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Using a metabolomics approach to investigate the sensitivity of a potential Arctic-invader and its Arctic sister-species to marine heatwaves and traditional harvesting disturbances

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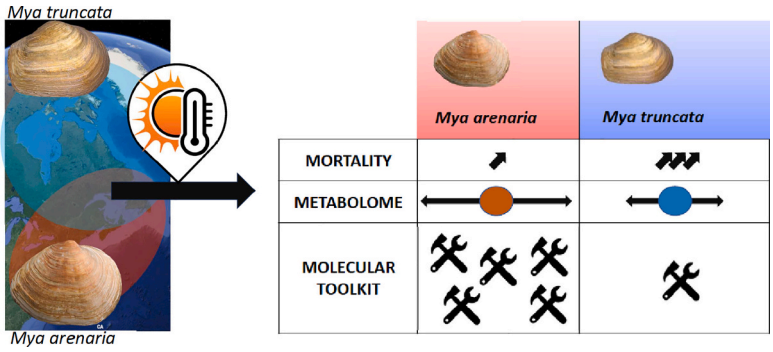
HIGHLIGHTS

- Clams of different climatic ranges show marked differences in heatwave tolerance.
- The polar clam is more vulnerable to future heatwaves than the temperate clam.
- Greater heatwave tolerance is underpinned by greater metabolomic reprogramming.
- Higher tolerance may grant the temperate clam the ability for poleward expansion.
- Traditional harvesting (single event) was not found to affect clam survival.

GRAPHICAL ABSTRACT



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ABSTRACT

Coastal species are threatened by fishing practices and changing environmental conditions, such as marine heatwaves (MHW). The mechanisms that confer tolerance to such stressors in marine invertebrates are poorly understood. However, differences in tolerance among different species may be attributed to their geographical distribution. To test the tolerance of species occupying different thermal ranges, we used two closely related bivalves the softshell clam *Mya arenaria* (Linnaeus, 1758), a cold-temperate invader with demonstrated potential for establishment in the Arctic, and the blunt gaper *Mya truncata* (Linnaeus, 1758), a native polar species. Clams were subjected to a thermal stress, mimicking a MHW, and harvesting stress in a controlled environment. Seven acute temperature changes (2, 7, 12, 17, 22, 27, and 32 °C) were tested at two harvesting disturbance intensities (with, without). Survival was measured after 12 days and three tissues (gills, mantle, and posterior adductor muscle) collected from surviving individuals for targeted metabolomic profiling. MHW tolerance differed significantly between species: 26.9 °C for *M. arenaria* and 17.8 °C for *M. truncata*, with a negligeable effect of

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harvesting. At the upper thermal limit, *M. arenaria* displayed a more profound metabolomic remodelling when compared to *M. truncata*, and this varied greatly between tissue types. Network analysis revealed differences in pathway utilization at the upper MHW limit, with *M. arenaria* displaying a greater reliance on multiple DNA repair and expression and cell signalling pathways, while *M. truncata* was limited to fewer pathways. This suggests that *M. truncata* is ill equipped to cope with warming environments. MHW patterning in the Northwest Atlantic may be a strong predictor of population survival and future range shifts in these two clam species. As polar environments undergo faster rates of warming compared to the global average, *M. truncata* may be outcompeted by *M. arenaria* expanding into its native range.

1. Introduction

Anthropogenic pressures threaten marine ecosystems via global change, habitat destruction and fragmentation, natural resource extraction, non-native species, and pollution (IPBES, 2019; IPCC, 2022). Studying how these pressures impact marine invertebrates from polar ecosystems presents logistical limitations, thus they are often more poorly understood than tropical or temperate species. Polar species, however, may face greater rates of environmental change than those from temperate ecosystems (IPCC, 2022). The biogeographic distributions of Arctic marine invertebrate populations are greatly constrained by environmental conditions (Bozinovic et al., 2011; Calosi et al., 2010; Chown and Gaston, 1999; Sunday et al., 2012). As such, global change may potentially shift species distributions (Parmesan, 1996; Pinsky et al., 2013; Sunday et al., 2012) with consequences for the long-term persistence of species and ecosystem functioning.

Increased greenhouse-gas emissions are driving changes in average ocean temperature conditions (Hansen et al., 2010; IPCC, 2022), leading to the increasing intensity of extreme climatic events, such as heatwaves (IPCC, 2022; Meehl and Tebaldi, 2004). Marine heatwaves (MHW, as defined by Hobday et al., 2016) have doubled in frequency since 1982 (Hoegh-Guldberg et al., 2018). These extreme events may pose a greater threat to species and communities than gradual, average ocean warming (Frölicher and Laufkötter, 2018; Smale and Wernberg, 2013; Thibault and Brown, 2008). Ecosystems may face losses of key species that are unable to adapt or disperse towards more favourable environments (Sunday et al., 2015; Walther et al., 2002). Areas that have experienced notable MHW include the Northeast Pacific Ocean (Bond et al., 2015), the Northwest Atlantic Ocean (Mills et al., 2013), the Mediterranean Sea (Olita et al., 2007), and the Arctic Ocean (Simpkins, 2017), at times setting off mass mortality events (Garrabou et al., 2009). These mortality events are likely induced in part by temperatures that exceed species' physiological tolerance thresholds (Sorte et al., 2011; Stillman, 2002, 2019). According to the climate extreme hypothesis (CEH), extreme climatic events may constrain species ranges (Bozinovic et al., 2011; Smale and Wernberg, 2013; Bennett et al., 2021) and drive the evolution of their physiological tolerance (Buckley and Huey, 2016; Grant et al., 2017; Bennett et al., 2021). Defining species' physiological tolerance limits is key to predicting climate warming effects (Somero, 2010; Huey et al., 2012; Bozinovic and Pörtner, 2015) and should factor into conservation strategies (Somero, 2010; Seebacher and Franklin, 2012).

Marine coastal ecosystems provide food security and support economies worldwide (Anderson et al., 2011; Pauly et al., 2005). Marine ecosystems and the species that inhabit them are predicted to be impacted not only by global climate change, but also by other anthropogenic stressors, such as aquatic non-native invasive species (Christensen et al., 2014; Mora et al., 2009). Most aquatic organisms in these ecosystems are ectothermic and therefore temperature is important to their physiology and bioenergetics (Kelley, 2014; Rahel and Olden, 2008). Global warming is also expected to impact their biogeography, with increasing temperatures leading to potential displacements of native cold-water species by novel or invasive southern species (Csapó et al., 2021; Goldsmit et al., 2020). However, methods to predict which species may become invasive remain elusive (Kelley, 2014), although contrasting closely related species may provide important insights (Rehage et al., 2020).

Several types of invertebrate physiological responses to environmental stressors have been documented (Harley et al., 2006; Poloczanska et al., 2013; Przeslawski et al., 2015), ranging from sub-cellular to whole organism-level coordinated responses (Hofmann and Todgham, 2010; Kassahn et al., 2009). For example, invertebrate physiology is highly dependent on environmental temperature (Moyes and Schulte, 2015), as temperature drives biochemical rates (Kingsolver, 2009) disrupts oxygen distribution within organisms (Angilletta Jr., 2009), and alters the functional properties of both membranes (Hazel and Williams, 1990) and proteins (Somero, 1995). Adaptive processes underpin the range of temperatures under which organisms can survive (Angilletta Jr., 2009; Hochachka and Somero, 2002). The generalist vs. specialist (eurytolerant versus stenotolerant) paradigm suggests evolutionary selection for wide versus narrow thermal windows in contrasting types of environments (Somero, 2005). Thermal generalists outperform thermal specialists in highly variable environments (Gilchrist, 1995). The greater plasticity found in their tolerance mechanisms should not only make them less vulnerable to climate warming (Magozzi and Calosi, 2015; Munday et al., 2013; Somero, 2005), but also provide them a competitive edge as their range shifts northward (Parmesan, 1996). Beyond a species' given thermal tolerance window, the cellular metabolic machinery (i.e. enzymes and membranes) is likely compromised and unable to compensate for the negative impacts due to temperature stress (Bowler, 1987; Hightower, 1991). Quantifying these thresholds is valuable to predict marine species' vulnerability to climate warming. A novel perspective to thermal thresholds may be achieved by focusing on cellular physiology through metabolomics. This approach allows for the identification of biomarkers associated with stressor-related metabolic responses and for integrating these into a hierarchical physiological framework (Weckwerth, 2003). Metabolomics may be used to tease apart the underlying metabolic changes associated with temperature responses and potential differences in adaptive capacity within and between populations or species (Lin et al., 2006; Bundy et al., 2008; Sun et al., 2022).

Here, we take an integrative approach to evaluate the effects of combined stressors on invertebrate physiology to develop our understanding of cellular mechanisms used to cope with global change. Specifically, this study examines the responses of the temperate (generalist and highly invasive – Lasota et al., 2014) softshell clam *Mya arenaria* (Linnaeus, 1758) and the polar (cold-water specialist – Sleight et al., 2018) blunt gaper *M. truncata* (Linnaeus, 1758) to MHW and harvesting disturbance. We assessed mortality and sampled tissues from the surviving individuals for metabolomic profiling. It was hypothesised that *M. arenaria* would show a greater tolerance and thermal plasticity to MHW treatments. Harvesting disturbance was predicted to have a negative effect on survival and shift the clam metabolome with compensatory responses of the metabolism, and that the combined effect of both treatments would yield a synergistic negative effect. By evaluating the responses of these congeners to MHW and harvesting, we provide a case study for the conservation of coastal marine species supporting food systems in both temperate and northern communities facing the growing threat of anthropogenic stressors.

