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Elevated aerial temperature modulates digestive enzyme activities in *Mytilus californianus*

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ABSTRACT

The marine intertidal mussel *Mytilus californianus* aggregates to form beds along the Pacific shores of North America. As a sessile organism it must cope with fluctuations in temperature during low-tide aerial exposure, which elevates maintenance costs and negatively affects itsoverall energy budget. The function of its digestive gland is to release enzymes that break apart ingested polymers for subsequent nutrient absorption. The effects of elevated aerial warming acclimation on the functioning of digestive gland enzymes are not well studied. In this study we asked whether digestive gland carbohydases and proteases could be overstimulated in warm condition to possibly mitigate the costs related to the heat-shock response. We compared mussels acclimated to a + 9 °C heat-shock during daily low-tide aerial exposure to mussels acclimated to isothermal tidal conditions in a simulated intertidal system. The results showed fairly consistent activities of cellulase, trypsin, and amino-peptidase across tidal variation and between thermal treatments; however, amylase activity was lower in warmed versus cool mussels across low and high-tide. We also observed the expression of heat-shock genes in gill tissue during warm tidal conditions, suggestive that moderate temperatures during aerial exposure can induce a stress response.

1. Introduction

The mussel *Mytilus californianus* is a sessile intertidal species that aggregate to form beds along the west coast of North America. It is an important foundation species because it recycles nutrients within the intertidal zone and serves as critical habitat for hundreds of associated organisms (Menge et al., 2008; Suchanek, 1992). Because it is stationary it must cope with the prevailing environment thereby making it a sentinel of global change (Helmuth et al., 2006). Therefore, understanding its physiology provides insight into nearshore community dynamics and how sessile populations may respond to current and future environmental conditions.

As residents of the marine intertidal zone, mussels are exposed to fluctuating conditions where marine and terrestrial environmental conditions converge. As ectotherms, the body temperature of mussels is closely linked to thermal fluctuations of the local environment which is driven primarily by the interaction of aerial temperatures and tidal cycles (Helmuth and Hofmann, 2001). Mussels feed during high-tide submergence, thus the temporal ebb and flow of the tide determines variation in feeding intervals. Because tidal height varies day-to-day, mussels affixed to high shore heights are exposed to pronounced aerial-temperatures, solar radiation, and have less feeding opportunities compared to individuals settled on low shore-levels (Connor et al., 2016; Helmuth, 1998). Air exposure time varies throughout the season due to transitions between diurnal and mixed-semidiurnal tides, where high-shore mussels can be exposed to air (and heat) for 12 h or more in their most extreme ends of the tidal cycle (Gracey et al., 2008). In addition to the metabolic challenges of warming, incidences of high body temperatures during aerial exposure can lead to cellular protein damage (Gracey et al., 2008; Halpin et al., 2004) which requires ATP-costs to repair (Lindquist and Craig, 1988). Simultaneously, mussels close their shell-valves during low-tide and switch to low ATP-yielding fermentation pathways that are subsequently modulated by temperature (Connor and Gracey, 2012; De Zwaan, 1977; Kluytmans et al., 1978). Hence, variations in nutrient acquisition, body temperature, and metabolism affect the total energy budget of mussels and ultimately, the amount of resources that can be allocated toward positive growth over temporal scales (Widdows and Hawkins, 1989). To this end, growth-rate has a strong influence on the life-cycle of mussels. Growth determines mussels' ability to compete for space on the shore, escape predation, and

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reproduce (Bayne et al., 1983). Therefore, observing the effects of temperature on the biochemistry of nutrient acquisition is important for understanding the physiological underpinnings that modulate their ecology.

Within local shoreline regions, the growth rate of mussels vary across tidal and wave-exposure gradients (Connor et al., 2016). Mussel size increases from high to low shore-height levels (tidal-gradient) and seaward (wave-exposure-gradient) at any shore-level (Connor and Robles, 2015). Hence, the high-shore-wave-sheltered microenvironments are the hottest and most energetically stressful because they impose high thermal-maintenance costs coupled with low food-intake. Using digestive enzyme activities of the digestive gland as a proxy of nutrient intake, a recent laboratory and field-based study of *M. californianus* suggests that mussels can alter their digestive enzyme activities to partially compensate for the energetic demands of physiological stress (Connor et al., 2016). In Mytilus spp. there is a positive relationship between digestive enzyme activity with food intake, and a negative relationship between gut residence time (GRT) with food intake (Bayne et al., 1989; Connor et al., 2016; Fernández-Reiriz et al., 2001). These results suggest that, like other animals, mussels use a yield-maximization strategy (low intake but high GRT) when food is scarce and a rate maximization strategy when food is abundant (high intake but low GRT;(German et al., 2015)). High- vs low-food availability is encountered when comparing high- vs low-shore mussels, with the latter provided more access to food (Connor and Robles, 2015; Connor et al., 2016). However, measurements of digestive enzyme activities in the field revealed higher than predicted cellulase activity in mussels high on the shore during the low-tide sampling period (Connor et al., 2016). These results suggest that the quantity and type of enzyme expressed in mussels is not only activated by food intake but is also driven by the energy demands associated with elevated temperatures indicating a potential scavenging strategy for more recalcitrant organic matter in high-shore mussels (Connor et al., 2016). We make this suggestion because if elevated cellulase activities in high-shore mussels merely reflect warming increasing the rates of reactions for all proteins, then all digestive enzyme activities should be elevated in high-shore mussels, yet that isn't the case. Cellulase was the only enzyme (of those measured) to have elevated activity in the high-shore mussels (Connor et al., 2016). While these results support the hypothesis that mussels possess a dynamic digestive enzyme regulatory system in order to maximize assimilation efficiency during energetic stress (i.e., heat stress), the precise environmental cues that modulate this system requires further examination. For example, other enzymes may also be elevated in heat stressed mussels, but this function may occur during the high-tide period, during which mussels are actively feeding; collecting mussels from the rocky intertidal zone during high tide is dangerous and isn't done with any frequency.

