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Physiological and biochemical responses to acute environmental stress and predation risk in the blue mussel, *Mytilus edulis*

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ABSTRACT

The effects of climate change and associated increases in temperature on organisms are a major focus of scientific research, but how these impacts play out within ecological contexts is complex and hence often ignored. For example, the influence of predation risk (nonconsumptive effects, NCEs) can alter behavior, creating scenarios where individual physiological responses depend on the interaction between abiotic conditions, such as temperature, and the presence of risk in the environment. Yet a mechanistic understanding of how the interplay among abiotic and biotic stressors, especially NCEs, shapes the short-term physiological performance of intertidal organisms remains limited. From both physiological and biochemical perspectives, we explored the short-term interaction between temperature, feeding history, and predation risk from a predatory snail (*Nucella lapillus*) on the intertidal mussel (*Mytilus edulis*). We measured heart rate, key aerobic (citrate synthase) and anaerobic (cytosolic malate dehydrogenase) metabolic enzymes, and total antioxidant capacity to elucidate metabolic strategies utilized by mussels in short-term, multi-stressor events. After 60 min of continuous exposure to increased temperature and predation risk, heart rate and aerobic capacity were primarily impacted by temperature, whereas total cytosolic malate dehydrogenase activity displayed an antagonistic relationship in response to the combined effects of feeding history and predation risk. In contrast, total antioxidant capacity displayed a three-way interaction among all treatments (feeding history, temperature and predation risk), driven by opposing thermal responses between fed and starved mussels in the absence of risk. Our results suggest that although mussels are fairly tolerant of acute stress events, the interaction of feeding history and predation risk may prevent them from launching a coordinated stress response when thermal stress is high.

1. Introduction

The effects of global climate change have been extensively documented, revealing impacts to both abiotic and biotic processes that are essential to community structure and ecosystem function (Queirós et al., 2014; Ruckelshaus et al., 2013; Wernberg et al., 2011). Indicators or metrics of physiological state can serve as powerful tools to assess the vulnerability of ecologically important organisms under current and future scenarios (Gunderson et al., 2016). They can also help identify the causal physiological and ecological mechanisms that structure communities and ecosystems in response to global change (Denny and Helmuth, 2009). The influence of an organisms' physiological state on species interactions such as competitive ability (Pechenik et al., 1998) and predator avoidance (Strobbe et al., 2010) has been relatively well-studied, particularly in regard to the role of energetic/nutritional state (Fitzgerald-Dehoog et al., 2012; Lesser et al., 2010; Matassa and Trussell, 2014). Further, a number of studies have examined differences in how species interaction strengths are affected by changes in abiotic conditions such as temperature (Poloczanska et al., 2016). Yet, we still know relatively little about how and when simultaneously applied multiple biotic and abiotic stressors affect organisms' physiological functioning.

Perhaps the largest gap in our understanding is the potential physiological influence of predation risk on prey response—often referred to as nonconsumptive effects (NCEs) or “the fear of being eaten” (Trussell et al., 2006)—and the interaction between NCEs and other stressors. NCEs can strongly affect prey physiology (Hawlena and Schmitz, 2010a, 2010b; Stoks et al., 2005) and fitness (Creel et al.,

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2007), and these individual-level changes can scale up to impact communities and ecosystems (Schmitz, 2009; Trussell and Schmitz, 2012). Moreover, these effects can be highly context dependent; for example, resource availability (Donelan et al., 2017) or prey energetic state (Matassa et al., 2016) can strongly affect the strength of non-consumptive effects on prey foraging and physiology by affecting the cost/benefit calculus of antipredator behavior (e.g., refuge use, Lima and Bednekoff, 1999). While there are several hypotheses to explain observed changes in prey physiology under predation risk including increased metabolic demands (Rovero et al., 1999), enhanced production of heat shock proteins (Pauwels et al., 2005), and upregulation of antioxidant enzymes (Slos and Stoks, 2008), these mechanisms remain relatively unexplored in the context of other stressors that are likely to accompany environmental change, including temperature.