2. Materials and methods

2.1. Specimen collection, transport, and husbandry

Softshell clams, *M. arenaria*, and blunt gapers, *M. truncata*, were collected from August to October 2020 at two locations in the Lower Saint Lawrence estuary: intertidally at Métis-sur-Mer, Québec, Canada (48° 40′ 4.6092" N, 68° 1′ 5.9484" W) and SCUBA diving (~ 10 m) at Godbout, Québec, Canada (49° 19′ 25.626" N, 67° 35′ 17.034" W), respectively. Individuals with shell lengths between 50 and 70 mm were selected, maintained under humid conditions to prevent desiccation, and transported in 20 L containers (200 individuals *per* container) to the Maurice-Lamontagne Institute (MLI) in Mont-Joli, Québec, within 10 h of collection.

Upon arrival at MLI, individuals were checked for overall condition and those that displayed abnormalities (e.g. broken valves) or were unresponsive (i.e. inability to retract siphons or close valves) were excluded from the experiment. Approximately 500 healthy individuals of each species were then separately placed into randomly assigned holding tanks (polyethylene fish pan) containing ~20 cm of washed sand (num. 70, Groupe Bellemare, Trois-Rivières, Québec). Clam burying behaviour was noted during this phase (buried/not buried). Tanks were set up in a semi-open recirculating system where they were supplied with 1 L m $^{-1}$ of water coming from a 750 L header tank maintained at 6 °C (ambient temperature at collection sites) by a heat pump (Gell'air, Mont-Joli, Québec). Water in the header tank was continuously renewed with 0.5 L min^{-1} of sand-filtered estuarine water pumped from 2 km offshore. Clams were acclimatized to these conditions for at least 21 d prior to the start of the experiment and were fed daily (at 16 h00) with a commercial algal mixture (Shellfish Diet 1800, Reed Mariculture Inc., San Jose, CA, USA) containing five marine microalgae at 1.2 mL *per* animal according to the manufacturer's recommendations.

2.2. Experimental design, system, and protocol

We used a fully crossed factorial experimental design to investigate the combined effect of MHW and harvesting disturbance on the survival and tissue-specific metabolome profiles of *M. arenaria* and *M. truncata*. This included seven temperature levels (2, 7, 12, 17, 22, 27, and 32 °C) crossed with two harvesting disturbance conditions (i.e. with harvesting disturbance and without harvesting disturbance), yielding 14 distinct treatment levels for each species. Each treatment level had four independent tank replicates, totaling 56 experimental tanks. Each experimental tank was filled with ~20 cm of sand and set at a constant flow rate of 1 L m $^{-1}$. Estuarine water was pumped into two 750 L header tanks, each fitted with a sand filter, and held at either a high (35 °C) or low (1 °C) temperature using four independent Gell'air heat pumps. As above, water in the header tanks was continuously renewed with 0.5 L min^{-1} of sand-filtered estuarine water pumped from 2 km offshore. Water was pumped from the header tanks to the experimental tanks. Temperature was controlled independently in each experimental tank with proportional-integral-derivative (PID) controllers (REX—C100, XNY International, Wenzhou, China). Each PID controlled a three-way valve that supplied experimental tanks with a mixture of high and low temperature water to achieve target temperatures.

Eight randomly selected individuals of each species (16 total) were transferred to each experimental tank and allowed to acclimatize for 14 d prior to beginning experimental treatments. At the time of the transfer, shell morphometrics (length, height, and width) were recorded, clams were labeled, and then hand-buried into experimental units. Average (± standard deviation, $n = 448$) lengths, heights and widths were 71.10 ± 7.37 mm, 44.63 ± 4.82 mm, and 29.75 ± 3.89 mm for *M. arenaria* and 59.83 ± 5.04 mm, 38.91 ± 3.81 mm, and 27.53 ± 2.84 mm for *M. truncata*.

"MHW" (sensu Hobday et al., 2016) exposure temperatures exceeded the 90th percentile calendar day average and were sustained for a minimum duration of 5 d. Of the seven temperature levels, only 22–32 °C inclusively were technically considered MHW based on the local summer sea surface temperature maxima at each collection location – currently around 16–17 °C (Chin et al., 2017; NASA JPL, 2021). However, the unknown seawater-sediment thermodynamic transfer of heat may delay the warming-exposure of these sediment-dwelling organisms. Despite this limitation, we considered temperature treatments 2–17 °C were within the annual range of temperatures experienced at the collection sites. This design thus tested the species' physiological responses within and beyond the bounds of the species' recent thermal histories, in conditions of increased MHW intensity projected by various studies.

A temperature exposure duration of 12 d was selected based on recent average MHW duration of 5–7 d in the study region (Jeong et al., 2016; Lau and Nath, 2012) and projected increases in heat-wave duration of 3.6 d by 2041–2070 (Lau and Nath, 2012), of 1–10 d by 2040–2069 (Jeong et al., 2016) or 8.4 d for each 1 °C $^{-1}$ increase in mean global surface temperature (Perkins-Kirkpatrick and Gibson, 2017). The temperature level range was chosen to encompass the entire thermal range of both species and beyond with the upper limit (32 °C) selected based on the reported upper lethal temperature limit (LT50-24 h) (30.9–34.4 °C) for adult *M. arenaria* (Kennedy and Mihursky, 1971). The lower temperature tolerance in these species is unknown, but likely approaches the freezing point based on their known distribution; given experimental system limitations, the lowest temperature used in this study was 2 °C.

The 12 d MHW treatment (hereafter, "*temperature*") included an initial 3 d step-wise ramping period, during which the temperature was increased or decreased at a stable rate to reach the target temperature. Ramping was conducted for 8 h, then paused 16 h overnight and restarted the next day for a total of 24 ramping hours during the 3-d ramping period. This metric was used to determine the ramping rate at each treatment level. From an initial temperature of 6 °C, the theoretical ramping rates were − 0.17, 0.04, 0.25, 0.46, 0.67, 0.88 and 1.08 °C h $^{-1}$ for treatment levels 2, 7, 12, 17, 22, 27 and 32 °C respectively.

The harvesting disturbance treatment (hereafter, "*harvest*") used in the current stud was similar to previous studies investigating harvesting disturbance effects for other bivalves: softshell clam (Beal and Vencile, 2001), eastern oysters and hard clams (Lenihan and Micheli, 2000), *Ruditapes* spp. clams (Beck et al., 2015), and Venus clams (Ballarin et al., 2003). In the current study, *harvest* consisted of a single non-lethal disturbance where the substrate in each tank was completely turned over, effectively removing clams from the substrate, and exposing them at the surface. This was achieved by emulating professional clam diggers, using a professional grade clam rake with four tines each approx. 25 cm in length (BACK-HOE-4, KB White Company, Marblehead, ME, USA). The treatment was applied once immediately following the 3-d ramping period and did not coincide with feeding times.

In all eight tanks exposed to the 32 °C treatment, the exposure period was terminated due to 100 % mortality within 10 d. Tanks that displayed significant temperature variation during either acclimation, ramping, and/or exposure periods were removed from analyses ($n = 14$). The final experimental units retained for tissue sampling (41 tanks), the resulting mortality and the average physical-chemical parameters during acclimation, ramping, and exposure periods are given in Suppl. Table I.

2.3. Monitoring physical chemical parameters

Physico-chemical properties of the sea water were monitored using a multiparameter probe (HI-98194, Hanna Instruments, Padova, Italy) to measure temperature, an NBS pH meter (914, Metrohm AG, Herisau, Switzerland, with an iUnitrode PT1000 probe, Metrohm AG, Herisau, Switzerland) to measure pH_{NBS}, a refractometer (DD H2Ocean, MOPS Aquarium Supplies, Hamilton, ON, Canada) to measure salinity, and a

handheld oxymeter (FSG02, PyroScience GmbH, Aachen, Germany) to measure dissolved oxygen (DO). Temperature (°C) was measured daily at all stages (acclimatization, pre-exposure, temperature ramping, temperature exposure), while pH_{NBS}, salinity, and dissolved oxygen (%) were measured every 3 d at all stages except temperature ramping. Each experimental unit was equipped with an automatic temperature logger (HOBO 8 K Pendant® Temperature/Alarm Data Logger, Onset, Massachusetts, USA) to record data at 15 min intervals during pre-exposure, temperature ramping, and temperature exposure. Average temperature ramping rates (+ SE) were − 0.13 ± 0.003, 0.05 ± 0.002, 0.25 ± 0.003, 0.40 ± 0.005, 0.64 ± 0.006, 0.81 ± 0.004, and 0.94 ± 0.007 °C h^{-1} for temperature treatments 2, 7, 12, 17, 22, 27, and 32 °C, respectively. All seawater conditions during pre-exposure, ramping, and exposure periods are summarized in Suppl. Table I.

2.4. Mortality assessment and dissection

Mortality was assessed at the end of the experimental phase (12 d). Clams were gently removed from the substrate, their siphons prodded and the mantle stroked following Kennedy and Mihursky (1971). Responsiveness was assessed visually based on the ability of individual clams to retract their siphons or adduct the shells around the exposed mantle. The absence of these responses was interpreted as death. Mortality was assessed in each tank and compiled for each treatment level ($N = 656$ mortality-survival measurements).

The surviving specimens (treatments 2–17 °C for *M. truncata* and 2–27 °C for *M. arenaria)* were dissected on ice to obtain tissue samples for metabolomic analyses. Three individuals *per* species were processed from each experimental unit ($N = 13$–19 individuals *per* treatment for *M. truncata* and 12–21 individuals *per* treatment for *M. arenaria*). Three tissues (gill, mantle, and posterior adductor muscle – hereafter, "muscle") were dissected from each individual to assess tissue-specific metabolomic profiles. Tissues were excised, blotted to remove excess water, weighed, immediately flash frozen in liquid nitrogen and stored at −80 °C for subsequent analyses.