In the current study we examined whether acclimation to moderately elevated air temperature under diurnal tidal conditions can lead to changes in digestive enzyme activity of digestive carbohydrases (amylase and cellulase) and proteases (trypsin and aminopepsidase) in mussels. We measured the digestive enzyme activities in the digestive glands of mussels acclimated to simulated tidal fluctuations in the laboratory. Cellulose is a structural component of the cell walls of green algae (Domozych et al., 2012). Following Connor et al. (2016), we predicted that cellulase would be elevated in mussels subjected to repeated moderate heat-stress in order to more rapidly break-down cellulose for energy directly or as a means to more readily release biochemical constituents within ingested algal cells; that is, allowing heat-stressed mussels to scavenge more resources from their ingested food. Enhanced cellulase activity and the carbon energy sequestered in energetically stressed mussels may allow them to partially compensate for the trade-off of the costs of growth for repair costs by digesting an organic matter component that can be ignored in mussels experiencing cooler temperatures and more frequent feeding opportunities lower on the shore. Elevated activity of digestive enzymes to scavenge for needed but scarce nutrients follows the nutrient balancing principle (Clissold et al., 2010). Here, we take an extended view of this principle and postulate that mussels up-regulate specific enzymes to mitigate an overall nutritional demand (i.e., energy demand) caused in part by elevated metabolic rates, and the heat-shock response (HSR) activation (i.e., heat stress). In this context, to our knowledge, this is the first study of direct effects of moderate temperature stress on digestive enzyme activity in mussels acclimated to simulated tidal conditions in the laboratory. Subjecting mussels to chronic moderate stress (i.e. pejus temperature range) provides the environment to which complex compensatory reactions related to bioenergetics can be observed; rather than the highly predictive negative reactions related to high stress scenarios (i.e. pessimum temperature range) (Sokolova et al., 2012). In this context it is tempting to hypothesize that all digestive enzymatic activities would rise with elevated temperatures experienced by the animal, but this is not universally observed since rates of enzymatic reactions are a function of how much enzyme is present (or the isoform of enzyme present) and expression of digestive enzyme genes don't always increase with rising temperature since there are costs associated with protein synthesis that may be curtailed by stress mitigation (Connor et al., 2016; Frederick et al., 2022; Hani et al., 2018; Pelusio et al., 2021). Hence, we predicted that moderate heat exposure will lead to enhanced cellulase activity in the animal, since this is what was observed in the field under natural conditions (Connor et al., 2016). If temperature stress is the main cue that increases cellulolytic activity, then our experimental design should lead to measurements that mimic those observed for high-shore mussels by Connor et al. (2016).

2. Materials and methods

2.1. Experiments

Two experiments were performed. In the first experiment a total of 48 mussels (6.51 ± 1.21 cm in length) were collected at a wave exposed portion of shore, +0.40 m above mean lower low water (MLLW) in Crystal Cove State Park, Laguna Beach, California (33° 33′ N, 117° 49′ W). Epibionts were scrubbed off and mussels were depurated in a 189-L closed container of gravel-filtered seawater at ≈17 °C and 35 ppt salinity for three days. Mussels were then separated into two treatment (76-L) tanks (24 mussels per tank) that shared a sump. In each tank, 16 mussels were placed on a shelf in the upper half of the tank that was subjected to a simulated tidal-regime that exposed animals to air for 12 h a day. One tank subjected mussels to an isothermal condition and the other tank subjected mussels to a temperature shock during low-tide (Fig. 1). This regime mimicked once per-day air-exposure that high-shore, wave-sheltered mussels might experience during mixed semi-diurnal tides (Connor et al., 2016). The remaining 8 mussels (per tank) were placed at the bottom of the tank where they remained submerged in flowing water throughout the course of the experiment. A 12:12 light-dark cycle was used to simulate day and night intervals. Mussels were acclimated for 30 days (Fig. 2).

Using a series of water pumps, and a computerized timer, the tanks produced a single high and low-tide. Low-tide occurred at 6 am and high-tide commenced at 6 pm. One tank subjected mussels to ambient air (≈17 °C) during low-tide. A second tank subjected musses to warm air produced by a set of ceramic heat emitters controlled by a Proportional-Integral-Derivative computer (Omega, Norwalk, CT) at a rate of 1.5 °C/h up to ≈26 °C at each daytime low-tide (Fig. 2, Fig. S1). This 9+ degree aerial heat-shock simulates heating regimes of high-shore regions along the coasts of North America (Connor et al., 2016; Helmuth and Hofmann, 2001). A peristaltic pump controlled by a computer added liquid microalgae (Shellfish Diet™, Reed Mariculture) to the sump daily. The Shellfish Diet™ nutrient composition comprised of 52% protein 22% carbohydrate and 16% lipid. The estimated concentration of particulate organic matter was ≈5 mg/L (see methods in Connor et al., 2016). A foam filter pump was turned on for 3 h daily to