Under climate change, species are experiencing potentially novel suites of environmental conditions (Ackerly et al., 2010) as both mean and extreme temperatures are already rapidly changing in many ecosystems (Harley et al., 2006); as a result, studies have documented, and predicted, widespread effects on organismal metabolism and physiology (Somero, 2010). But quantifying and recreating environmental conditions in controlled experiments is far more complex than is often recognized, and a growing number of studies have shown that a focus only on climatic means (versus extremes) will almost certainly fail to capture the processes by which changing environmental conditions affect organisms in nature (Bates et al., 2018; Helmuth et al., 2014; Lynch et al., 2014). For example, while projected increases in global temperature on the order of 1–3 °C or greater have been forecasted (IPCC, 2013), heat waves well above this magnitude already occur over shorter time scales (Hobday et al., 2016). At local scales, the range of environmental conditions experienced by plants and animals can be even greater than regional predictions. For example, even in temperate zones, intertidal organisms frequently experience body temperatures that far exceed air temperatures (Helmuth et al., 2016) and in shallow (< ~20 m) subtidal environments, coastal water temperatures can be much warmer than those recorded by offshore buoys and satellites (Pfister et al., 2007).

Using a multi-factorial laboratory mesocosm experiment, we examined the interactive effects of a suite of key environmental stressors (water temperature, predation risk, and feeding history) on short-term physiological and metabolic responses using two key interacting rocky intertidal species, a predator (the snail *Nucella lapillus*) and its prey (the mussel *Mytilus edulis*). Specifically, we measured impacts of interacting stressors on *M. edulis* physiology, focusing on heart rate, the production of metabolic enzymes (citrate synthase and cytosolic malate dehydrogenase) and total antioxidant capacity. The rocky intertidal zone has long been a model system for exploring organism-environment and inter-organismal interactions (Paine, 1994) because of strong spatial and temporal variation in abiotic stressors (e.g., temperature, desiccation). *M. edulis* is a dominant space occupier on rocky shores around the globe and is often subjected to extreme conditions, especially in the upper mid-intertidal zone, where mussel body temperatures during low tide can exceed 40 °C in air (Helmuth et al., 2016) and 25 °C in shallow tidepools and depressions (Helmuth and Hofmann, 2001; Miller and Dowd, 2017). Mussels are key prey for a variety of species across different trophic levels (Smith and Jennings, 2000), including the carnivorous snail, *N. lapillus* (Hughes and de B Dunkin, 1984), which was used in this study to generate predator risk cues. For this study, we focused on the full range of water temperatures that occur in the low intertidal zone (Helmuth et al., 2016) where predation risk is high and aerial exposure is minimal (Zippay and Helmuth, 2012) because this is where interactions between these two species exert a strong influence on rocky shore community structure and dynamics (Menge, 1976; Petraitis, 1998). In mussels, heart rate changes rapidly (Aagaard et al., 1991; Burnett et al., 2013; Bakhmet, 2017) and can be a reliable indicator of thermal tolerance (DeFur and Mangum, 1979) because it increases to a temperature-triggered breakpoint after which it declines. Mussel heart rate also elevates in response to predator cues (Rovero et al., 1999) and is reduced under low food conditions (Bayne, 1976). Citrate synthase (CS), a rate-limiting enzyme involved in the tricarboxylic acid (TCA) cycle, is a well-known indicator of cellular aerobic capacity (Dahlhoff and Stillman, 2002; Kawall et al., 2002) and cytosolic malate dehydrogenase (cMDH), also an indicator of metabolic capacity (Dahlhoff and Stillman, 2002; Ulrich and Marsh, 2006), plays an important role in anaerobic metabolism. Total antioxidant capacity is an indicator of cellular defense as antioxidants protect against cellular damage caused by reactive oxygen species (ROS) that are generated as a natural by-product of metabolic processes during periods of thermal stress (Alves de Almeida et al., 2007; Lesser, 2006).

Our study focuses on these responses after acute exposure to predation risk, temperature, and feeding history in order to better understand how mussels cope with rapid environmental changes (e.g. daily low tides or the onset of a heatwave) and how instantaneous responses might entrain different energetic pathways and strategies. For example, it is currently unclear whether metabolic processes (e.g. enzymatic activity) respond instantaneously to environmental changes or if there is a time lag between whole organismal and biochemical responses. We hypothesize that under baseline conditions, temperature and feeding history will affect physiological and biochemical pathways in a relatively predictable manner (see discussion of experimental metrics above), although the exact response time for these metrics is less well established. After the addition of predation risk, we expect the responses of all metrics to be less predictable and anticipate non-additive and complex interactions between stressors across response metrics. Examining the similarities and differences in response across metrics, will enable us to better understand the short-term challenges and metabolic trade-offs that organisms make when launching an coordinated stress response.