2.5. Metabolite extraction

Metabolite extraction and quantification methodologies were adapted from Hsiao et al. (2018) and performed at Iso-BioKem Laboratories (Rimouski, Québec, Canada). First, each sample was freeze-dried for 24 h at −50 °C. Each sample was then separated into two equal parts, weighed, and transferred into tissue homogenizing tubes containing 1.4 mm ceramic beads (Precellys 2 mL Soft Tissue Homogenizing Ceramic Beads Kit, Bertin Technologies SAS, Montigny-le-Bretonneux, France). Samples were then homogenized for 30 s at 6000 rpm at −4 °C using a 3D tissue homogenizer (Precellys24, Bertin Technologies SAS, Montigny-le-Bretonneux, France). Metabolites were extracted separately in positive and negative phase stock solutions from the two parts of each sample, respectively. The positive phase solution contained 200 mM of ammonium formate (Amm Fm) at pH 3 in liquid chromatography – mass spectrometry grade H_2O (H_2O-MS). The negative phase solution contained 100 mM ammonium acetate (Amm Ac) at pH 9 in H_2O-MS. The internal standards (ISTDs) were phenylalanine-d_8 and fumarate-d_4 for positive and negative phases, respectively. The two sample parts were extracted from homogenized tissues by adding 1600 μL of 50:30:20 acetonitrile:isopropanol:phase stock solution + ISTD (1 μg mL^{-1}) to each tube and vortexing. The tubes were then centrifuged at 31300 *g* for 5 min at 4 °C. A volume of 100 μL of the supernatant was transferred to a vial and stored at −80 °C until analysis by liquid-chromatography.

2.6. HPLC-QqQ-MS targeted analysis

Liquid chromatography – mass spectrometry analysis was performed using a high-performance liquid chromatographer (HPLC) (1260 Infinity II, Agilent Technologies, Palo Alto, CA, USA) coupled with a 6420 Triple Quad mass spectrometer (1260 Infinity II, Agilent Technologies, Palo Alto, CA, USA). The column used in both positive and negative modes was the Agilent InfinityLab Poroshell 120 HILIC-Z column (2.7 μm, 100 × 2.1 mm) (Agilent Technologies, Palo Alto, CA, USA). In positive mode, metabolites were eluted from the column according to a gradient mobile phase containing positive phase A (20 mM Amm Fm pH 3, H2O-MS) and positive phase B (20 mM Amm Fm pH 3, 90:10 ACN: H2O-MS), with a flow rate of 500 uL min^{-1} and a sample injection volume of 10 uL (Table 1). Column temperature was set at 30 °C. The positive linear gradient procedure was conducted as follows: 100 % phase B from 0 to 11.5 min, 70 % phase B from 11.5 to 12 min, and 100 % phase B from 12 to 17 min. In negative mode, the gradient mobile phases contained negative phase A (10 mM Amm Ac, 5 μM deactivator pH 9, H2O-MS) and negative phase B (10 mM Amm Ac, 5 μM deactivator pH 9, 90:10 ACN: H2O-MS), with a flow rate of 250 μL min^{-1} and a sample volume of 10 uL. Column temperature was set at 30 °C. The negative linear gradient procedure was conducted as follows: 90 % phase B from 0 to 12 min, 60 % phase B from 12 to 16 min, and 90 % phase B from 16 to 24 min. Unlike in positive mode, these negative phase solutions contained 5 μM of InfinityLab deactivator additive (Agilent Technologies, CA, USA). Prior to negative phase analysis, a H_3PO_4 wash (0.5 % H3PO4 90:10 ACN: H2O - ultra pure) was run through the pump at 5 mL min^{-1} for 5 min and through the system at 10 μL min^{-1} for ≥12 h.

The mass spectrometry electrospray ionization (ESI) parameters for positive and negative phases were identical for gas temperature (340 °C), gas flow (13 L min^{-1}), nebulizer (30 psi), capillary voltage (3500 V), scan type (multiple response monitoring), cycle time (500 ms), MS1 and MS2 resolution (unit), and cell accelerator voltage (7 V). Precursor ions, product ions, retention time, and QQQ parameters for each compound are summarized in Suppl. Table II.

Metabolites were quantified by creating calibration curves designed with standards for each analysed metabolite. First, metabolite stock solutions with concentrations of 1 mg mL^{-1} were made with specific solvents (0.1 % formic acid, 1 M HCl, or 0.1 % NH_4OH) for each compound, and a given volume (50 or 500 uL) was frozen at −80 °C, then freeze-dried for 24 h at −50 °C. Metabolites and their corresponding solvents and volumes are summarized in Suppl. Table III. After freeze-drying, 1 mL of extraction solvent with ISTD was added to the freeze-dried stock metabolite and vortexed. From this original concentration, a series of 5-fold dilutions was applied to obtain eight calibration concentrations. The resulting concentrations ranged from 6.4 to 5 10 e^5 ng mL^{-1} for the metabolites alanine, betaine, glycine, proline, and sarcosine, and from 0.64 to 5 10 e^4 ng mL^{-1} for the remaining 42 metabolites. Quantification was performed using MassHunter QQQ quantitative analysis (Quant-my-Way) from Agilent Technologies. The metabolites *alanine*, *cystine, succinyl-coa, glucose, NADP*, and *NADPH* displayed >50 % missing values across all samples and were removed from analysis.

Stock metabolites were purchased from various manufacturers, including Sigma-Aldrich (St. Louis, MO, USA), Cayman Chem (Ann

Table 1
Summary of the results of the generalized linear mixed-effects model (GLMER) testing the effects of the fixed variables *temperature* (T), *harvest* (H), *species* (SP), and their interactions on *Mya arenaria* and *Mya truncata* mortality (N = 656). Significant terms are given in bold font.

	Estimate	SE	*Z*-value	P_r (>|z|)
Intercept	**−10.22**	**2.47**	**−4.13**	**< 0.001**
T	**0.39**	**0.10**	**3.69**	**< 0.001**
H	2.39	2.87	0.83	0.41
SP	−7.05	4.31	−1.64	0.10
T:H	−0.10	0.13	−0.84	0.40
T:SP	**0.62**	**0.24**	**2.61**	**< 0.01**
H:SP	4.73	4.60	1.03	0.30
T:H:SP	−0.26	0.26	−1.02	0.31

Arbour, MI, USA), and Cambridge Isotope Laboratories (Tewksbury, MA, USA). The manufacturers for each reagent are summarized in Suppl. Table IV.

2.7. Bioinformatics and statistical analyses

All analyses were performed in R (v4.1.1) and RStudio (v1.4.1717) and plots produced with the ggplot2 package (Wickham, 2016). Mortality and metabolomics datasets were made publicly available through PANGAEA (Beaudreau et al., 2024).

Temperature, harvesting, and species effects on mortality were assessed in isolation and combined using a generalized linear mixed effects model (glmer) with the variables *temperature* (continuous), *harvest* (categorical), *species* (categorical) as main effects and a random *tank* effect of the experimental units assuming a binomial distribution of the dependant variable (mortality) and a *logit* link function using the *lme4* package (Bates et al., 2015). Goodness of fit was assessed using receiver operator curves with the *pROC* package (Robin et al., 2011). Assuming a non-linear temperature effect, mortality data were fit using a multiple change points (*mcp*) model whereby temperature changepoints were estimated for each species according to the two-phase curve (plateau – changepoint – binomial curve) using the Bayesian-analysis *mcp* package (Lindeløv, 2020). The difference between species' temperature changepoints was evaluated using Savage-Dickey estimates of the Bayes factor with the curves plotted based on the predicted values of the *mcp* models.

Metabolomics data were analysed in different phases. First, a permutation analysis of variance (PERMANOVA) was applied to all 481 samples to test the treatment effects on the response of 42 metabolites using the *adonis2* function (number of permutations = 999, distance method = Euclidean) in the *vegan* package (Oksanen et al., 2020). The fixed independent variables were assumed to be categorical for this analysis (*temperature, harvest, species, and tissue*) with the *tank* effect as a random variable. The *temperature* treatment was considered as a categorical variable to facilitate multivariate analysis and for ease of interpretation. *Tissue* (mantle, gill, muscle) was nested within each specimen. Assumptions of multivariate homogeneity of group dispersion were evaluated using the *betadisper* function. This assumption was not met for the factors *species* ($F_{1, 479}$, $p < 0.001$) and *tissue* ($F_{2, 478}$, $p < 0.001$).

PERMANOVA analyses showed the factor *species* to account for a large proportion of the variation in the metabolome. The three *tissue* datasets were further split according to common temperature treatments found between species (i.e. 2, 7, 12, and 17 °C), to explore the differences between species' metabolomes, yielding 12 separate *tissue x temperature* datasets. Similarly, the three *tissue* datasets were also split according to *species*, to explore the differences within species' metabolomes across different temperatures, yielding six separate *tissue* × *species* datasets. Prior to evaluating the effects of *species*, *temperature* and/or *harvest* on the metabolome, each above-mentioned dataset was transformed according to the following steps. The missing data in each dataset were imputed according to a random forest method using the *missForest* package (Stekhoven and Bühlmann, 2012). Each dataset was then centered log-ratio transformed (CLR) with the *mixOmics* package (Rohart et al., 2017) and centered and Pareto-scaled with the *MetabolAnalyze* package (Nyamundanda et al., 2010). The random effects of *tank*, the individual morphometrics (*length, width*, *height* in mm), and the behaviour of individuals (*buried or not*) in the acclimatization phase prior to the experiment were controlled by applying a linear mixed-effect model transformation to each metabolite using the *lmm2met* package (Wanichthanarak et al., 2019) to reduce the noise commonly associated with metabolomics studies. Once complete, multivariate normality (kurtosis and skew) of the transformed data was evaluated using the *semTools* package (Jorgensen et al., 2021). Despite transformation, the assumption of multivariate normality was not met in any of the datasets ($p < 0.05$) but was nonetheless retained due to its beneficial normalizing effect (albeit non-significant) on metabolite histogram distributions (Suppl. Fig. 1).

