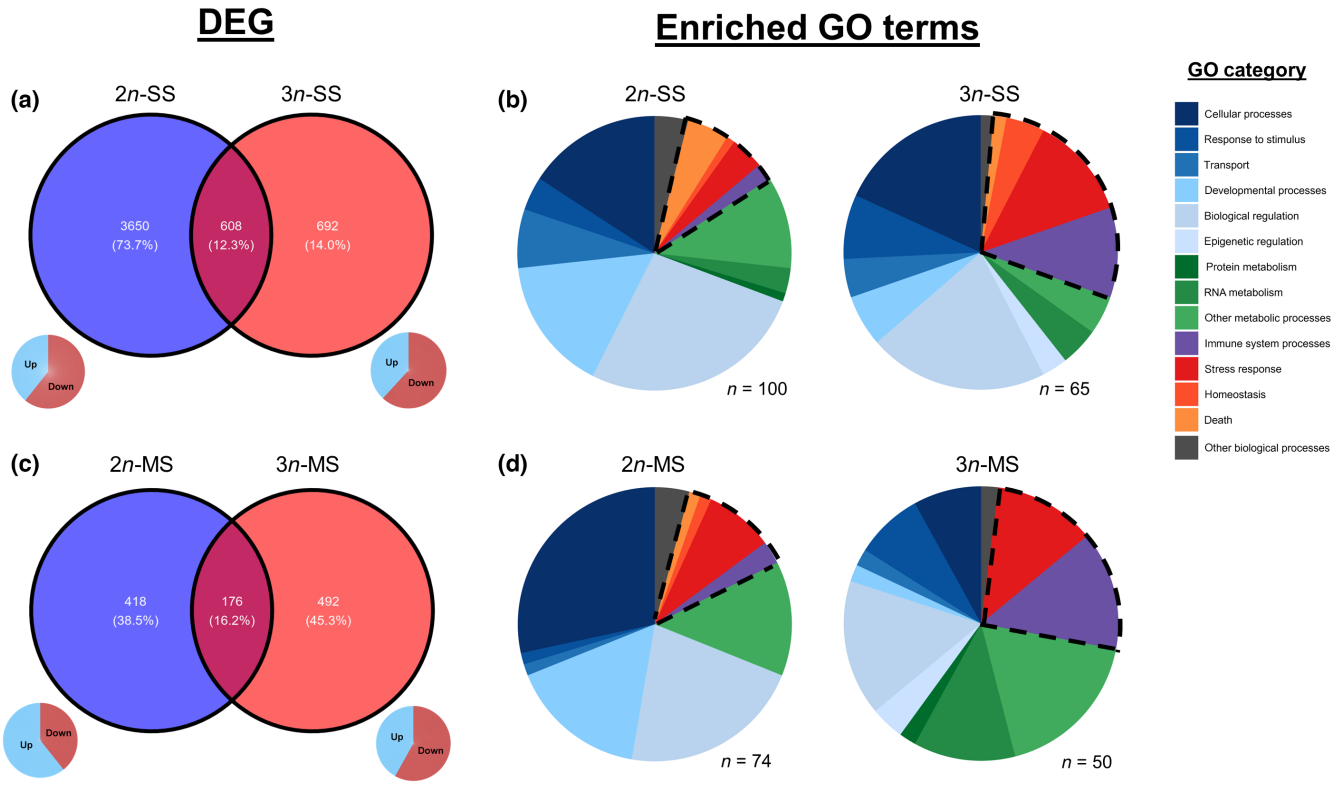

FIGURE 8 Venn diagram of overlapping and contrasting significant differentially expressed genes (DEGs) across ploidy within the single stressor (a) and multiple stressor (c) treatment. (b) Pie charts of significantly enriched GO term categories associated with unique DEG within diploid (2*n*) and triploid (3*n*) Pacific oysters after exposure to a single (b) or multiple stressors (d).