2. Materials and methods

2.1. Experimental design

To test how temperature, feeding history and predation risk interact to drive physiological and metabolic changes in the mussel, *M. edulis*, we conducted a fully-factorial laboratory mesocosm experiment with four levels of submerged body temperature (17, 25.5, 28.5, 31.5 ± 1 °C), two conditions of food history (fed and starved), and two levels of predation risk (presence or absence of water-borne risk cues from *Nucella lapillus*). The resulting 16 treatment combinations were each replicated 3–8 times (Appendix A Table 1) with some replicates lost due to deviations in temperature outside experimental conditions or electronic interference in heart rate measurements (see below). Temperatures used in the experiment were ecologically relevant to the site of collection including extreme temperatures found in tide pools (12–26 °C, Appendix B Fig. 1), and water-retaining crevices and depressions where mussels are often found (> 31.5 °C).

We collected mussels (n = 200, 40–50 mm in shell length with 46.44 ± 2.88 mm [mean ± SE]) from the low intertidal zone on a wave-exposed shore (Bathing Beach in Nahant, Massachusetts, USA (42°25′00.0”N, 70°54′23.4”W) in late June 2017. All invertebrates used in the experiment were collected from private property at Northeastern University with permission from the University in accordance with state regulations. Mussels were then cleaned, tagged and allowed to acclimate in flow through seawater tables at Northeastern University's Marine Science Center in Nahant, Massachusetts, USA for 25–30 days in flowing seawater at the ambient water temperature at the time of collection, 15–17 °C. Previous studies examining starvation in *M. edulis* have utilized a similar time period for starvation (Bayne, 1973; Bayne and Scullard, 1977; Widdows, 1973). A stepdown system was used to achieve 0.35 μm filtration to remove diatoms, unicellular algae and particulate organics for the starved mussels (n = 100), and water supplied to the fed mussels (n = 100) was filtered coarsely at 100 μm