Transformed metabolite data were analysed by principal component analysis (PCA) using singular value decomposition with the *prcomp* base function and the *screeplot* function to evaluate the proportion of variation among treatment groups explained by each component and to select the two first components in the models. The two first components explained most of the variation (> 50 % of total) and were plotted using the *ggplot2* package with the *species-, temperature-,* and/or *harvest-*–groupings overlaid for visual interpretation (frame type = 't'). Once distinct groupings were identified with PCA, a partial least squares discriminant analysis (PLS-DA) was applied using the *mixOmics* package. To validate the PLS-DA results, a k-fold approach and *Area Under the Curve* (AUC) of the receiver operating characteristic curve was used for model cross-validation with the *perf* function in the *mixOmics* package (5-fold, 50 repeats), and classification error rates plotted and evaluated using Mahalanobis and centroid distance measures. Models were not overfit as the classification error rate decreased consistently with each new component until it reached a plateau (Suppl. Fig. 2). Additionally, the *auroc* function in the *mixOmics* package measured the model's ability to classify samples into their respective treatment grouping using the AUC. The number of components sufficient to minimize the classification error rate and the AUC are reported with the main results. Generally, three or four components were sufficient to minimize classification error rate *per* the Mahalanobis distance metric (Suppl. Fig. 2). Consequently, following model validation, PLS-DA results were plotted with three components in the projections. Where group separation began to occur in *M. arenaria*, the AUC measurements for model classification at 22 and 27 °C were 0.6657 and 0.999, 0.793 and 0.998, and 0.778 and 0.984 for gill, mantle, and muscle, respectively. This indicates good model performance for *M. arenaria* tissues, especially at 27 °C. Where group separation began to occur in *M. truncata*, the AUC measure at 17 °C was ~1, 0.959, and 0.923 for gill, mantle, and muscle, respectively. This indicates good model performance for *M. truncata* tissues, especially at 17 °C. The metabolites most involved in group separation were extracted with variable importance in projection (VIP) scores according to the cut-off value (> 1) in every component using the *vip* function in the mixOmics package. The PLS-DA analyses showed results that were highly similar to those of the PCA analyses. Thus, the PLS-DA plots were moved to the Suppl. material (Suppl. Fig. 3).

Species tissue-specific metabolome responses were used to define the upper temperature response of the metabolome. To explore the changes within these tissues at increasing temperature levels, significantly differentially expressed metabolites (SDMs) (i.e. those that varied significantly among temperatures) were identified. Metabolites that were significantly up- or down-regulated ($p < 0.05$) compared to the 7 °C treatment were selected because this temperature most closely resembled the average and collection temperatures at the collection sites as well as the acclimatization temperature in the laboratory. This was achieved using unpaired *t*-tests corrected for false discovery rate (FDR) with the *t_test* function in the *rstatix* package. Selected SDMs were then used for pathway analysis.

Pathway analysis was performed using the *FELLA* package, a diffusion-based algorithm that considers a list of SDMs and places them into knowledge-based biological networks that allow for pathway crosstalk (Picart-Armada et al., 2018). The algorithm was set to 10,000 iterations and output limited to 1000 nodes. A study-specific network was built by running the diffusion algorithm through a reference network with the input of SDMs tested against a background set of all metabolites assayed in the HPLC-QqQ-MS targeted analysis. *Mercenaria mercenaria*, a model bivalve available in the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2000) was used to build the reference network. In addition to housing the original input metabolites, the resulting network generated a list of significantly affected metabolites, enzymes, reactions, modules, and pathways. The visual representation of this network was trimmed according to the elements of interest, namely the input metabolites and pathways, and then improved aesthetically using Cytoscape (Shannon et al., 2003).

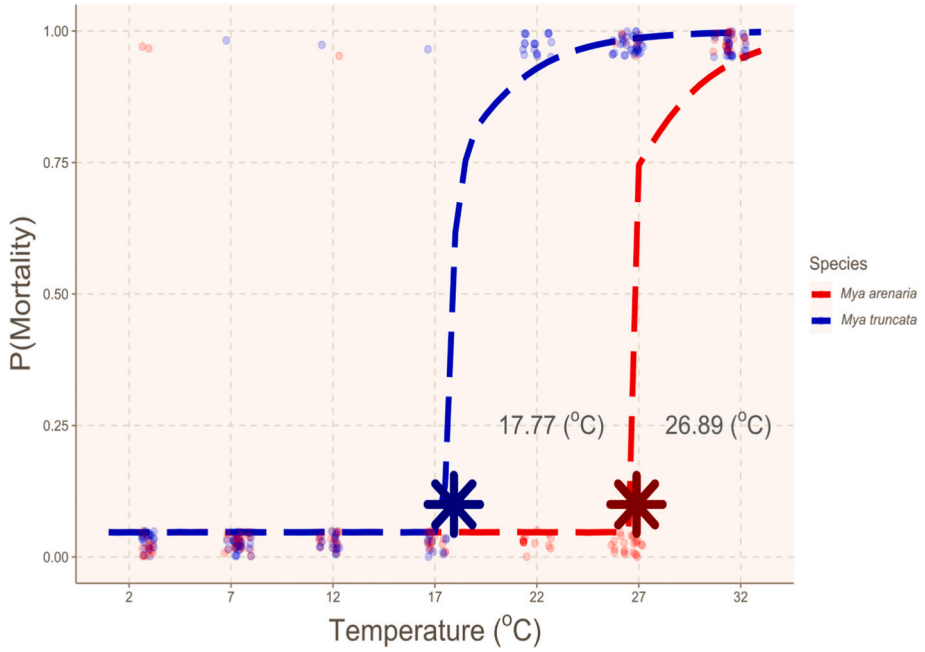

Fig. 1. Probability mortality curves predicted for temperatures ranging between 2 and 32 °C in *Mya arenaria* and *Mya truncata*. Curves and breakpoints (⁎) were estimated according to multiple change point (*mcp*) analysis ($N = 656$). Points represent individual clams.

3. Results

3.1. Mortality

After 12 d of experimental exposure to different temperature changes (including MHW scenarios) and harvesting disturbance, both *M. arenaria* and *M. truncata* experienced significant mortality. In total, 255 *M. arenaria* and 170 *M. truncata* survived exposure to the different conditions, out of an initial 328 individuals *per* species. Survival was not impacted by harvesting treatments but was by MHW. All *M. arenaria* died at 32 °C and at the 22–32 °C for *M. truncata* (Suppl. Table I). Low temperatures (2–17 °C) did not significantly affect mortality for either species (i.e. 100 % survival), while a temperature increase beyond 27 °C for *M. arenaria* and 17 °C for *M. truncata* increased mortality considerably. Variation in thermal sensitivity between species was indicated by the significant *temperature* × *species* interaction (Z-value = 2.61, p = 0.009; Fig. 1, Table 1). Breakpoints for survival were 26.89 °C for *M. arenaria* and 17.77 °C for *M. truncata* (Fig. 1), a difference of 9.11 °C (Savage-Dickey Bayes = 0).

3.2. Metabolite profiles

The metabolomes varied largely as a function of *species*, *tissue*, and *temperature*. The effect of *harvest* was also significant although only as a function of *temperature* and no clear pattern could be drawn from our results. Multivariate PCA analyses of species' tissue metabolomes at identical temperature treatments showed a consistent pattern (Fig. 2), with alanine, serine, and threonine explaining the most variation between species, and with betaine explaining the most variation among individual samples within species. The significantly differentially expressed metabolites between species' tissues can be found in the Suppl. material (Suppl. Fig. 4). This section will thus be focused on interpretations of temperature treatment responses separated by species and tissues.

Significantly up- or downregulated metabolites at temperatures approaching each species' upper thermal limit are summarized in Table 2. Full metabolite-specific comparisons are available in Suppl. Fig. 5. Significant variation in response to temperature was detected in *M. arenaria* tissues for 6 to 35 metabolites, with between 2 to 21 being downregulated and 4 to 23 upregulated across all tissues and temperature treatments (Table 2). *Mya truncata* had between 6 to 21 metabolites that varied significantly among temperature treatments, with between 3 to 12 being downregulated and 3 to 12 being upregulated across all tissues (Table 2).

Follow-up analysis of the effects of single variables and their interactions across all 481 clams and 42 metabolites (Table 3), revealed that tissue-specific metabolomes differed by species (significant *tissue* × *species* interaction, pseudo-$F_{2,421} = 3.87$, $p < 0.01$). The effect of *harvest* on the metabolome varied as a function of *temperature* (significant *harvest* × *temperature* interaction, pseudo-$F_{5,421} = 2.66$, $p < 0.001$). The effect of *species* (pseudo-$F_{1,421} = 129.83$, $p < 0.001$), *tissue* (pseudo-$F_{2421} = 22.74$, $p < 0.001$), and *temperature* (pseudo-$F_{5,421} = 5.44$, $p < 0.001$) were also significant, in decreasing order of effect significance.

Multivariate PCA analyses showed separation among individuals by *temperature* treatment groups as temperature approached the breakpoint of each species (Fig. 3). For *M. arenaria*, the separation began at 22 and 27 °C with the first and second components explaining >65 % of the variation in the metabolite levels for each tissue. For *M. truncata,* the separation began at 17 °C with the first two components explaining >57 % of variation in metabolite levels for each tissue. In contrast, the *harvest* groupings did not show a clear or consistent pattern of separation, and the overall effect appeared absent (Suppl. Fig. 6).