4.1 | Triploid oysters display a higher mortality following multiple stress exposure

While the thermotolerance of *C. gigas* is well studied, the impact of polyploidy on thermal tolerance limits remains unclear. Thermal limits in oysters vary across populations and can be plastic, with recent work demonstrating shifts in tolerance can occur following prior exposure to MHWs (Ding et al., 2020; He et al., 2021). Despite this variability, diploid Pacific oysters generally switch between aerobic and anerobic metabolism between 36 and 43°C when submerged, with 100% mortality observed after this range is exceeded (Ghaffari et al., 2019). A small, but growing, number of laboratory experiments suggest that triploids have a lower critical thermal maximum than diploids; For example Li et al. (2022) found that triploid *C. gigas* display a 1.51-fold higher cumulative mortality rate than diploids (57.05% vs. 37.73%) following acute heat shock (18–28°C) and a 3.25-fold higher mortality rate (83.7% vs. 25.73%) following chronic heat exposure. However, field assessments of triploid and diploid oyster survivorship over the course of a growing cycle suggest that summer mortality is more complicated than would be expected from differences in thermal tolerance limits alone.

Triploid *C. gigas*, *C. virginica*, and *C. hongkongensis* have been shown to exhibit lower (Gagnaire et al., 2006; Qin et al., 2019),

higher (Bodenstein et al., 2021, 2023; Guévélou et al., 2019; Houssin et al., 2019; Matt, 2018; Matt et al., 2020; Wadsworth, 2018; Wadsworth et al., 2019), and comparable (Dégremont et al., 2012; Ibarra et al., 2017) mortality rates when compared with diploids depending on the environmental conditions present. For example Wadsworth et al. (2019) found higher cumulative mortality in triploid *C. virginica* than diploids across growing sites within the Gulf of Mexico; mortalities were associated with sudden drops in salinity (<5 ppt) and high temperature (>30°C). The presence of multiple stressors preceding mortality events is consistent with both Guévélou et al. (2019) and Matt et al. (2020), who have noted that *C. virginica* triploid mortalities within Chesapeake Bay, VA are not correlated with the presence of elevated seawater temperature alone. Instead, common farm stressors, including tumbling stress and desiccation, as well as hyposalinity have been shown to contribute to triploid mortality (Bodenstein, 2019; Bodenstein et al., 2021, 2023). These results are consistent with the findings of this study that suggest that the synergistic impact of multiple environmental stressors motivates triploid mortality.

4.2 | Summer mortality as a reproductive disorder

While energy allocation to gametogenesis may play a role in the initiation of summer mortality events, we found no evidence of an interaction between reproductive investment and mortality. To replicate the seasonal timing of heatwaves, experiments took place in summer (June–July) and were intentionally timed to coincide with the period of peak reproductive output. Summer mortality has also been interpreted as a reproductive disorder that is caused by large shifts carbohydrate storage and utilization leading up to spawning (Koganezawa, 1975; Mori, 1979); as such, reproductive effort has been associated with higher mortality rates during the spring and summer in diploids, especially under warming and in the presence of marine pathogens (Huvet et al., 2010; Wendling & Wegner, 2013). Histological examination of the reproductive tissue of triploids in this study confirmed early (stage 1) or no gonad development (stage 0) throughout the experiment; the majority of diploids had mature gonads (stage 3) upon arrival at the laboratory and after single stress exposure, with evidence of spawning after multiple stress exposure (Figure 1d). Given that the mortality rate of triploids exceeded that of diploids following multiple stress exposure, it is unlikely that reproductive state was the motivating factor that impacted stress tolerance. However, it should be noted that although triploid oysters are commonly referred to as sterile, they can produce gametes (Allen & Downing, 1990; Jouaux et al., 2010; Matt & Allen, 2021), a phenomenon that may be enhanced by warm seawater temperatures (Normand et al., 2008). Future studies would benefit from an in depth investigation of whether the incomplete formation or rupture of gonads during gametogenesis contributes to increased rates of bacterial infection and mortality within triploids (De Decker et al., 2011).