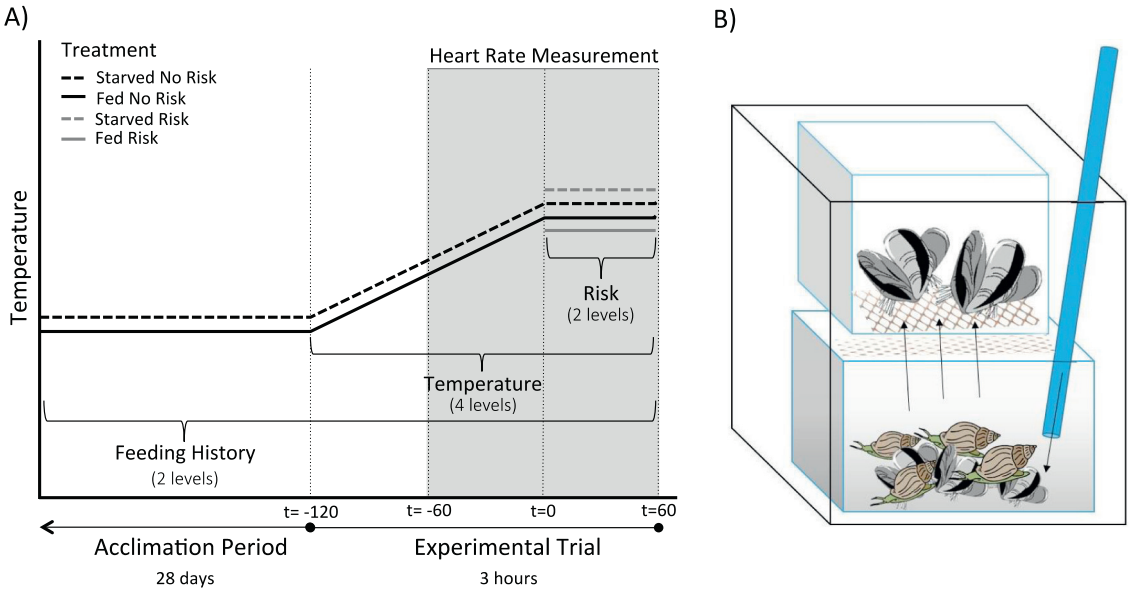

Fig. 1. (A) Timeline for a sample treatment combination from one experimental temperature showing the introduction of the feeding history treatment—Fed (solid lines) and Starved (dashed lines) during the acclimation period at 16 °C. Fed and starved mussels were held at the same temperatures despite distance between lines. Following acclimation, mussels were grouped into four temperature treatments and ramped to the target temperature for 2 h ($t = -120$ to $t = 0$). To manipulate risk, predators (gray) and no predator controls (black) were added at $t = 0$. Two sets of mussels were sacrifed at $t = 0$ (mussels 1–100) and $t = 60$ (mussels 101–200) for enzyme assays, while heart rate was continuously montored ($t = -60$ to $t = 60$) on mussels 101–200. (B) A sample schematic of mesocosm set-up, with seawater flowing down the blue hose, across predator cue and up to the experimental mussels, before flowing out of a spigot. Top container held two *M. edulis* and a temperature logger. Bottom container held either 25 *N. lapillus* and 30 juvenile *M. edulis* mussels (risk) or juvenile mussels alone (absence of risk). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

allowing them to feed continuously. To ensure that the fed mussels were satiated, we supplemented their diet with live *Isochrysis galbana* fed by moving the mussels to separate aerated baths for 2 h (20,000 cells/ml) every 1–2 days. During these time periods, the starved mussels were also relocated to control for movement and facilitate cleaning of the holding containers.

After the acclimation period, experimental trials manipulating predation risk and temperature were conducted on fed and starved mussels over the course of 1 week (Fig. 1A). We staggered replicates of each treatment combination across the day and week to eliminate potential time of day and time in laboratory biases, and included the experimental trial as a block in statistical models when appropriate. At the start of the experimental trials, two mussels with the same feeding history were transferred to an open mesh basket (10 × 10 × 7 cm) in experimental mesocosms (14 × 14 × 16 cm) filled with 0.35 μm filtered seawater at 16 ± 1 °C. Each mesocosm also contained another plastic chamber (10 × 10 × 7 cm) that sat upstream (Fig. 1B) of the experimental mussel basket and contained either 25 *N. lapillus* (2–3 cm shell length) and 30 mussels (1–2 cm shell length) for food (presence of risk) or 30 mussels alone (absence of risk). Filtered seawater flowed into the *N. lapillus* predator chamber in each mesocosm, picking up *N. lapillus* risk cues that then flowed into the basket containing the experimental mussels. These experimental units were then semi-submerged in larger water baths (64 × 45 × 36 cm) such that each bath held one replicate of the four feeding x predation risk treatment combinations ($n = 4$ mesocosms per bath per trial). Each bath was maintained using a combination of aquarium heaters (50 W and 100 W), chillers (Coralife, Franklin, WI), and a submersible pump to target one of four temperatures: a control temperature (17 °C) and three increasingly stressful temperatures (25.5 °C, 28.5 °C, and 31.5 °C) based on initial breakpoint trials (Appendix A Table 2) Although we tried to target specific temperatures, the flow through system for temperature control created some variation in the thermal treatments with the control treatment at 16–18 °C instead of 15–17 °C. Tidbit temperature data loggers (Onset Computer Corporation, Bourne, MA) were used to continuously record water temperature in each mesocosm for the duration of the experiment (Appendix B Fig. 2). The final temperature traces were used to assign individual replicates to the target temperature bins. Mussels experienced a 2-h ramping period to the target temperature, mirroring changes in temperature at low tides, at which time the predator cues were introduced. Temperatures were then held constant at the target temperature after the predator addition (Appendix B Fig. 1).

We destructively sampled mussels at two time points during the experiment: 1) immediately following the 2-h ramp period and before the predator addition ($t = 0$) and 2) 30 min after predator addition ($t = 60$). At each time point, we opened one mussel from each mesocosm and dissected the adductor muscle, which was immediately frozen individually in liquid nitrogen and stored at −80 °C until biochemical analyses were conducted. Because of the destructive nature of these dissections, different mussels were used at each time point. Mussel adductor tissue is the main muscular system that functions in valve closure and plays a critical role in feeding abilities, defense against predation, and aerial emergence response for bivalves (Freeman, 2007; Livingstone, 1978), making it the most appropriate tissue for identifying metabolic shifts in *M. edulis*. At the conclusion of the multi-factorial experiment, an additional test was conducted using additional mussels from the same collection site acclimated under fed conditions to determine if circadian rhythms could impact measured biochemical responses (see Appendix C.1).