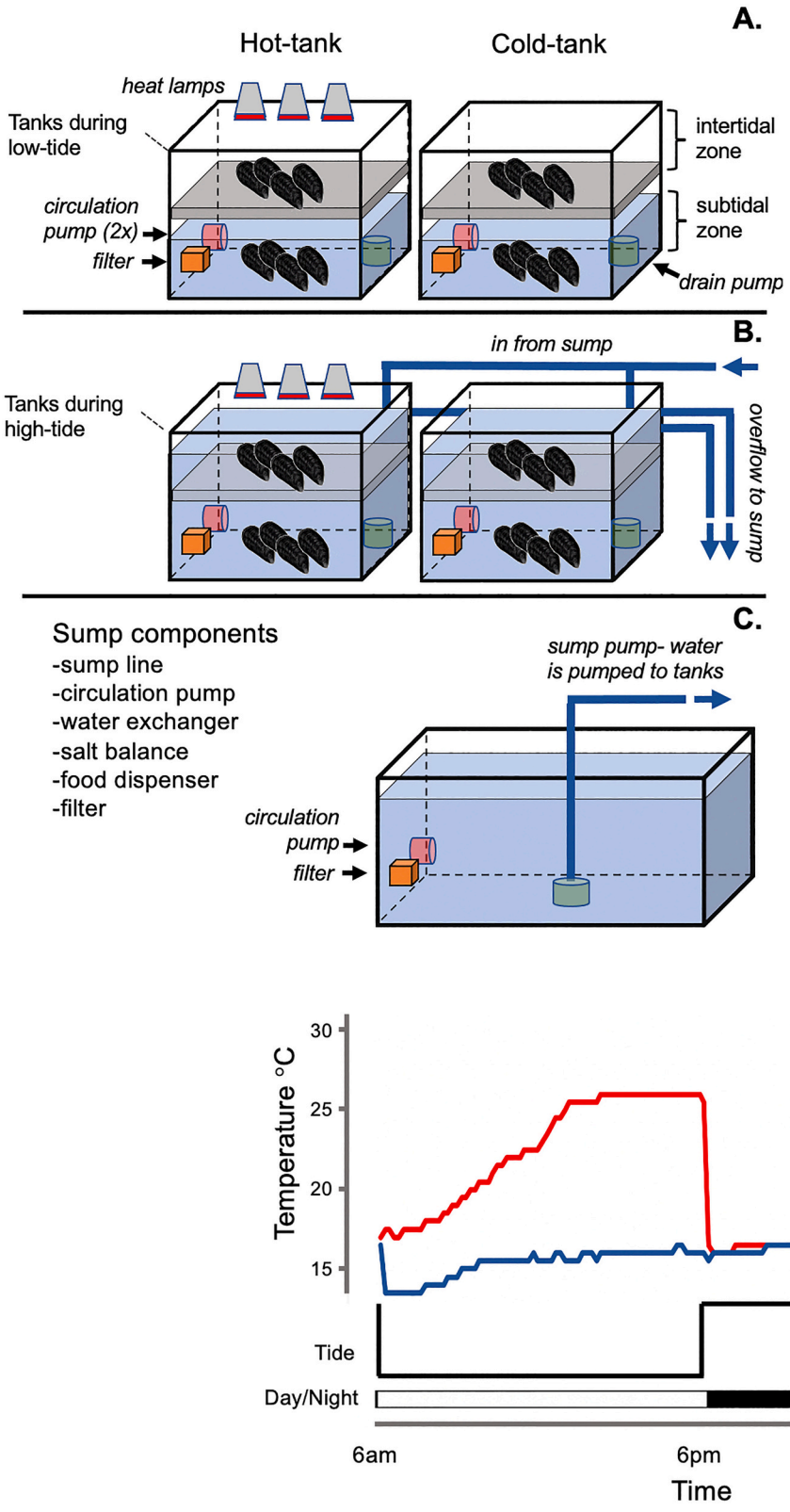

Fig. 1. Intertidal simulation system (exp. 1). Two 76-L tanks were established side by side in a cold room (≈17 °C) and shared the same seawater (35 ppt) through a sump tank. The treatment tanks contained an intertidal and subtidal section. A) Mussels within the intertidal section were subjected to air once a day using drain pumps that transferred water to the sump once a day. The hot tank was equipped with heat-lamps that raised the temperature of the surrounding air to ≈26 °C. Mussels within the subtidal section remained submerged throughout the experiment. B) During high-tide water is continuously pumped into the experimental tanks. C. Water maintenance occurred in the sump. The timing of events within the tank system was controlled by computer.

Fig. 2. Temperature signatures between the hot and cold tank. An example of the temperature of a single day. The cold tank was stable at ≈17 °C over the tidal course. The air-temperature in the hot tank was raised to ≈26 °C during low-tide at a rate of 1.5 °C an hour. The high-tide water temperature was ≈17 °C similar to that of the cold tank.

remove uneaten food and feces, and weekly water changes replaced half of the water volume from each tank. On the 30th day of acclimation, the intertidal mussels were collected at the end of the daytime low-tide period (6 pm), and the end of the night-time high-tide period (6 am). The subtidal mussels were also collected at 6 am. This scheme generated six groups; low-tide in the heated tank (L_H), low-tide in the cold tank (L_C), high-tide cold (H_C, represented in each tank since neither tank was warmed during high tide), and constant cold submergence (CCS, which

represents the submerged mussels in both tanks). Digestive glands and gill tissue from each mussel were dissected, frozen on dry ice, and stored at −80 °C. Digestive gland was sampled as it is related to digestive enzymes secretion and the break-down of ingested polymers. Gill tissue was used to measure differential expression of stress markers between animals from the cold and warmed tanks. The use of gill tissue to analyze environmentally modulated gene expression in mussels has an extensive history and its use in this study allows for a comparison of stress in mussels between tank environments and to performance observed in previous studies (Connor and Gracey, 2020, 2011; Gracey and Connor, 2016; Place et al., 2012, 2008).

2.2. Digestive enzyme activity measurements

2.2.1. Carbohydrase assays: Amylase assay & cellulase assay

Frozen digestive gland tissues were weighed and homogenized in 25 mM maleate buffer, pH 6.5 to form a 5 or 6× dilution using a Polytron PT 10–35 homogenizer at 3000 rpm. Homogenates were centrifuged at 9400 x*g* for 2 min at 4 °C. Supernatants were pipetted into small aliquots (100–200 μL) at −80 °C for storage until immediate use in spectrophotometric assays of digestive enzyme activities. All enzyme assays were measured using a BioTek Synergy H1 Hybrid spectrophotometer equipped with a monochromator (BioTek, Winooski, VT, USA).

Following German and Bittong (2009) substrate was made by mixing concentrations of 1% soluble starch (amylase) or 0.5% carboxymethyl cellulose (cellulase) with 25 mM maleate and 1 mM $CaCl_2$, pH 6.5. In 1.5 mL centrifuge tubes. For amylase activity, 50 μL of substrate was combined with 50 μL of diluted homogenate (5 μL homogenate: 45 μL buffer). For cellulase activity, 100 μL of substrate was combined with 100 μL of diluted homogenate (10 μL homogenate: 90 μL buffer). Homogenate controls (buffer and homogenate), and substrate controls (buffer and substrate) were run simultaneously to control for background absorbances generated by the homogenate and substrate, respectively. Assays were incubated at 17 °C for 30 min for amylase, and for 2 h of constant shaking for cellulase. After incubation, the reactions were halted by adding 20 μL 1 N NaOH to amylase tubes and 40 μL 1 N NaOH to cellulase tubes. Somogyi-Nelson reagent A was added to all tubes. 50 μL of substrate for amylase and 100 μL of substrate for cellulase were then added to the homogenate blank tubes. After 10 min of boiling and then cooling on ice, Somogyi-Nelson Reagent B was added to all tubes (see German et al., 2004 for ingredients in Somogyi-Nelson reagents). 100 μL of each solution was transferred to new vials of 300 μL nanopure water to be centrifuged (17 °C, 8000 rpm) in the Eppendorf 5430 Centrifuge for 5 min. Supernatant absorbance was measured at end-point using a spectrophotometer (BioTek Synergy H1) at 650 nm wavelength.