4.3 | Triploid mortality as a physiological syndrome

The "MOREST," a multi-disciplinary program coordinated by IFREMER to investigate oyster mortalities within France, concluded that summer mortality may be a "physiological syndrome" that results from energetic limitation under multiple stressor scenarios (Samain & McCombie, 2008). While this may be true for diploids as well, the results of physiological assays employed in this study suggest that triploids may experience a greater degree of energetic limitation than diploids following exposure to multiple stressors. In this study, diploid and triploids displayed similar metabolic rates within the control and single stressor treatment, a result that is in agreement with other studies in *C. gigas* that found no difference in resting metabolic rate (RMR) across ploidy at 22 and 27°C (Farrell et al., 2015), and 30°C (Shpigel et al., 1992). However, after multiple stress exposure, triploids did not significantly increase their oxygen consumption, while diploids increased and maintained an elevated metabolic rate for up to 8 days following stress exposure (Figure 3). The failure of triploids to increase their metabolic rate could be interpreted as metabolic depression, which can be protective over short durations by effectively reducing ATP and protein turnover rates to maintain energy balance. However, failure to increase respiration following a stressful event can result in the accumulation of anaerobic metabolites and the discontinuation of important ATP-demanding processes that can, in turn, increase mortality rates (Guppy & Withers, 1999; Lesser, 2016; Zittier et al., 2015).

Triploids failing to increase their metabolic rate under stress is consistent with a transition into "pessimum" or a state of time-limited survival (Sokolova et al., 2012). Within this range, all available metabolic energy is reallocated to somatic maintenance; using this mechanism, metabolic demand is reduced to "wait out" an environmental stressor. Given the short application of the secondary stressor in this experiment, one possibility is that triploids entered this state and failed to recover due to the dysregulation of metabolic pathways responsible for energy homeostasis. For example, the metabolic gene transcript with the largest difference in expression across ploidy in this experiment following multiple stress exposure was glyoxylate/hydroxypyruvate reductase (GRHPR). GRHPR is a dual activity enzyme that is responsible for two cellular roles: the excretion of glyoxylate, the toxic product of purine metabolism in marine invertebrates, and the maintenance of D-glycerate, an important precursor to the gluconeogenic pathway which is responsible for the maintenance of glucose homeostasis to meet enhanced energy demand in during periods of stress (Lassalle et al., 2016; Noguchi et al., 1982). GRHPR expression was significantly depressed in triploids relative to diploids following multiple stress exposure, along with other putative contributors to the gluconeogenic pathway (e.g., putative malate dehydrogenase 1B). Potential interactions between ploidy and stress and their impact on glucose homeostasis could potentially explain observed physiological differences and warrants further study.

In addition to metabolic rate, triploids experienced a significantly greater decrease in Na^+/K^+ ATPase enzyme activity following multiple stress exposure (Figure 4A). Na^+/K^+ ATPase is a ubiquitously

expressed multifunctional transmembrane protein complex that is essential for numerous physiological processes such as osmotic and ionic regulation (Crane, 1977; Skou, 1957; Wright & Manahan, 1989) and is commonly used an indicator of energetic limitation following environmental and oxidative stress in marine invertebrates (Bianchini & Wood, 2003; Haque et al., 2019; Wheatly & Henry, 1987). Na^+/K^+ ATPase-mediated physiological processes are energy intensive, accounting for up to 77% of larval oxygen consumption in the sea urchin *Strongylocentrotus purpuratus* (Leong & Manahan, 1997). While the observed decrease in Na^+/K^+ ATPase enzyme activity could indicate triploids have entered a state of energetic limitation, it should be noted that this cannot be confirmed without additional information. The portion of total Na^+/K^+ ATPase that actively contributes to osmoregulation can vary due to several factors, including the presence of cofactors, enzyme turnover rate, and enzyme binding affinity (Hochachka & Somero, 2002). All these factors are temperature dependent and subject to biochemical adaptation as the climate continues to change (Somero, 2010, 2012). For these reasons, future studies would benefit from measuring the physiologically active fraction (in vivo activity) of Na^+/K^+ ATPase in addition to total enzyme activity (in vitro activity, as presented in this study) to ascertain the relationship between the two measures and determine whether triploids are indeed metabolically constrained.