2.2. Heart rate

The mussels used for heart rate measurements were the same mussels that were sacrificed for $t = 60$ biochemical analyses. Mussel heart rates were measured continuously throughout the experiment using non-invasive infrared sensors (Newshift, Portugal) attached to the outer shell near the dorsal edge of the mussel for best detection of heartbeat (Burnett et al., 2013). The sensors were attached using Fun Tak mounting putty (Loctite, USA) and superglue (Loctite, USA). All

mussels were given a 12–18 h recovery period in an ambient holding tank between the application of the sensors and the start of a given experimental trial. At the start of each trial, the heart rate sensors were each connected to an amplifier (Newshift, Portugal) that transmitted the signal to a USB-powered oscilloscope (Picoscope 2000 series: Pico Technology, UK) connected to a computer. All waveforms were saved using the Picoscope software at a sampling rate of 1500 S/s and saved using a random identifier to allow for blind analysis. We visually assessed each waveform for integrity and discarded those that were unreadable due to excessive noise or sensor detachment. Usable waveforms were manually counted in 5-min intervals, taking the average beats per minute (BPM) of three continuous minutes within that period when possible. Baseline data ($t = 0$) data for heart rate was calculated as the average heart rate during the last hour of ramping ($HB_{baseline}$). Changes in heart rate were calculated for each mussel by subtracting the baseline average heart rate for that individual from the average individual heart rate for the hour after predators were added at $t = 0$ (HR_{after}) for each mussel.

2.3. Total enzymatic and antioxidant capacity activity

To assess aerobic capacity (Dahlhoff and Stillman, 2002), we used a similar protocol to Drake et al. (2017) and Collins et al. (2019) to measure total citrate synthase (CS) activity in the adductor muscle of each experimental mussel at $t = 0$ and $t = 60$ min (see Drake et al., 2017 and Collins et al., 2019; for details see Appendix C.1). Enzymatic activity was measured using a microplate spectrophotometer (BioTek Synergy HT, Winookski, VT) with a pathlength of 1 cm set to read 412 nm at 25 °C for 30 min using a kinetic sweep to detect the production rate of 5-thio-2-nitrobenzoic acid (TNB). The extinction coefficient for TNB was 13.6 mM^{-1} cm^{-1}. Using BioTek Gen5 software, the maximum slope of change in absorbance was calculated by subtracting the mean background rate from the mean enzymatic rate for each sample. Citrate synthase specific activity (in micromoles of oxaloacetate oxidized per minute) is expressed as international units per gram of wet mass (U g^{-1} WM). To standardize samples between different plates, a sample of mussel adductor tissue was run on every plate (in triplicate) to quantify levels of citrate synthase activity relative to the experimental sample.

Two forms of malate dehydrogenase exist in invertebrates: a mitochondrial isoform (mMDH) that functions as part of the aerobic TCA cycle and a cytosolic form (cMDH) that is important in anaerobic metabolism and maintaining cytosolic redox balance (Basaglia, 1989). It has been shown that cMDH is present in high concentrations in most invertebrate muscles (Lazou et al., 1987), and we attained similar results to Dahlhoff et al. (1991) and found that over 90% of the MDH in adductor muscle was the cytosolic form (Appendix C.2). To assess anaerobic capacity, we measured total cytosolic malate dehydrogenase activity levels in the adductor muscle of *M. edulis*. The same protein homogenates, dilutions and volumes were used as above with the procedure being similar except the MDH assay buffer differed in its composition (200 mM imidazole pH 7.15, 0.2 mM NADH) and 0.2 mM oxaloacetate was quickly added to the 96-well plate. Background and enzymatic activity were measured in the same way as above except at a wavelength of 340 nm using a kinetic sweep to measure the rate of NADH oxidation, which had an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The change in absorbance and specific activity (U g^{-1} WM) calculations for cMDH were conducted similarly to those for citrate synthase (see above).

Antioxidant capacity is a measure of the cellular defense capabilities and comprises a full suite of components, including vitamins, proteins, lipids, etc. (Costantini and Verhulst, 2009). We measured total antioxidant capacity of the adductor muscle from *M. edulis* at $t = 0$ and $t = 60$ using a commercial kit following the manufacturer's instructions (Cayman Chemical # 709001, Ann Arbor, Michigan). The plate was shaken for 5 min at room temperature and absorbance was then monitored at 750 nm using a microplate spectrophotometer (Synergy HT, BioTek, Winookski, VT). The average absorbance of the standards as a function of the final Trolox concentration (μM) was plotted for the standards curve in each run. The total antioxidant concentration of each sample was calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation: Antioxidant Capacity (mM) = [(Sample average absorbance) - (y -intercept)/Slope] x Dilution.