2.2.2. Protease assays

2.2.2.1. Aminopeptidase assay. Following Roncari and Zuber (1969), substrate was made by dissolving 0.005 g of L-Alanine-p-nitroanilide hydrochloride (ApNA) in 25 mM maleate buffer at pH 6.5. In a microplate, 99 uL of pre-incubated ApNA solution at 17 °C was combined with 1 uL of homogenate. Homogenate control was made by combining 99 uL of buffer with 1 μL of homogenate. Assays were incubated at 17 °C for 30 min. Absorbance was measured at end-point using a spectrophotometer at 410 nm.

2.2.2.2. Trypsin assay. Following German and Bittong (2009), substrate was made by dissolving 0.01 g of N-alpha-benzoyl-L-Aranine-p-nitroanilide (BAPNA) in 25 mM maleate buffer at pH 6.5 with heat. In a microplate, 95 uL of pre-incubated BAPNA solution at 17 °C was combined with 5 uL of homogenate. Homogenate control was made by combining 95 uL of buffer with 5 μL of homogenate. Assays were incubated at 17 °C for 30 min. Absorbance was measured at end-point using a spectrophotometer at 410 nm.

2.3. Enzyme activity calculations

The unit conversion equation was used to calculate for carbohydrase, aminopeptidase, and trypsin enzyme activity in U (1 μmol L^{-1} reducing sugar or p-nitroaniline liberated per minute, respectively) per gram of wet weight digestive gland tissue (see German et al. (2011)).

Net absorbance was found by subtracting the absorbance of the homogenate blank from the absorbance of the sample. An extinction coefficient was extrapolated through a glucose standard curve for amylase and cellulase, through a p-nitroaniline standard curve for aminopeptidase and trypsin.

2.4. Expression of chaperone genes in gill tissue

The collected gill tissue from the first experiment was destroyed in a freezer failure. Hence the second experiment was deployed to acclimate mussels to tidal environments and confirm the heat-shock response (HSR) induced by the experimental system in experiment one. This second experiment was slightly different due to COVID restrictions that didn't exist during the initial experiment. For the second experiment, 18 mussels (9 per tank) were acclimated to environmental regimes (warm and cold) that were similar to the first experiment (12 h daily low-tides that began at 6 am) on the day of sampling where high-tide was moved to 12 pm (i.e. a five-hour low-tide) (Fig. S2). Mussels in the hot tank were acclimated to a daily ≈9+ degree heat shock that started at 10 am and ramped to ≈24 °C with a rate of 2 °C/h (slightly faster than experiment 1). On the day of sampling, the heat shock began at 7 am. A temperature of ≈15 °C was maintained during low-tide in the cold tank and during high-tide in both tanks. Mussels (6.53 ± 0.82 cm) were collected from a rocky headland on San Buenaventura State Beach 34° 16′ N, 119° 16′ W and depurated for three days before being transferred to an intertidal tank. Mussels were acclimated for two-weeks to the tank conditions and were sacrificed for gill tissue harvesting. Animals were sacrificed ($N = 3$) at 7 am (low-tide start), 12 pm (low/high-tide transition), and 2 pm (high-tide). gill tissue was immediately frozen on dry ice. Mussels used in the experiments were collected from different sites that likely differed in environmental variables, and the lingering effects of acclimatization can alter the degree of trait performance in individuals post removal from their natural environment (Moyen et al., 2020b). Although the two experiments used mussels from different collection sites, our focus was to confirm diagnostically, the relative differences in molecular response between mussels acclimated to moderately warm or cold tidal environments, as has been previously reported (Connor and Gracey, 2020, 2011).

2.4.1. qPCR protocol

RNA was extracted from frozen tissue with Trizol (LifeScience) using an established protocol (Gracey et al., 2008). cDNA was synthesized from the resultant RNA (iScript cDNA synthesis kit, Bio-Rad). Gene expression was measured using 1 μL of cDNA in a a qPCR assay with a Bio-Rad CFX thermocycler. The qPCR profile used was—denature (95 °C), annealing (60 °C), extension (72 °C) for 40 cycles. We designed primers from *M. californianus* ESTs annotated in Gracey et al. (2008). The primers were designed for genes related to protein folding including genes (gene symbols are human nomenclature) inducible heat-shock protein 70 (*HSPA12A*), t-complex protein 1 (*TCP1*), t-complex protein 1 subunit eta (*CCT7*), and Tubulin-alpha (*TBA1A*) as control (Table 1). The heat-shock protein 70-12A gene is a member of the family of protein folding genes and consistently shown to activate under acute and repeated thermal stress in mussels (Connor and Gracey, 2020, 2011; Connor et al., 2016; Gracey et al., 2008; O'Brien et al., 2021). T-complex genes belong to the TCP1 ring-complex chaperonin which are involved in refolding a variety of proteins including tubulin and actin (Palumbo et al., 2015). Tubulin-alpha was previously used in qPCR reactions that

Table 1
Primer sequences and accession numbers for *M. californianus*.

Gene name	Forward (5′ – 3′) Reverse (5′ – 3′)	Size (bp)	GenBank Accession no.
t-complex protein 1 (*TCP1*)	Forward: AGACTGGCATGCAAAGAAGC Reverse: TGGCAGCATCAACAACCATG	162	GE749699.1
t-complex protein 1 subunit eta (*CCT7*)	Forward: GGAGGCGCAATAGAGATGGA Reverse: CACCTTGTGCATGTCTCTGA	190	ES396291.1
Heat shock protein 70 (*HSPA12A*)	Forward: AGACGCCGATATACAGTGGG Reverse: CGACAGTACAATGAGGCAGC	150	ES389649.1
Tubulin-alpha 1A (*TUB1A*)	Forward: TCCAAGACACGGCAAATACA Reverse: TTGAAACCAGTTGGACACCA	205	ES735904.1

validated microarray data (Gracey et al., 2008). CFX Maestro software (Bio-Rad) was used to record Ct scores. The scores were used to determine gene expression using the relative quantity method (RQ) ($2^{\Delta Ct}$) (Pfaffl, 2007). This method is very sensitive to small changes in Ct scores and leads to complex and skewed distributions of results. We deployed bootstrapping and randomization techniques to overcome the lack of a standard distribution (Pfaffl et al., 2002). Bootstrapping (37 permutations) was performed for all genes and the randomized Cts of the target genes were matched with those of the reference gene and RQ was subsequently determined. The number of permutations used allowed for pairs to match at least twice.