4.4 | Triploids exhibit signs of transcriptional dysregulation following multiple stress exposure

In this study, we observed major differences in gene expression across ploidy that only manifested after stress exposure (Figure S6; Figure 5f). It is worth noting that differences in gene expression do not always translate to the protein level, and the functional consequences of dysregulated expression cannot be assumed to impact physiology. From this perspective, DEG analysis has the potential to identify ploidy-specific differences in important biological pathways that warrant further study. Polyploidy has been suggested to result in the dysregulation of key biological processes due to changes in gene dosage, the genetic architecture of regulatory networks, incomplete silencing, epigenetic instability, and/or other downstream impacts of chromosome set duplication (Comai, 2005; Kim et al., 2011). Genome level changes, such as gene dosage, can impose energetic "costs" that may only manifest when multiple stressors are present. For example, *C. virginica* is remarkably tolerant to a range of environmental stressors, but the simultaneous exposure of two or more stressors has been shown to reduce the tolerance to any single factor (Cherkasov et al., 2006; Ivanina et al., 2012; Kurochkin et al., 2009). Synergistic effects of multiple stressors have been between warming and salinity (Jones et al., 2019; Marshall et al., 2021; McFarland et al., 2022) and hypoxia and ocean acidification (Gobler et al., 2014) in oysters.

In response to multiple stress exposure, both diploids and triploids drastically increased the expression of the molecular chaperones HSP68, HSP70-B2, HSP27, and BAG3 (Figure 6d). However, out of the top 50 stress-related genes expressed in both ploidy, 74% displayed lower, insignificant, or decreased expression within triploids (Figure 7b). Additionally, functional enrichment analysis of ploidy-specific gene sets indicated that GO terms associated with stress and immune processes were significantly enriched in triploids compared to diploids (Figure 8). These results are consistent with the findings of (Li et al., 2022), who found an overrepresentation of inflammatory response and apoptosis gene expression following acute temperature stress in triploid *C. gigas*. In this study, triploids notably exhibited dysregulated expression of several classes of stress response proteins, including molecular chaperones and heat shock proteins, ubiquitin ligases, and other proteins that regulate apoptosis. Diploids had higher expression levels across six heat shock proteins following multiple stress exposure. HSPs are a broad class of molecular chaperones that are capable of mediating heat-induced cellular damage by preventing protein misfolding (Meistertzheim et al., 2007). Similarly, six E3 ubiquitin-protein ligases species were observed to have variable expression across ploidy, out of which four had higher expression in diploids. E3 ubiquitin-protein ligases are known to protect against apoptosis by regulating p53 protein which plays a pivotal role in the initiation of cell division/death (Pan & Blattner, 2021).

In addition to stress response genes, a variety of genes associated with pathogen recognition, innate immunity, and the metabolism of xenobiotics were either dysregulated or highly elevated within triploids when compared with diploids following multiple stress exposure. For example, a suite of complement C1q-like proteins (C1QL) and complement C1q tumor necrosis factor-related proteins (C1QTNF) were elevated in triploids more than diploids (Figure 7b). C1q superfamily members has been shown to play a critical role in innate immunity, pathogen recognition, inflammation, apoptosis, damage repair, and cell survival through the apoptosis NF-κB pathway (Kishore et al., 2004; Zhang et al., 2014). Alternatively, triploids decreased the expression of a variety of immune-related transcript when diploids either did not alter their expression or increased them, including multiple epidermal growth factor-like (MEGF) domains protein 6 isoform X3 and 10, MyD88, and tumor necrosis factor ligand superfamily member 10 (TNFSF1). MEGF domain proteins are exclusively found in hyalinocytes and have been identified to play a role in pathogen recognition in the *C. gigas* immune system (Wang et al., 2015). Similarly, MyD88 and TNFSF1 play central roles in the toll-like receptor and interleukin-1 receptor signaling pathways, both of which regulate the immune system, differentiation, proliferation, and cell death (MacEwan, 2002).

4.5 | Ploidy altered the expression of mitochondrial and metabolic enzymes under stress

In addition to stress and immune response proteins, significant differences in the expression patterns of a variety of genes that regulate mitochondrial function and aerobic metabolism were observed after multiple stress exposure when DEG were generated with their

respective ploidy as a background (see Tables S7 and S8). For example, elevated expression of methyltransferases and other proteins involved in stress-related mitochondrial biogenesis and proliferation was observed in diploids, but not triploid, such as rRNA methyltransferase 2 (Rorbach et al., 2014), and mitochondrial genes implicated in heat and oxidative stress tolerance, including NADH–ubiquinone oxidoreductase (Downs & Heckathorn, 1998), 39S ribosomal protein L19 (Chen et al., 2020) and isocitrate dehydrogenase [NADP] (Jo et al., 2001). The elevated expression of genes within pivotal regulatory roles in the TCA cycle was also only observed in diploids, including cytochrome P450, cytochrome b-c1, and cytochrome c oxidase. A muted transcriptional response of triploids is in agreement with prior work that suggests that triploid *C. gigas* are less sensitive to environmental cues than diploids (Duchemin et al., 2007) and mount a delayed gene regulatory response to heat shock within laboratory assays (Li et al., 2022).