Baseline values for enzymatic and antioxidant data were determined as their value at $t = 0$. Because we could not take repeated measurements on individual mussels, changes in enzymatic and antioxidant responses after predator addition ($t = 60$) were calculated by taking the average value from each feeding history x temperature treatment combination at $t = 0$ and subtracting that average from the value for each individual mussel sampled at $t = 60$.

2.4. Statistical analyses

Baseline analyses ($t = 0$) before predator addition were analyzed using feeding history and temperature as fixed factors. Heart rate ($HR_{baseline}$) was analyzed using a two-way ANOVA with feeding history and temperature as fixed factors and the interaction (feeding history x temperature). For the baseline biochemical data, we ran three separate mixed-model ANOVAs for enzyme activity (total CS and cMDH) and total antioxidant levels with feeding history and temperature as fixed factors, the interaction (feeding history x temperature), and experimental block as a random factor.

After predators were added, we assessed the change in heart rate, CS, cMDH activity and total antioxidant capacity over the course of each experimental trial ($t = 0$ to $t = 60$). For the change in heart rate, we conducted a three-way ANOVA with feeding history, temperature and predation risk as fixed factors, all possible two-way interactions (feeding history x temperature, feeding history x risk, temperature x risk), and the three way interaction (feeding history x temperature x risk). Changes in CS and cMDH activity, and total antioxidant capacity were analyzed using three separate mixed-model ANOVAs with feeding history, temperature and predation risk as fixed factors, all possible two-way fixed-factor interactions (feeding history x temperature, feeding history x risk, temperature x risk), the three way fixed-factor interaction (feeding history x temperature x risk) and experimental block as a random factor.

All data were evaluated for normality and homoscedasticity. To meet the assumptions of ANOVA, all response variables at $t = 0$ were log transformed and the log of the proportional change in heart rate [log ($HR_{after}/HR_{baseline}$)] was used for the statistical tests for change in heart rate. Model residuals were analyzed for significance across blocks to determine if experimental block should be included as random factor; block was included for all biochemical models but not for the heart rate models. All linear models and assumptions tests were completed using JMP Pro 13.0 (SAS Institute Inc., Cary, NC, USA) Type III sums of squares and REML variance estimates for mixed models. Multiple comparisons on significant effects and interactions are reported using the Fisher-Hayter method to account unequal sample sizes (Hayter, 1984), and the results of other pairwise analyses are found in Appendix A Tables 3–7. Q-values for Fisher-Hayter tests were manually calculated and *p*-values for each comparison were calculated using the 'ptukey' function in R (R Core Team, 2019). For total antioxidant capacity we also ran four Least-Square means contrasts in JMP that were corrected using the Holm-Bonferroni method. Although we did not have a priori hypotheses, posterior analyses of the three way interaction involved 120 pairwise comparisons which impeded our ability to analyze the treatments involved in the interaction. Contrasts (four total) were run for each pair of Fed and Starved treatments at each temperature in the 'no risk' treatment only and are reported where significant.

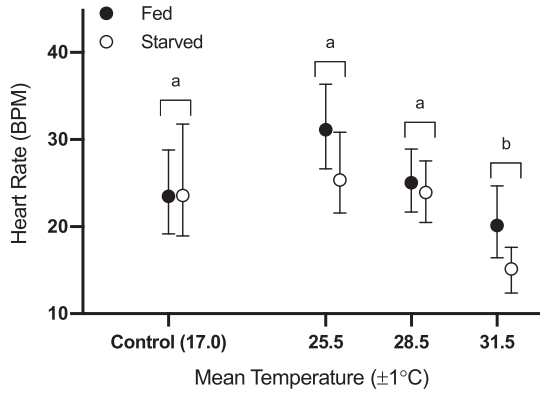

Fig. 2. Baseline: Feeding history and temperature impact initial heart rate of mussels. Initial heart rate (BPM) of *Mytilus edulis* maintained under fed (solid circles) or starved (open circles) conditions in the lab for 3 weeks (see methods) across all temperature treatments (17 °C, 25.5 °C, 28.5 °C, and 31.5 °C). Initial measurements occurred before the addition of predation risk from the snail *Nucella lapillus*. Values are back-transformed least square means ± 95% CIs from the log scale to the scale of the predictor variable. Different letters denote significant differences ($p < .05$) between temperature treatments based on Fisher-Hayter post-hoc tests. Although feeding history significantly lowered heart rate, the interaction (feeding history x temperature) was not significant and therefore not compared using FH tests. Sample sizes for each treatment combination given in Appendix A Table 1.