2.5. Statistics

Statistical analyses were performed with SPSS (version 26). Levene's test was performed on digestive enzyme activity data for each enzyme. Digestive enzyme data was log-transformed if groups were heterogeneous. An analysis of variance (ANOVA) followed by Tukey's Honesty Significant Difference (HSD) test was used to determine variation in mean enzyme activity in the three different tidal-height positions (low-tide, high-tide and subtidal) between heat and no heat exposure

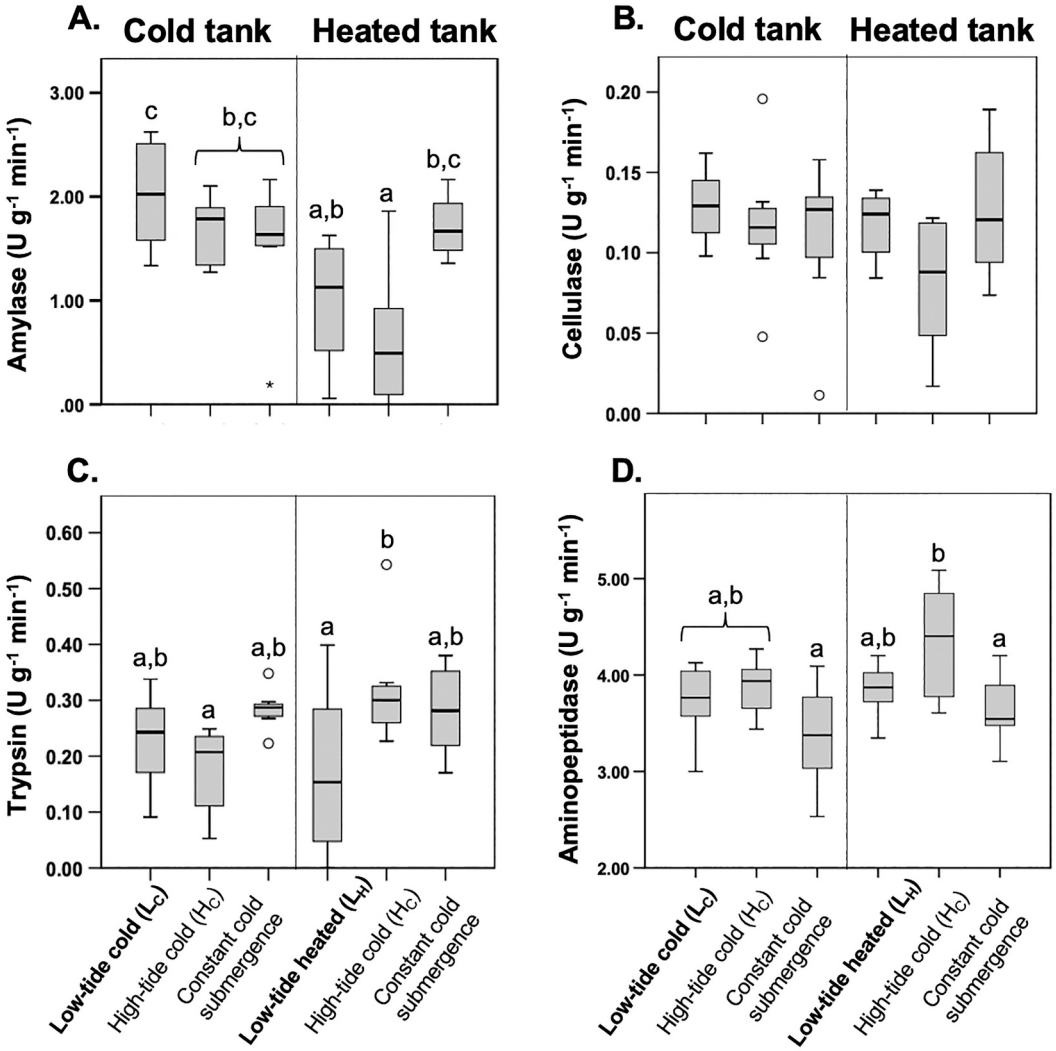

Fig. 3. Digestive enzyme activities. Mean digestive enzyme activities of A) amylase ($N = 48$), B) cellulase (N = 48), C) aminopeptidase (N = 48) and D) trypsin (N = 48) under laboratory stimulated tidal positions and temperature conditions. The conditions include low-tide heat (L_H), low-tide cold (L_C), high-tide cold in the heated tank (H_C heat), high-tide cold in the cold tank (H_C cold), and constant cold submergence (CCS) in the cold or heated tanks. Tukey's test was used to identify significant differences ($p < 0.05$) in the mean enzyme activities (U g^{-1} min^{-1}) between the conditions. The difference is indicated by letters (a, b, and c) on graphs. Boxplots show lower and upper quartiles (box), and median (horizontal line within each box).

treatment. An ANOVA and Tukey's test was used to identify differences in gene expression between treatment groups. A p-value <0.05 was used to identify statistically significant variation.

3. Results

3.1. Digestive enzyme activities

Levene's test on raw amylase data rejected homogeneity between treatment groups, therefore the activity values were log-transformed. Amylase activity was significantly lower in the L_H and the H_C mussels from the heated tank compared to L_C and H_C mussels in the cold tank ($P < 0.001$; Table S1, Fig. 3). For example, the mean amylase activities of the intertidal mussels were > 2-fold in the cold tank in comparison to the heated tank. No differences in amylase activity were detected between subtidal, constant cold submergence (CCS) mussels in the two tanks. The slight variation in amylase activity among low-tide, high-tide, and subtidal animals suggest an effect of tide on enzymatic activities. The range of amylase activity across groups was 0.001–2.62 U g^{-1} min^{-1}.

Cellulase enzyme activity showed no significant differences in means between all of the experimental groups ($p > 0.05$) (Table 1. Fig. 3). However, the average range in activity between H_C mussels in the two treatments (0.13 U g^{-1} min^{-1}) was >2 times higher than the average range between the L_H and L_C groups (0.06 U g^{-1} min^{-1}). The average range between L_H and L_C mussels was on par with that of the average between constant cold submergence mussels in the two tanks (0.14 U g^{-1} min^{-1}). The activity ranged between 0.01 and 0.20 U g^{-1} min^{-1}.