PLS-DA analyses indicated similar trends (Suppl. Fig. 3) as those reported above for the PCA analyses with PC1 group separation in *M. arenaria*, occurring at temperatures as low as 22 °C for the gill and muscle and 27 °C for the mantle and at 17 °C for all tissues in *M. truncata*. Metabolites with VIP scores > 1 were considered to contribute most to group separation in PC1, as reported in Table 4. On this basis, the following metabolites contributed to separating temperature treatments for all three *M. arenaria* tissues: a-aminoadipic acid, histidine, and threonine. The following metabolites were involved in separating temperature treatments based on VIP scores for all three *M. truncata* tissues*:* arginine, glycine, proline, and tyrosine. When comparing similar tissues between species, the VIP metabolites were: (i) a-aminoadipic acid, ATP, glutamine, histidine, and serine for gills; (ii) arginine, serine, and threonine for mantle; and (iii) lysine, NAD,

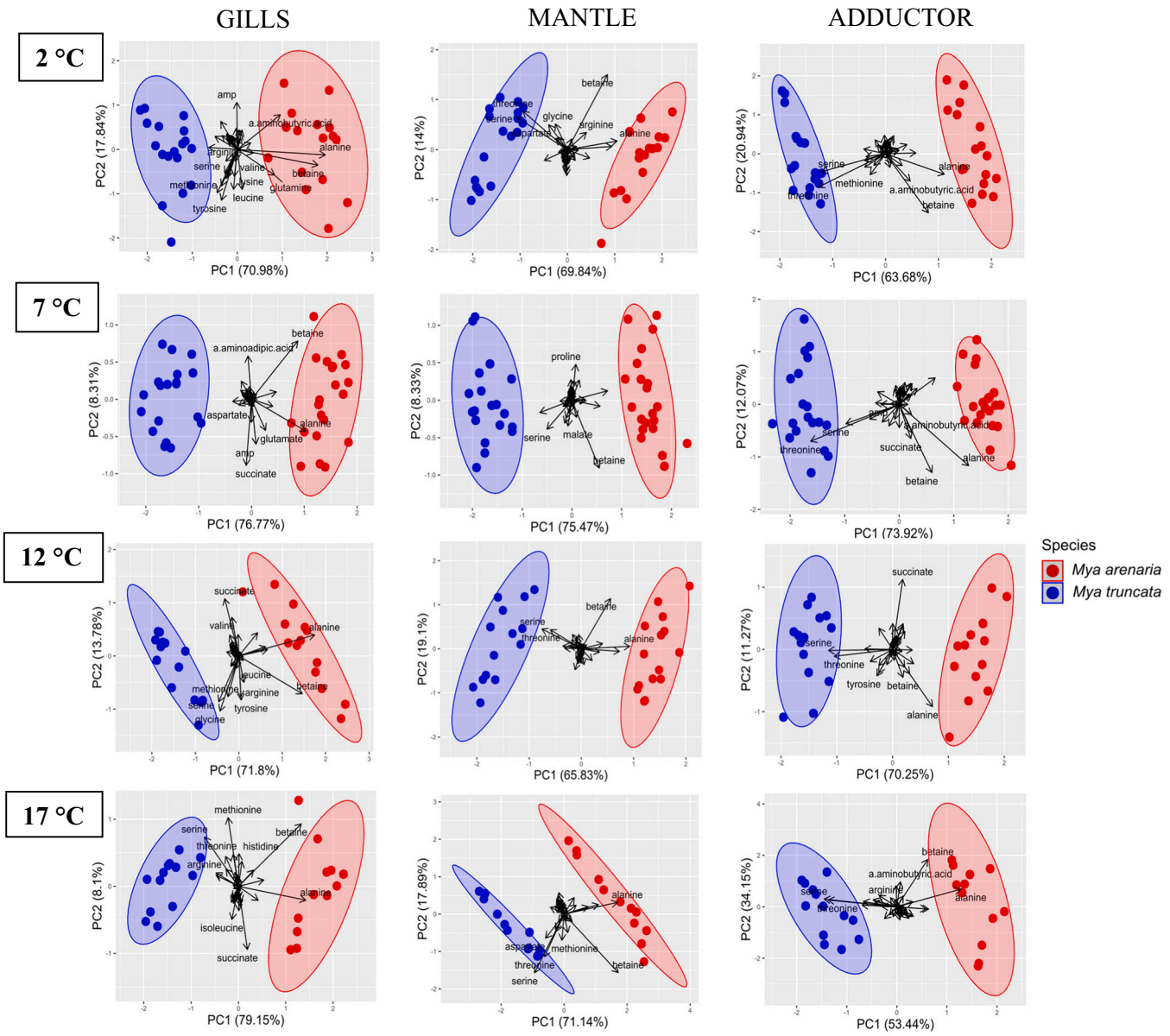

Fig. 2. Principal component analyses (PCA) plots displaying the PC1 and PC2 of the targeted metabolome (42 metabolites) comparing the gills, mantle, and muscle tissues of *M. arenaria* and *M. truncata* at all four common temperatures (2, 7, 12, and 17 °C). Points represent individual clams.

phenylalanine, proline, threonine, tryptophan, and valine for muscle. Full VIP score results for all components are reported in Suppl. Table V.

3.3. Metabolic pathways responding to temperature

Based on species- and tissue-specific networks constructed using SDMs associated with the upper temperature response (Table 2), 17, 27, and 7 pathways were found to be affected by *temperature* in *M. arenaria* and 6, 6, and 3 pathways in *M. truncata* for mantle, gills, and muscle tissue, respectively (Fig. 4). Comparisons across tissue-specific networks between species revealed that the endocytosis and phosphatidylinositol (PI) signalling pathways were uniquely altered in the gill tissue of both *M. arenaria* and *M. truncata*. Full pathway analysis results at all temperature levels can be found in the Suppl. Table VI.

4. Discussion

This study reports striking differences in MHW tolerance and metabolomic plasticity between two partly sympatric clam species with different climatic niches, and a negligible impact of harvesting disturbance. These findings have important implications for predicting biogeographic distributional shifts in coastal waters and provide indicators for management strategies faced with coinciding climatic and direct human stressors. Our results suggest that the temperate-polar specialist blunt gaper (*M. truncata*) is more vulnerable to MHW than its congener the temperature generalist softshell clam (*M. arenaria*) by nearly 9 °C in MHW intensity and that this difference may be rooted in its cellular physiology. These species undergo differing metabolic remodelling near their respective thermal limits to withstand short-term temperature extremes. Our findings indicate that tissue-specific metabolic pathways underpin the observed differences in whole-organism thermal stress responses between the temperate-polar specialist and its temperature generalist and highly invasive congener. This is expected to have strong implications on the likelihood of biological invasion into the native range of *M. truncata* under global warming and future conditions of MHW intensification (Goldsmit et al., 2020).

Table 2
Normalized and multivariate-transformed levels of individual metabolites near the upper thermal limit in *M. arenaria* (27 °C) and *M. truncata* (17 °C). Arrows indicate a significant ($p < 0.05$) increase (green up arrow) or decrease (red down arrow) in the metabolite level as compared to the 7 °C treatment according to unpaired t-tests with FDR correction. Metabolites from positive (POS) and negative (NEG) phase analysis are separate.

	SPECIES	*M. arenaria*			*M. truncata*		
	TEMPERATURE	27 °C	27 °C	27 °C	17 °C	17 °C	17 °C
	TISSUE	Gills	Mantle	Muscle	Gills	Mantle	Muscle
POS	α-aminoadipic.acid	↑	↑	↑	↑		
POS	α-aminobutyric.acid	↓		↓	↑		
POS	alanine	↓		↓		↓	
POS	AMP	↓					↓
POS	arginine	↓	↑	↓	↑	↓	↓
POS	aspartate	↓	↓	↓	↓	↑	
POS	β-aminoisobutyric	↓	↓	↓		↑	↓
POS	betaine			↓		↓	↓
POS	FAD	↓	↓	↓			
POS	glutamate	↑		↓	↓		
POS	glutamine	↓	↓	↓	↓	↓	↓
POS	glycine	↓		↓	↓	↓	↓
POS	histidine	↑	↑	↑	↑		
POS	hydroxyproline		↓	↓		↑	
POS	isoleucine		↓			↑	↑
POS	leucine	↑	↓	↑		↑	↑
POS	lysine		↑	↑			↑
POS	methionine	↑					
POS	NAD		↓	↓	↓		↓
POS	phenylalanine	↑	↑	↑	↑	↑	↑
POS	proline	↑	↓	↓	↓	↑	↑
POS	se rine	↑	↑	↑	↑	↑	
POS	threonine	↑	↑	↑		↓	↓
POS	tryptophan	↑		↑	↓	↑	↑
POS	tyrosine			↑	↓	↑	↑
POS	valine	↑	↑	↑		↑	↑
NEG	acetyl.coa	↓	↓	↓			
NEG	ADP	↓	↓	↓	↓		
NEG	aketoglutarate		↓				
NEG	ATP	↓	↓		↓		
NEG	cis.aconitate	↓	↓	↓			
NEG	citrate	↓	↓	↓	↓		
NEG	d.fructose.1.6.biphos.trisod.	↓	↓		↓		
NEG	fumarate	↓	↓				
NEG	glucose.6.phosphate	↓	↓	↑			↓
NEG	lactate		↑	↑	↑	↑	
NEG	malate		↓	↑	↓		
NEG	nadh	↓		↓		↓	
NEG	oxaloacetate	↓	↓	↓			
NEG	phosphoenyl.pyruvate	↓	↓				
NEG	pyruvate			↑			↑
NEG	succinate	↓		↑	↓	↓	↓