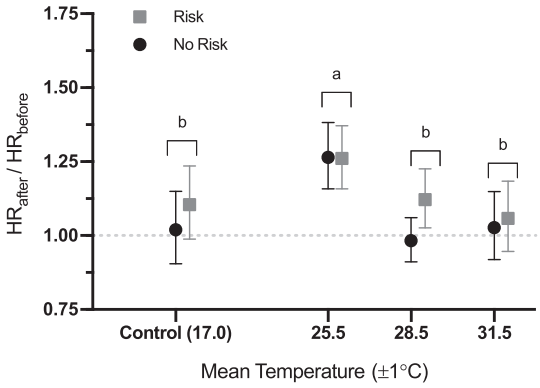

Fig. 3. After predator addition temperature drives heart rate elevation in mussels more than risk. Change in heart rate (BPM) for *Mytilus edulis* in the absence (black circles) and presence (gray squares) of predation risk from the snail *Nucella lapillus* across all temperatures (17 °C, 25.5 °C, 28.5 °C, and 31.5 °C) after the addition of risk. Analyses were run on the log of the proportional change in heart rate [$\log(HB_{before}/HB_{after})$]. Values are back-transformed least square means ± 95% CIs from the log scale to the scale of the predictor variable. Different letters denote significant differences ($p < .05$) between temperature treatments based on Fisher-Hayter post-hoc tests. The dotted line at zero indicates no change from baseline condition. Sample sizes for each treatment combination given in Appendix A Table 1.

3. Results

3.1. Heart rate

At $t = 0$, before predators were added, both temperature ($F_{3,68} = 9.109$, $p < .001$) and feeding history ($F_{1,68} = 4.181$, $p = .0448$) affected mussel heart rate (Fig. 2) and these effects were additive (temperature x feeding history: $F_{3,68} = 1.007$, $p = .395$). Mussels exposed to the highest temperature had a 29% lower heart rate than those at all other temperatures —17 °C (Fisher-Hayter: $p = .015$), 25.5 °C (Fisher-Hayter: $p < .0001$) and 28.5 °C (Fisher-Hayter: $p = .001$); no other differences were detected. On average, starved mussels had a 12% lower heartbeat rate than fed mussels across all temperatures. After exposure to predator risk cues, temperature continued to affect mussel heart rate ($F_{3,60} = 8.334$, $p = .001$; Fig. 3). Mussels exposed to 25.5 °C had 26% higher heart rates compared to baseline, the highest increase in heart rate compared to all other temperatures (Fisher-Hayter: 17 °C, $p = .003$; 28.5 °C, 0.0001; 31.5 °C, $p = .0008$). Mussels in the presence of risk increased heart rates by 14% compared to those in the absence of risk, regardless of feeding history, but this pattern was not significant ($F_{1,60} = 2.797$, $p = .099$, Fig. 3).

3.2. Citrate synthase (CS) activity

There was no significant effect of temperature ($F_{3,83.2} = 1.754$, $p = .162$) on total CS activity at $t = 0$, though feeding history showed a marginal effect ($F_{1,77.5} = 3.005$, $p = .087$) with starved mussels showing 7% lower total CS activity than fed (Appendix B Fig. 4A). However, at $t = 60$, when replicates had been exposed to a full hour of temperature treatments and predator risk cues, total CS activity changed in response to temperature ($F_{3,75.4} = 4.758$, $p = .0043$; Fig. 4). At 31.5 °C, all treatment combinations decreased total CS activity by an average of 10% relative to their baseline condition, which was significantly lower than the change in CS activity at 17 °C (Fisher-Hayter: $p < .001$), and 28.5 °C (Fisher-Hayter: $p = .002$). CS activity in mussels held at 17 °C, 25.5 °C, 28.5 °C did not differ significantly from each other (Fisher-Hayter: $p > .05$) and showed an average increase of 7% above baseline for all treatment combinations. Neither feeding history ($F_{1,69.6} = 1.708$, $p = .196$), predation risk ($F_{1,69.6} = 0.117$, $p = .733$), nor their interaction significantly affected CS activity (feeding history x predation risk: $F_{1,70.2} = 0.955$, $p = .332$).