Variation in protease activity across groups was limited, but the activities of trypsin and aminopeptidase each varied significantly among the groups ($p < 0.01$; Table S1). Trypsin activity in the H_C mussels from the heated tank was higher and more variable than all other groups (Fig. 3). This variation appeared to be driven by a single high value in a H_C mussel from the heated tank, and the treatment groups were not significantly different from each other following its removal. Analysis of aminopeptidase enzyme activities revealed heterogenous variance between treatment groups, therefore they were log-transformed.

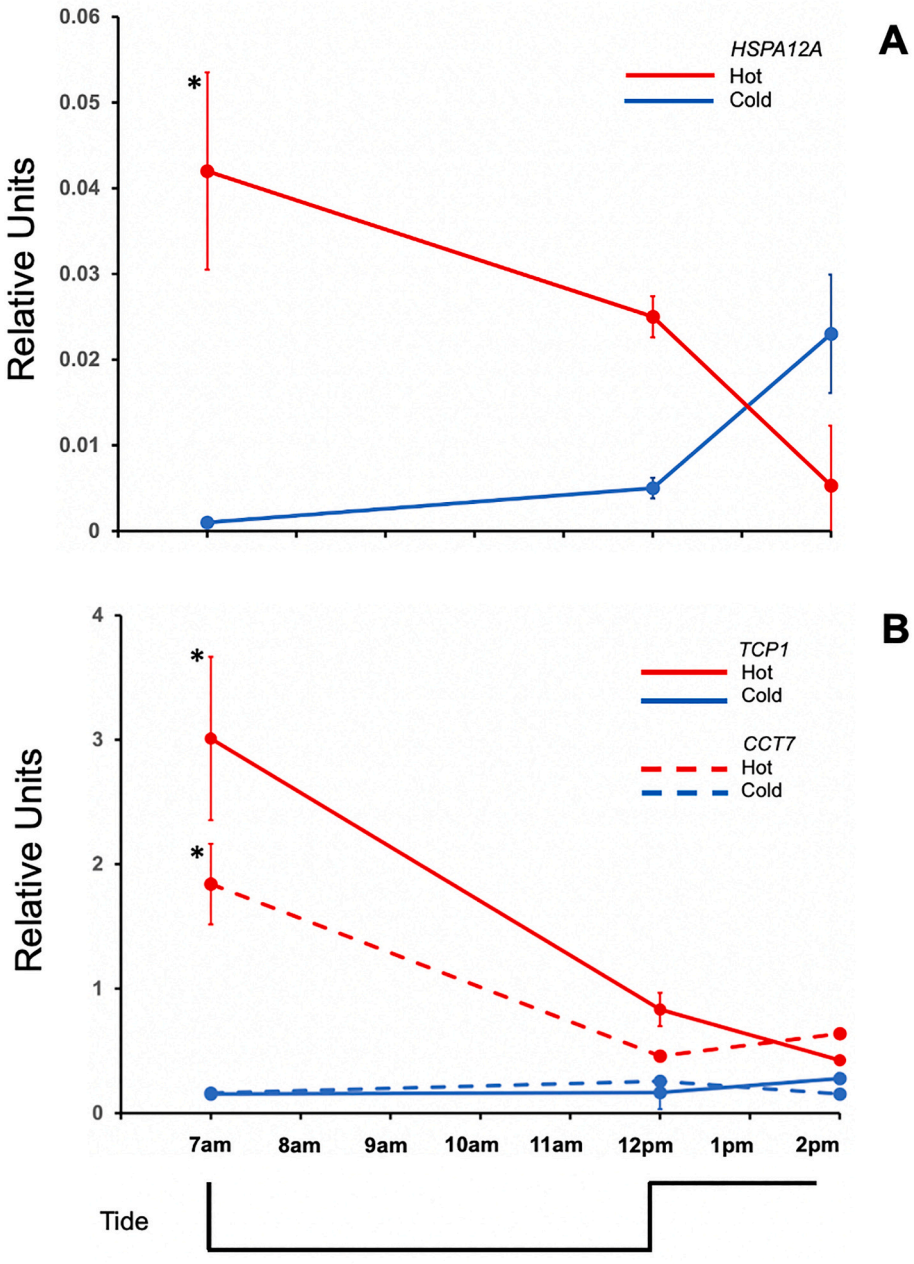

Fig. 4. Temporal Gene expression. Mean gene expression over time in gill tissue of mussels following acclimation to cool and warm tidal fluctuations in laboratory conditions (Fig. S2). Gene expression was measured at 7 am, 12 pm (low-tide time points), and 2 pm (high-tide time point). The measured genes were A) *HSPA12A* B) *TCP1* (solid line) and *CCT7* (dashed line). Blue lines represent cool conditions and red lines represent warm conditions. Each point represents mean gene expression following 37 permutations of target gene expression from 3 animals. The error bars represent 1 S.E. of the mean value. Asterisks represent significant differences between hot and cold treatment at the stated time-point as determined by ANOVA and Tukey's test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Aminopeptidase activity was highest in the H_C mussels from the heated tank compared to all other groups (Table S1, Fig. 3.). This was likely driven by the slightly lowered mean activity of the constant cold submergence mussels (Table S1). Lastly, no variation of aminopeptidase activity between constant cold submergence mussels was detected.

3.2. Expression of chaperone genes in gill tissue

Transcript abundance of protein folding genes was higher in mussels from the heated tank versus those from the cold tank (Fig. 4). As predicted, *HSPA12A* was particularly activated in mussels that were subjected to warming. There was a $>$ 40-fold difference in HSPA12A transcript abundance between hot and cold acclimated mussels at the commencement of low-tide. The 7 am mean transcript abundance of *HSPA12A* in mussels acclimated to cold and hot treatments was 0.001 and 0.042 relative units respectively. Transcript abundance in warmed mussels waned during peak day-time aerial temperature of $\approx$ 26 °C and submergence. Transcriptional patterns for T-complex chaperonin genes in warm acclimated animals were like that of the HSP-70 expression. They displayed peak abundance at 7 am followed by depressed expression. The 7 am mean transcript abundance of *TCP1* between cold and hot treatments was 0.15 and 3.01 respectively and for *CCT7* it was 0.16 and 1.84 relative units respectively.