Table 3
Summary of results from the permutation analysis of variance (PERMANOVA) with the effects of the fixed variables *temperature* (T), *harvest* (H), *species* (SP), *tissue* (TS), and their interactions on *Mya* spp. clam metabolome (42 metabolites) ($N = 481$). Degrees of freedom (df), R^2 values, F-statistic value and associated *P*-values for each treatment term are reported, with significant results highlighted in bold.

	df	R^2	*F*-value	*P-value*
T	**5**	**0.04**	**5.44**	**< 0.001**
TS	**2**	**0.07**	**22.74**	**< 0.001**
SP	**1**	**0.19**	**129.83**	**< 0.001**
H	1	0.00	0.15	0.303
T:TS	10	0.02	1.15	0.197
T:SP	3	0.00	1.01	0.380
T:H	**5**	**0.02**	**2.66**	**< 0.001**
TS:SP	**2**	**0.01**	**3.87**	**< 0.010**
TS:H	2	0.00	1.26	0.239
SP:H	1	0.00	0.52	0.558
T:TS:H	10	0.01	0.53	0.907
T:TS:SP	6	0.00	0.36	0.962
T:SP:H	3	0.01	1.48	0.172
TS:SP:H	2	0.00	0.20	0.937
T:TS:SP:H	6	0.00	0.43	0.922

4.1. Mortality

Of the two congeners in this study, the thermal generalist *M. arenaria* showed a much greater tolerance to MHW stress when compared to the thermal specialist *M. truncata*. This difference in upper thermal tolerance has ecological implications related to their potential global redistribution under ocean warming. *Mya arenaria* was expected to exhibit a wider thermal window considering its broad biogeographic distribution and climatic range, including its native range along Atlantic coast from southeastern United States to eastern Canada and its invasive range, which includes the west coasts of Canada and northern Europe including Iceland. In contrast, *M. truncata* solely inhabits cooler Arctic and sub-Arctic waters. No previous study has assessed the upper thermal limit of *M. truncata*, whereas the LC50 (24 h) of *M. arenaria* ranges from 30.1 to 32.5 °C (Kennedy and Mihursky, 1971) although this upper thermal limit is expected to decrease with increasing duration of exposure. Our results suggest *M. truncata* is unable to withstand a MHW lasting 12 days (or more) at temperatures exceeding 18 °C. Thus, with future MHW intensification, *M. truncata* may undergo population decline, particularly at its southern edge with the potential for local extinction. Examples of such range contractions are projected for numerous polar invertebrates based on correlative species distribution models (Alabia

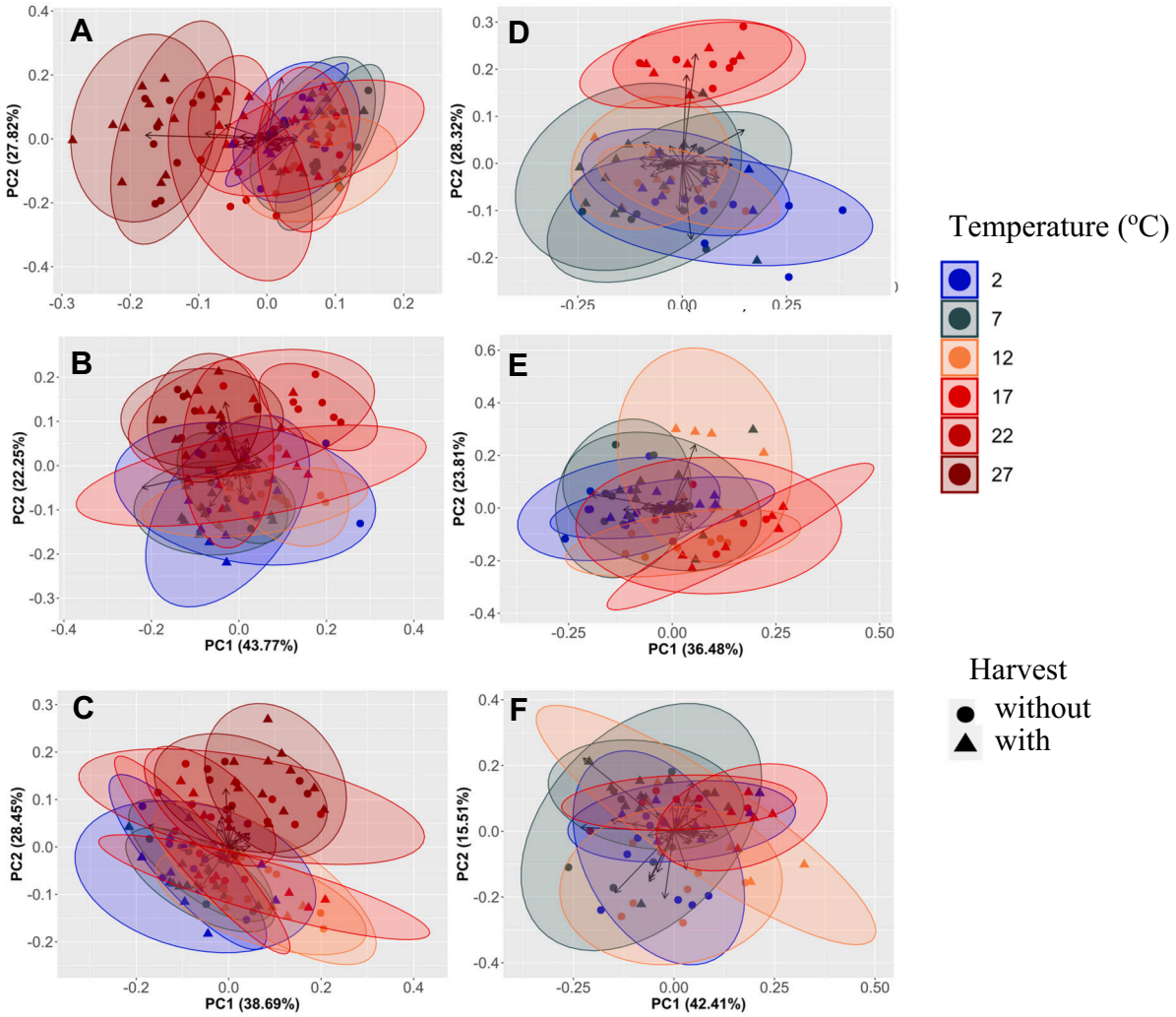

Fig. 3. PCA plots displaying the PC1 and PC2 of the targeted metabolome (42 metabolites) for different temperature treatments in the gills, mantle, and muscle tissues of *M. arenaria* (**A**, **B**, and **C**) and *M. truncata* (**D**, **E**, and **F**). Each plot displays the grouping variable *temperature* (2, 7, 12, 17, 22, and 27 °C, if available) and *harvest* (with, without). Points represent individual clams.

et al., 2018). While these types of models can inform biogeographic redistribution scenarios, sub-lethal impacts of MHW help predict actual performance and ultimately population dynamics (Beckerman et al., 2002) as they reflect organisms' physiological responses as they approach their tolerance limits.

4.2. Metabolome

The exposure to simulated MHW levels elicited physiological changes at the cellular level, which provides a mechanistic explanation for the species' tolerance levels and indicates that the effects may persist, ultimately affecting individuals' performances and survival. The shifts in metabolome profiles suggest a coordinated response to MHW across several tissues, with unique species- and tissue-specific responses. The main defining differences between species' metabolomes were explained by the levels of three amino acids: alanine, serine, and threonine. Within species, *Mya arenaria* displayed an extensive cellular response at multiple sub-lethal temperature levels, whereas *M. truncata* exhibited limited ability to adjust its metabolome at extreme temperatures. Aside from α aminoadipic-acid, ATP, and NAD+, the metabolites most engaged in the species- and tissue-specific temperature responses are amino acids.

Amino acid metabolism operates at several levels of stress response through bioenergetic substrates (Wood, 2001; Zurburg and De Zwaan, 1981), protein turnover (Houlihan, 1991; Wood, 2001), antioxidant defenses (Atmaca, 2004; Newsholme et al., 2012), and immunological signalling and protection (Grohmann and Bronte, 2010; Z. Huang et al., 2020; McGaha et al., 2012). Histidine is involved in antioxidant responses (Kohen et al., 1988; Chen et al., 2021; Wang et al., 2021) and inflammatory signalling (Novák and Falus, 1997; Delitheos et al., 2010) and significantly increases in all *M. arenaria* tissues and the gills of *M. truncata.* Glycine, serine, and threonine may provide energy substrates (Meister, 1965), antioxidant protection (Hu et al., 2015; Meister and Anderson, 1983), or improved membrane fluidity (Chung et al., 2018; Zwingelstein et al., 1998). Branched-chain amino-acids (BCAAs), which include isoleucine, leucine, and valine, increase with MHW intensity in all tissues except in *M. arenaria* mantle, which has been previously reported in aquatic ectotherms (Y. Wang et al., 2019; Xu et al., 2017). Proline, hydroxyproline, glutamate, glutamine, and arginine are involved in common pathways (Meister, 1965), and three of these contribute significantly to the MHW response in our study animals. Proline and glutamine may confer alternate sources of energy production (Phang and Liu, 2012) and contribute to general stress response (Liu and Phang, 2012; Natarajan et al., 2012). Tyrosine consistently increases in the adductor muscle tissue in both our study species, while phenylalanine increases in all tissue metabolome profiles in both species; changes in these metabolites may confer tolerance to MHW through catecholamines (i.e. adrenaline, noradrenaline, dopamine) (Hirashima

Table 4
List of most impactful metabolites emerging from the PLS-DA analysis according to VIP scores (> 1) extracted from PC1. Metabolites are organized according to similarities within species and across gills, mantle, and muscle tissues. VIP scores are included in brackets (for full output, see Suppl. Table V).