Citrate synthase enzyme activity (Figure 4B), but not gene expression (Figure S7), was elevated in diploids and triploids following multiple stress exposure. Citrate synthase (CS) is the rate-limiting enzyme of the TCA cycle and regulates oxidative ATP production through mitochondrial respiration (Ciccarone et al., 2017). Total CS activity has been found to be positively correlated with respiration rate within a variety of marine invertebrates (Dahlhoff et al., 2002; García-Esquivel et al., 2001, 2002), including *C. gigas* (Moran & Manahan, 2004), and decreases in response to starvation and acute heat shock in mussels (Dahlhoff et al., 2002; Torossian et al., 2020). In triploid oysters, CS gene expression has been shown to be elevated across five tissue types (Qin et al., 2018) and is correlated with high glycogen content (Li et al., 2017). However, studies in other taxa have produced mixed results. For example, triploid zebrafish displayed the same CS enzyme activity following temperature stress (van de Pol et al., 2021), while triploid white sturgeon displayed a marked decrease with respect to diploids (Leal et al., 2019). In this study, we observed no difference in CS activity across ploidy, with triploids elevating CS activity following multiple stress exposure without significantly increasing their metabolic rate. While the decoupling of CS activity with respiration in triploids could be indicative of metabolic dysregulation, as was previously noted for Na^+/K^+ ATPase, the physiological consequences of an enzyme's activity are difficult to interpret without information about how much of that enzyme is physiologically active. Nevertheless, the potential for polyploidy to prevent or slow the transition from aerobic to anaerobic metabolism through the dysregulation of important genes that function in ATP generation and turnover is an interesting hypothesis that warrants further study.

5 | CONCLUSION

Our results contribute to a growing body of work that suggests that polyploidy negatively impacts environmental stress tolerance. A comparison of mortality rates across ploidy within this study supports the prevailing view that temperature stress alone is unlikely to motivate triploid mortality events observed in aquaculture. Instead, our work suggests that triploids exhibit enhanced mortality following exposure to multiple environmental stressors that can be present over the course of a tidal cycle during a heatwave. Following exposure to multiple environmental stressors in succession, triploids display evidence of metabolic depression, changes in the activity of metabolic enzymes, and distinct differences in the expression of gene transcripts related to cellular stress response, immunity, and metabolism. Together their results demonstrate that ploidy impacts the genomic and physiological stress response of Pacific oysters and provides a basis for future study into the potential causes of observed differences in performance. Understanding what motivates the susceptibility of triploid m to extreme climate events such as heatwaves is needed to ensure food security within future climate scenarios, especially as triploidy becomes an increasingly popular tool to elicit reproductive control across a wide range of taxa used within marine aquaculture.

AUTHOR CONTRIBUTIONS

Matthew N. George, Mackenzie Gavery, Brent Vadopalas and Steven Roberts conceived of the study, analyzed data, and wrote the manuscript. Mackenzie Gavery secured funding for the study. Matthew N. George oversaw the completion of hatchery experiments, conducted respirometry measurements, sampled tissue, performed RNA extractions, and analyzed sequencing results. Brent Vadopalas and Olivia Cattau assisted with tissue sampling during hatchery experiments. Olivia Cattau completed the citrate synthase enzyme assay. Delaney Lawson completed the histology analysis. Mollie A. Middleton completed the Na^+/K^+ ATPase enzyme assay.

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CONFLICT OF INTEREST STATEMENT

The authors have no financial or non-financial competing interests associated with this research or the publication of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at https://doi.org/10.5281/zenodo.7693092 (George et al., 2023). Tag-seq data are available under the NCBI BioProject ID 913164. Supporting tables and figures are provided together with this manuscript.

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