4. Discussion

Intertidal mussels possess flexible digestive systems sufficient to maximize assimilation efficiency within fluctuating environment conditions of the intertidal zone (Bayne et al., 1988; Connor et al., 2016; Elvin and Gonor, 1979). We previously observed elevated ex vivo cellulase activity and heat-shock gene expression in mussels residing in the characteristically warm microhabitats of the upper-intertidal-zone (i.e. high on the shore); which includes limited feeding times for mussels in this intertidal region (Connor et al., 2016). Hence, we predicted that elevated digestive enzyme activity in mussels residing in upper regions of the intertidal acts to provide resources for the ATP demands of protein folding, maintenance of ion gradients, protein turnover, and other aspects imposed on metabolism by elevated temperatures. We hypothesized that elevated cellulase activity in high-shore mussels may be a form of nutrient balancing (Clissold et al., 2010), or scavenging. Unlike the Adaptive Modulation Hypothesis (Karasov, 1992), which posits a match between nutrient load in the diet and digestive enzyme activities against that nutrient, Nutrient Balancing suggests elevated enzymatic activity toward a limiting nutrient (Clissold et al., 2010), or against a nutrient (e.g., cellulose) that might be ignored under better feeding and temperature conditions, like those experienced lower in the intertidal zone. Specifically, we hypothesized that elevated temperatures experienced by mussels high on the shore generated a scenario where sufficient digestion of cellulose is required to meet the energetic demands imposed on the mussels by elevated temperature, which would include the heat shock response (HSR).

We subjected mussels to daily moderately elevated (pejus) aerial temperatures in laboratory conditions that were sufficient to trigger the onset of an ATP demanding stress response in gill tissue. Indeed, the temperatures used in this experiment induced expression of chaperone-function genes (Fig. 4). This HSR is in line with the normal molecular functioning of thermally exposed gill, supported by previous transcriptomic and proteomic expression laboratory studies of the genus *Mytilus* (Connor and Gracey, 2020, 2011; Lockwood et al., 2010). Importantly, the HSR is activated under moderate temperatures despite the fact that it is energetically costly, and that certain enzymes do not require their assistance since they can tolerate extreme temperatures (Lockwood and Somero, 2012). However, the level of stress endured by the experimental mussels cannot be quantified precisely in our study because temperature hardening has been shown to occur in *M. californianus* in temporal observations of the HSR (Buckley et al., 2001; Moyen et al., 2020a). We found a high abundance of the heat-shock gene transcripts at the beginning of low-tide suggestive that peak expression occurred during late-evening and after our early-evening sample point. This inference is in line with a study by Gracey et al. (2008) that revealed heat-shock gene transcripts in *M. californianus* were sustained through high-tide following elevated aerial temperatures. There are many heat-shock genes in the transcriptomes of intertidal bivalves, and they are expressed at different times of the day, depending on environmental conditions (Connor and Gracey, 2020; Zhang et al., 2012). Furthermore, thermal hardening may depress the sensitivity or change the timing of HSR components including gene expression and we did not assess the level of hardening that may have occurred in the experimental mussels (Georgoulis et al., 2021). Nonetheless, the experimental mussel subjects appeared to sense and respond to elevated temperature changes during low-tide under our simulated tidal conditions.

Our hypothesis of increased cellulase activity in mussels exposed to warm temperatures was not supported. This suggests that temperature itself is not the main variable affecting activity levels of cellulase in the digestive gland of *M. californianus*. Although the shellfish diet we used to feed our mussels contains green algae (Sakamoto and Toyohara, 2009), which would have cellulose in its cell walls (Painter, 1983), it is possible that natural detritus in the ocean contains more cellulose than was in the shellfish diet (Antonio et al., 2010; Duarte et al., 2005; Parsons and Strickland, 1962). Thus, it could be that cellulose is a viable nutrient for mussels to scavenge in nature, but not necessarily under the conditions in our experiment. However, higher concentrations of cellulose containing detritus from ground macroalgae (*Enteromorpha*) and plant debris (*Juncus*) did not lead to an increase in cellulase activity in the guts of common cockle (*Cerastoderma edule*; Arambalza et al., 2018). Because cockle are not closely related to *Mytilus* in the bivalve phylogeny, a similar experiment to that done in the cockle using *Mytilus* is required for a more robust comparison to the present study (Plazzi et al., 2011).

Variation in cellulase levels may also be the result of algal composition. For example, Ibarrola et al. (1998) showed that *C. edule* continuously adjusted amylase, cellulase, laminarinase, and protease levels across seasons, which temporally tracked changing algal composition along the coast. Researchers found higher enzyme activities during Spring-Summer than Autumn-Winter in correlation with levels of chlorophyll *a* and particulate organic matter in the water. To this end, mussels in our previous field study (Connor et al., 2016) were evaluated at a single time-point during summer which could have masked any seasonal temperature, salinity, and other physical and biological effects. Long-term multi-sampling seasonal studies may be necessary to resolve inferences of cellulose scavenging by *M. californianus* in nature. Finally, the ingestion and establishment of cellulolytic bacteria in the guts of mussels may contribute to their cellulose digestive activity and this should be investigated in future studies that compare laboratory to wild-caught animals.

Unexpectedly, we found that mussels subjected to warm air during low-tide (L_H and H_C mussels in the heated tank) had a pronounced negative effect on amylase enzyme activity, but minimal effect on cellulase and protease activity, in comparison to mussels from the cold treatment (Fig. 3). In metazoans, electrons eventually released from the oxidation of glucose liberated by amylase (and maltase) activity subsequently helps synthesize ATP, therefore the lower enzyme activity we observed in heat-challenged mussels suggests the opposite of possible enzymatic-mitigation of the ATP demands of elevated temperatures. Amylolytic activity would be expected to increase with increasing temperature (German et al., 2016; Seiderer and Newell, 1979). In contrast, Connor et al. (2016) did not find mean differences in amylase activity between mussels from warm and cool microhabitats on the same shore in a natural setting. However, the range of activity decreased along a thermal gradient in the field suggestive that thermal constraints may be imposed upon digestive enzyme activity in nature. In other words, in addition to experiencing less heat stress in general, some

individuals in cooler natural microhabitats have greater digestion capacity than a majority of mussels in nearby warmer microsites.