	M. arenaria			*M. truncata*		
	Gills	Mantle	Adductor	Gills	Mantle	Adductor
Within species	α-Aminoadipic.acid (1.58)	α-Aminoadipic.acid (1.16)	α-Aminoadipic.acid (1.45)	Arginine (1.7)	Arginine (1.79)	Arginine (1.54)
	Threonine (1.67)	Threonine (1.36)	Threonine (1.79)	Glycine (1.06)	Glycine (1.49)	Glycine (1.13)
	Histidine (1.44)	Histidine (1.13)	Histidine (1.26)	Proline (2.07)	Proline (1.5)	Proline (2.06)
				Tyrosine (1.48)	Tyrosine (1.77)	Tyrosine (1.9)
	α-Aminoadipic.acid (1.58)			α-Aminoadipic.acid (2.1)		
Gills	ATP (1.34)			ATP (1.02)		
	Glutamine (1.37)			Glutamine (1.18)		
	Histidine (1.44)			Histidine (1.58)		
	Serine (1.23)			Serine (2.13)		
Mantle		Arginine (1.03)			Arginine (1.79)	
		Serine (1.47)			Serine (1.13)	
		Threonine (1.36)			Threonine (2)	
Muscle			Lysine (1.67)			Lysine (1.6)
			NAD (1.17)			NAD (1.41)
			Phenylalanine (1.38)			Phenylalanine (1.72)
			Proline (1.31)			Proline (2.06)
			Threonine (1.79)			Threonine (1.85)
			Tryptophan (1.55)			Tryptophan (1.84)
			Valine (1.18)			Valine (1.57)
Remaining	ADP (1.36)	Acetyl.coa (1.42)	α-Aminobutyric.acid (1.47)	α-Aminobutyric.acid (1.87)	Alanine (1.61)	AMP (1.15)
	β-Aminoisobutyric (1.6)	ADP (1.52)	Aspartate (1.53)	Aspartate (1.24)	Betaine (1.25)	Betaine (1.02)
	Citrate (1.1)	Aketoglutarate (1.17)	β-Aminoisobutyric (1.65)	Glutamate (1.75)	Isoleucine (1.48)	Isoleucine (1.46)
	d.fructose.1.6.biphosphate. trisodium (1.18)	ATP (1.49)	Cis.Aconitate (1.02)	Succinate (1.27)	Lactate (1.11)	
	Glucose.6.phosphate (1.2)	cis.aconitate (1.4)	FAD (1.06)		Leucine (1.21)	
	Leucine (1.13)	Citrate (1.42)	Glutamine (1.15)		Methionine (1.03)	
	NADH (1.47)	d.fructose.1.6.biphosphate. trisodium (1.4)	Hydroxyproline (1.05)		Phenylalanine (1.62)	
	Oxaloacetate (1.01)	FAD (1.46)	Lactate (1.2)		Tryptophan (1.73)	
	Phenylalanine (1.01)	Fumarate (1.46)			valine (1.5)	
	Phosphoenyl.pyruvate (1.23)	Glucose.6.phosphate (1.31)				
	Tryptophan (1.62)	Hydroxyproline (1.22)				
	Valine (1.36)	Lysine (1.51)				
		Malate (1.41)				
		Phosphoenylpyruvate (1.09)				

et al., 2000; Nagatsu et al., 1964; Zhang et al., 2014). Tryptophan increases and nicotinic acid (NAD^+) decreases consistently in the adductor muscle. Tryptophan transits towards the kynurenine pathway (energy substrates and NAD^+) or the serotonin pathway (stress-sensitive neurotransmitters) (Yao et al., 2011). Decreased tryptophan concentrations in *M. truncata* gills suggests that these pathways cannot sustain sufficient levels of NAD+ or protective neurotransmitters. Lysine is a ketogenic metabolite through the saccharopine pathway (Higashino et al., 1971) and increases in the adductor muscle of both species in this study. It may be converted to α-aminoadipic acid, which increases in all *M. arenaria* tissues and in *M. truncata* gills, and is considered a marker for protein oxidation (Sell et al., 2007; Lee et al., 2019). This suggests that *M. arenaria* tissues undergo significant oxidative stress, whereas *M. truncata* shows oxidative stress primarily in its gill tissue.

Finally, energy substrates respond to MHW in *Mya* spp. ATP levels decrease in gills and are known to generally respond to environmental stress, accompanied by decreased adenosine diphosphate (ADP) levels (Calderwood et al., 1985; Caldwell and Hinshaw, 1994; Vetter and Hodson, 1982). Decreased concentrations of ATP and ADP suggest metabolic reduction and eventually depression at higher temperatures (Pörtner et al., 1998; Sokolova, 2013). When exposed to repeated sublethal MHW events, clams would be expected to accumulate negative impacts hampering population-level traits (Sokolova et al., 2012). Beyond individual metabolites, the ability of clams to modify their metabolome in response to MHW is shaped by changes in pathways.

4.3. Network analyses

Network analyses provide a synthetic mechanistic understanding of cellular stress responses, helping to overcome the limitations of interpreting individual molecular responses. In our study, we show tissue-specific functional classes of responses, including cell signalling, structural turnover, DNA repair and expression, and metabolism. Strikingly, we observe few cell signalling pathways in *M. truncata*, in contrast to *M. arenaria* which relies heavily on these functions. Based on our targeted metabolomics analysis, it appears that MHW affect numerous pathways, especially in *M. arenaria* gills. This may, in part, explain the greater MHW tolerance of *M. arenaria* and highlights how the less-responsive molecular toolkit in *M. truncata* may render it vulnerable to global change. However, further investigation is needed to explore different levels of the clams' molecular response (such as the transcriptome and the proteome for example) in order to support this.

We found minor overlap in tissue pathways affected by MHW stress across species. Endocytosis and phosphatidylinositol (PI) signalling pathways are expressed in the gills of both species. Endocytosis can coregulate with PI signalling through the PI3K enzyme and influence

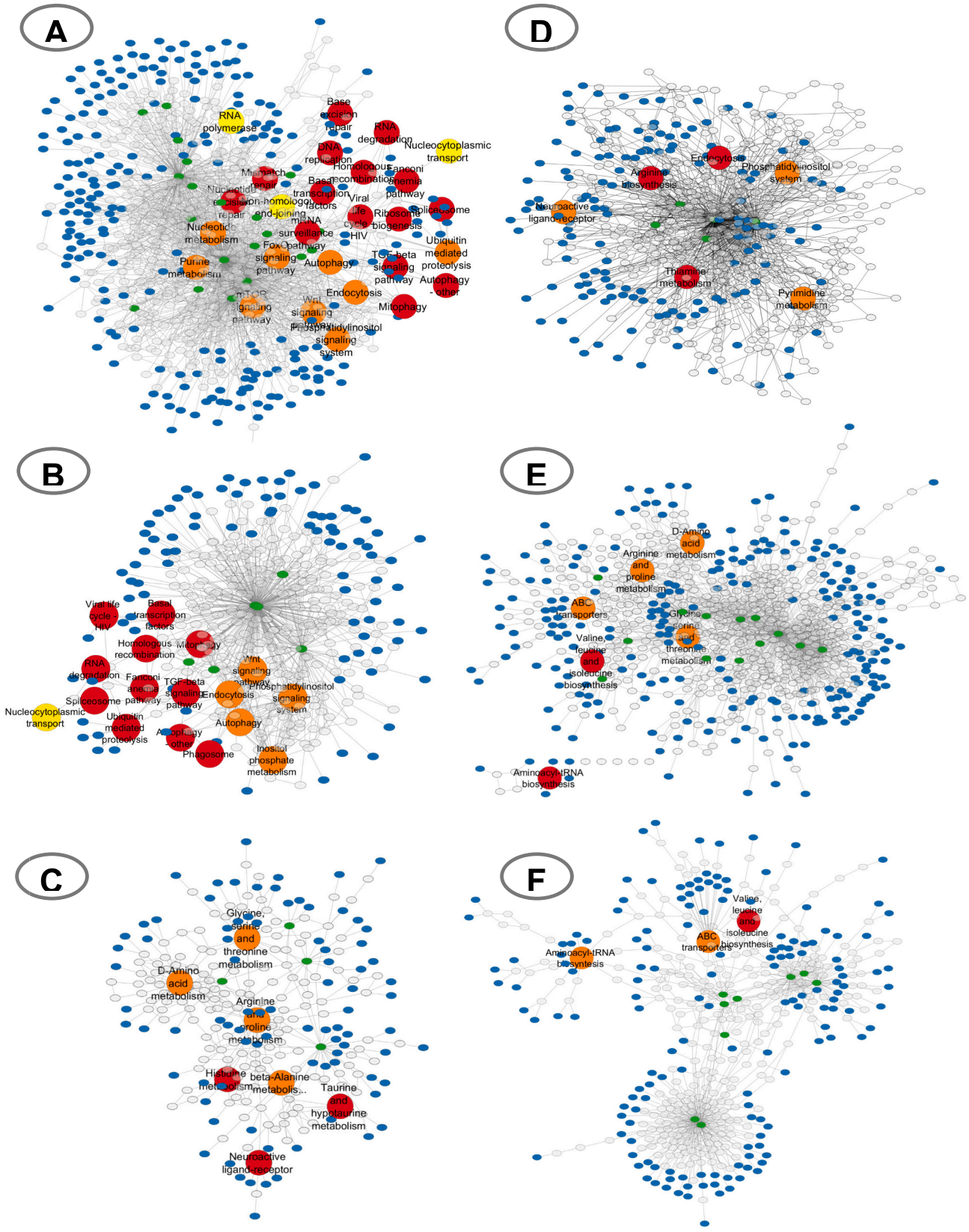

Fig. 4. Pathway analyses results showing the significant pathways expressed at the upper temperature treatment (relative to 7 °C) in the gills, mantle, and muscle tissues of *M. arenaria* (**A**, **B**, and **C**, respectively) and *M. truncata* (**D**, **E**, and **F**, respectively) based on a knowledge-based network and diffusion algorithm (*FELLA*). Nodes are either compounds, enzymes, reactions, modules or pathways. Terminal nodes are blue; most connected (> 10) nodes are green; most connected (>10) pathways are red; pathways are orange; and least connected (1) pathways are yellow. Pathways were labeled with text.

pro-inflammatory responses (Hawkins and Stephens, 2015). Endocytosis possesses a unique role in membrane remodelling (Moyes and Schulte, 2015) and showed greater activity with higher temperatures in fish (Padrón et al., 2000; Røsjø et al., 1994), greater recycling of defective membrane-bound proteins under heat-stress (Foot et al., 2017; López-Hernández et al., 2020), greater protein turnover, and greater cellular remodelling. Together, these pathways may be fundamental cellular processes that clams regulate in response to the negative impacts of MHW.