Cellulase may be more resistant to thermal perturbations than amylase. For example, cellulase had higher break-point temperatures than amylase in digestive gland homogenates of the blue mussel, *M. edulis* (Brock et al., 1986). Activity measurements of *M. edulis* cellulase and amylase generally increased from low (4 °C) to high temperatures, however amylase activity decreased between 24 and 28 °C, while cellulase activity was not negatively affected until it was subjected to higher temperatures (28 and 32 °C) (Brock et al., 1986). Thus, it is possible that cellulase and amylase temperature responses differ in *M. californianus* and other members of the blue mussel complex (German et al., 2016). Species specificity is important under pejus temperatures as shown by a study of abalone revealing amylase activity was lowered following a + 5 °C change in one of two abalone (i.e., not both species) from the same genus (Frederick et al., 2022). Alternative to our findings, Seiderer and Newell (1979) showed an increase in style-amylase activity in the divergent warm adapted intertidal South African Mytilidae mussel *Choromytilus meridionalis* acclimated in warm water (22 °C). In that study, mussels were acclimated in water at 8, 15, and 22 °C for 16 days. Results showed increased activity across the experimental temperature range, but high water-temperatures do not serve as an appropriate proxy-environment for thermal exposure in air. Lastly, gene expression influence on protein abundance may have also played a role in differential expression of amylase between cool and warm conditions. Empirical support for this inference is currently lacking and should be explored.

Warming can impact digestion simply by changing the amount of time over which the mussels can feed. For example, Anestis et al. (2007) showed a positive relationship between valve closure-duration and water temperature from 17 °C to 24 °C in closely related warm adapted mussel, *M. galloprovincialis*. Even when submerged, mussels do not feed during valve closure (Gracey and Connor, 2016), and hence, temperature effects on filtration can modulate ingestion and digestive enzyme activity. In this context, the mussels in our study were acclimated to daily cycles of immersion and aerial emergence which better reflects their natural feeding and thermal stress regimes within the intertidal zone and their interplay, than acute exposures to warm water implemented in previous studies. We did not follow how often the mussels in these experiments were closing their valves when submerged, but our own previous work showed that mussels spent time with valves closed, and in a quiescent state (low heart rate), even under constant submergence (Gracey and Connor, 2016). Whether heat exposed animals spend more or less time with valves closed when submerged is unknown, but a heat response can carry over, even to times of submergence in water, that could impact feeding time, and therefore, digestion (including digestive enzyme activities).

Similar to that of cellulase, protease activity was also sustained across thermal warming cycles in this study in agreement with the inference that consistent amino acid acquisition is necessary to maintain enzyme synthesis and structural cellular integrity when foraging is intermittent. Amino acids in general cannot be stored in model metazoans cells or specialized organs highlighting its importance and here we assume this also applies to bivalves. However, some amino acids can be temporarily stored as conjugated carnitine complexes in mussels during aerial exposure (Connor and Gracey, 2012). Nevertheless, a low-protein diet (low amino acid availability) triggers protein catabolism, including of muscles, which can be dangerous for mussels because the adductor muscle connects the shell valves that prevent exposure to deadly environmental elements. In line with this inference, Dowd and Jimenez (2019) showed larger adductor muscle fibers in high-shore mussels in comparison to low-shore ones. Furthermore, our previous laboratory and field experiments revealed consistent trypsin activity across algal concentration and temperature gradients, showing that protein was likely efficiently digested regardless of concentration in the diet (Connor et al., 2016). Additionally, Albentosa et al. (2012) found that protease activity was highest when mussels were energetically stressed by lowered food presentation and total intake. Pronounced protease stability following starvation, has also been shown in other genera (aquatic and terrestrial) (Abolfathi et al., 2012; Albentosa and Moyano, 2008). Finally, extreme thermal stress (>25 °C) experienced by *Mytilus* leads to pronounced HSP activation and potentially high protein turnover rates (Gracey et al., 2008; Koen and Bayne, 1989). It appears that the heat shock protein (HSP) response is activated during moderate temperatures as well, either to stabilize proteins or in anticipation of oncoming extreme temperatures. Protein preservation is a requirement for survival, hence the consistent activity of protease enzymes over temperature variation is in line with robust amino acid pool and protein synthesis maintenance (Moore et al., 2007).

4.1. Concluding thoughts

Mytilus californianus is subjected to large shifts in body temperatures and feeding regimes which can promote energy balance stress (high thermal-maintenance costs coupled with low food-intake). *Mytilus* may cope with this stress by maximizing nutrient acquisition through variable feeding and digestive related processes such as filtration rate, ingestion, assimilation, and gut residence time (Bayne et al., 1988). Because mussels are sessile and cannot migrate to avoid environmental stress or to forage for food, growth (cell size,division of somatic and sex cells) only occurs after maintenance costs (basal metabolism, protein turnover and stabilization, sodium-potassium pump activity, fatty acid synthesis) are managed. Hence, there is a physiological trade-off (Sibly and Calow, 1986) between growth and variables that comprise maintenance costs. Thaler et al. (2012) suggests a strong selective advantage of traits which mitigate trade-offs between processes that lead to growth/survival and stress/death. In the current study we revealed that carbohydrase digestion in the digestive gland of mussels is partially compromised during moderate aerial heat-stress. Amylase activity was lower and cellulase activity remained stable. Hence, these results uncovered a potentially novel thermal-stress phenotype (reduction in amylase activity, but robust cellulase activity) in *Mytilus* that should be considered in future environmental studies and predictions concerning changes in their physiological-ecology as global air-temperatures continue rise.

There are other considerations for optimizing energy reserves. For example mussels can absorb DOM through mantle tissue (Wendt and Johnson, 2006); however, this has not been evaluated in mussels under cycles of aerial heat stress. Furthermore, cellulose-hydrolyzing bacteria have been identified in gut flora of *Mytilus* and it is assumed that the contribution from these exogenous sources to total cellulase activity in the digestive gland is minor. However, no study has evaluated this allocation or how overall floral composition may alter it. To further this point, an evaluation of the gut microbiome of *M. edulis* showed a dominance of *Endozoicomonas* which possesses genes that predominantly encode for cellobiase (Neave et al., 2017). These complexities must be evaluated in future studies to attain an integrative perspective of how sessile animals maintain energetic homeostasis and survive in stressful environments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on https://german.bio.uci.edu/images/pdf/Pham_et_al_supplements.pdf.

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