Gills and mantle of *M. arenaria* had the greatest number of similar pathway responses to MHW. Of all functional classes expressed in the gills, DNA repair and expression are most prominent. The DNA replication pathway can slow or halt DNA synthesis (Osborn et al., 2002) commonly associated with heat stress (Velichko et al., 2012). DNA repair mechanisms are ubiquitous in heat-stressed ectotherms (Malev et al., 2010) and promote cell death (Zhou et al., 2001). DNA transcription is stress sensitive (Sadhale et al., 2007) and preserves proteostasis (Jamar et al., 2018). Removing defective RNA transcripts is essential to coping with heat stress (Cherkasov et al., 2013). In contrast, *M. truncata* expresses a single DNA repair and expression pathway, the aminoacyl tRNA biosynthesis pathway, in its mantle and muscle. Heat impacts nascent, growing, and mature tRNA (Huang and Hopper, 2016), and ROS affects tRNA enzymes, leading to misfolded and oxidized proteins (Ling and Söll, 2010). To survive the damaging effects of MHW, cells should efficiently target and remove these damaged structures through cell turnover mechanisms to prevent their interfering with normal cell functions.

Cell turnover mechanisms replace damaged enzymes or membrane structures and accompany the synthesis of cellular machinery. In *M. arenaria*, the autophagy, mitophagy, phagosome, and ubiquitin-mediated proteolysis pathways are altered in both mantle and gill tissues, while ribosome biogenesis is altered in gill tissue only. Although mitochondria are unquestionably targets of heat stress (Slimen et al., 2014), the role of mitophagy in heat stress is poorly understood. The phagosome pathway, uniquely expressed in the mantle, is intimately linked to autophagy (Shui et al., 2008) and is affected by heat stress in salmon (Shi et al., 2019) and oyster (Zhang et al., 2022). Of the two pathways uniquely expressed in *M. arenaria* gills in this study, ubiquitin-mediated proteolysis is involved in routine maintenance and cellular stress response (Schwartz and Ciechanover, 1992) and responds to heat shock (Abdelmohsen et al., 2009; Maor-Landaw et al., 2014), while the ribosome biogenesis pathway is suppressed as an initial signal of nucleolar stress (Golomb et al., 2014; Rubbi and Milner, 2003). There is evidence of both downregulation (Cherkasov et al., 2015) and upregulation (Mohamed et al., 2014; Quinn et al., 2011) of these pathways with heat stress. Cell turnover mechanisms also appears important to the MHW response of *M. truncata*. Amino acid levels can be partly linked to protein degradation and synthesis, and many pathways expressed in *M. truncata* are of this nature. Generally, the synthesis of these amino acids may serve many competing functions, as discussed above (see Section 4.2 Metabolome).

Metabolic pathways provide substrates and cofactors for metabolic activity and these requirements may be altered in clams experiencing MHW. Although the patterns of metabolic activity differ between species, the total number of these pathways detected is comparable. Purine metabolism salvages or synthesizes nucleotides, which may be needed for increased gene expression under heat stress (Jiang et al., 2020) and is uniquely expressed in *M. arenaria* gills while β-alanine metabolism and taurine and hypotaurine metabolism are uniquely affected in muscle of *M. arenaria*. Taurine and hypotaurine metabolism is present in *M. arenaria* muscle likely for their roles in tissue repair, immunity, and as potential antioxidants (Aruoma et al., 1988; Salze and Davis, 2015). Inositol phosphate metabolism is uniquely expressed in *M. arenaria* mantle tissue, likely due to its relationship to the PI signalling system discussed above (Tsui and York, 2010). Interestingly, the pathways of arginine and proline metabolism, D-amino acid metabolism, and glycine, serine and threonine metabolism are altered in both *M. arenaria* muscle and *M. truncata* mantle tissues, while thiamine metabolism and pyrimidine metabolism are altered in *M. truncata* gills. Thiamine metabolism may play a role in oxidative stress mitigation, autophagy, and endoplasmic-reticulum stress (Liu et al., 2017), while pyrimidine metabolism may serve to salvage or synthesize nucleotides for gene expression, as mentioned for purine metabolism (Jiang et al., 2020). These findings, and amino acid dynamics, suggest species-specific changes in metabolic pathways under MHW conditions participate in complex cellular responses that promote short-term survival of both species.

Pathways linked to cell signalling were found to be significantly utilised in both species' responses to MHW. In *M. arenaria*, this was apparent with the expression of the phosphatidylinositol (PI), the TGF-b, and Wnt signalling systems in the mantle and gills, mTOR and FoxO signalling uniquely in gills, and neuroactive ligand-receptor interaction signalling in muscle. Pathways linked to cell signalling were minimally involved in the *M. truncata* MHW response through the neuroactive ligand receptor interaction signalling pathway and the above-mentioned PI signalling system in gills. The TGF-b gene is involved with inflammatory response and is upregulated in heat-stressed fish (J.-L. Sun et al., 2020; Yang et al., 2021). Wnt-related genes display different expression patterns under heat stress (G. Huang et al., 2021; Risha et al., 2021; Yin et al., 2022), and may alter membrane characteristics (Risha et al., 2021). mTOR signalling in gills impacts RNA translation and mTOR transcription increases in ectotherms exposed to elevated temperatures (Chou et al., 2018; Pandey et al., 2021). FoxO signalling is associated with oxidative stress (Essers et al., 2005; Lehtinen et al., 2006) and FoxO transcripts are upregulated under heat stress (Eremina et al., 2021). The neuroactive ligand-receptor interaction signalling pathway is poorly understood but has been detected in various stress responses (Feng et al., 2022; Liu et al., 2018), including heat (Kim et al., 2017; Lu et al., 2018). Overall, signalling pathways appear to be central mechanisms in stress response for eurythermal species and may provide a framework for investigating integrated stress signalling.

A unique pathway expressed in *M. truncata,* both in the mantle and the muscle, is the membrane transport pathway of ABC transporters. These membrane proteins are involved in responses to environmental stress as found in studies involving echinoderms such as sea urchins (Echinoidea) (Marques-Santos et al., 2017) and sea cucumbers (Holothuroidea) (Huo et al., 2019). Considering the diversity and role of these transmembrane proteins, they merit further investigation.

Lastly, an unexpected pathway, viral life cycle HIV-1, affected *M. arenaria* gills and mantle, but is likely identified during pathway analysis as an artefact. This may be due to the relationship of the animal viral life cycle to pathways such as endocytosis, autophagy, phagosome and the general immune response of animals (Yatim and Albert, 2011).

5. Conclusions

The marine clam species studied here are integral components of temperate and polar coastal benthic ecosystems, environments which are increasingly subjected to anthropogenic stressors, but differ in their ability to cope with environmental stressors. We show that *M. arenaria* possesses a comparative advantage over *M. trunata* when faced with MHW, as reflected by their survival and by the extent of their cellular stress responses. On the other hand, single harvesting disturbance events can be tolerated by these clam species in the short term and do not appear to affect their physiological response to MHW. A more extensive study on harvesting stress would need to be done to determine these clam species' ability to tolerate longer or repeated harvesting disturbance events. Our study illustrates the complex, species- and tissue-specific mortality and metabolomic responses to a single MHW event according to their projected intensities in the North Atlantic regions of Eastern Canada. These congeners display marked differences in MHW tolerance. This may jeopardize population survival of *M. truncata*, which

is much more vulnerable as indicated by its inferior MHW tolerance of 17.77 °C. These differences can be explained in part by differences in their ability for tissue-specific metabolome remodelling. In fact, at their respective MHW limits, *M. arenaria* utilises a significantly greater number of signalling and DNA repair pathways than does *M. truncata*. The successful establishment and invasive characteristics of *M. arenaria* in Northern Europe and Western North America may, in part, be linked to these differences in metabolome plasticity, suggesting that this temperate invader may outcompete *M. truncata* in its native range as cold-temperate and Arctic thermal habitats continue to warm. These findings will be useful to inform clam species management and conservation practices, to refine distribution modelling for these species, while preserving sustainable traditional fishing practices under projected global change conditions. Finally, the study of cellular stress response mechanisms should increasingly be used to help define non-model marine invertebrates' ability to cope with global change stressors, as well as identify sub-lethal effects than can be used as early warnings of longer-terms fitness-related impacts to support management in preserving natural populations and stocks.

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Credit authorship contribution statement

Nicholas Beaudreau: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Tessa M. Page:** Formal analysis, Methodology, Validation, Visualization, Writing – review & editing, Investigation. **David Drolet:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing, Resources. **Christopher W. McKindsey:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Kimberly L. Howland:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Piero Calosi:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The datasets generated for this study are available on PANGAEA® Data Publisher, under OA-ICC data compilation, with the following digital object identifier: Beaudreau et al. (2024) https://doi.org/10.1594/PANGEA.964771.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170167.

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