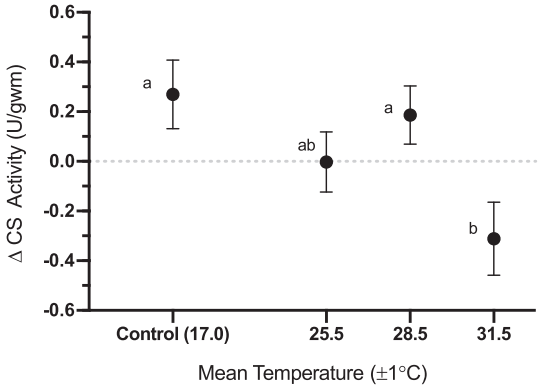

Fig. 4. Temperature impacts change in citrate synthase. Change in citrate synthase (CS) activity (U g^{-1} WM) at $t = 60$ in *Mytilus edulis* across all temperatures (17 °C, 25.5 °C, 28.5 °C, and 31.5 °C ± 1 °C) after the addition of predation risk from the snail *Nucella lapillus*. At $t = 60$ all treatments had been held at a steady temperature for 1 h. Values are mean change from baseline (see methods) ± SE. Different letters denote significant differences ($p < .05$) between temperature treatments based on Fisher-Hayter post-hoc tests. The dotted line at zero indicates no change from baseline condition. Sample sizes for each treatment combination given in Appendix A Table 1.

3.3. Cytosolic malate dehydrogenase (cMDH) activity

There was no significant effect of feeding history ($F_{1,78.2} = 0.2150$, $p = .644$) or temperature ($F_{3,82.8} = 0.563$, $p = .6410$) on total cMDH activity at $t = 0$ (Appendix B Fig. 4B). In contrast, at $t = 60$, there was a significant, antagonistic interaction between feeding history and predation risk on the change in total cMDH activity regardless of temperature (feeding history x predation risk: $F_{1,70.2} = 5.395$, $p = .0231$; Fig. 5). Fed mussels in the presence of risk and starved mussels in the absence of risk both showed a 7% increase in cMDH activity relative to baseline, while fed mussels in the absence of risk and starved mussels in the presence of risk showed a 3% decrease in total cMDH activity compared to the baseline. Temperature did not significantly affect

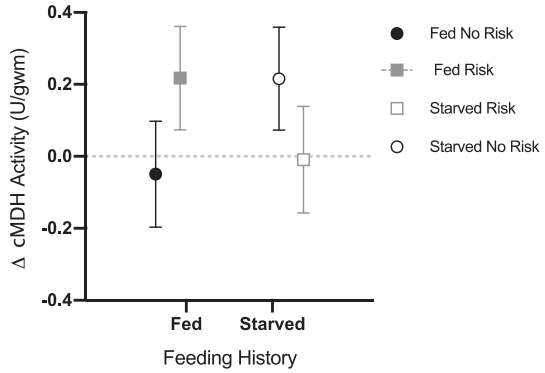

Fig. 5. Change in cytosolic malate dehydrogenase activity in response predation risk depends on feeding history in mussels. Change in cytosolic malate dehydrogenase (cMDH) activity (U g^{-1} WM) at t = 60 in *Mytilus edulis* adductor muscle from mussels in the absence (black circles) and presence (gray squares) of predation risk from the snail *Nucella lapillus* and maintained under fed (solid markers) or starved (open markers) conditions in the lab for 3 weeks (see methods). Values are mean change from baseline (see methods) ± SE. The dashed lines help visualize interaction and the dotted line at zero indicates no change from baseline condition. Sample sizes for each treatment combination given in Appendix A Table 1.

cMDH activity at t = 60 ($F_{1,75.4}$ = 1.357, p = .262), though patterns suggest that the effects of predation risk on cMDH activity tended to vary across temperature, ($F_{3,70.2}$ = 2.34, p = .079), with mussels exposed to risk showing an 11% decrease in cMDH compared to the baseline at 25.5 °C.

3.4. Total antioxidant capacity

There was no significant effect of feeding history ($F_{1,77.8}$ = 0.220, p = .640) or temperature ($F_{3,82.7}$ = 0.658, p = .580) on total antioxidant capacity at t = 0 (Appendix B Fig. 4C). At t = 60, however, there was a significant interactive effect of all three treatments on the change in total antioxidant capacity compared to baseline (temperature x feeding history x predation risk: $F_{3,71.8}$ = 3.104, p = .0145: Fig. 6A,B). Mussels in the absence of risk responded the most dynamically to temperature (Fig. 6A), while mussels in the presence of risk do not appear to change antioxidant capacity in response to temperature (Fig. 6B). However, at 25.5 °C, fed mussels in the presence of risk increased antioxidant production by 10% above baseline (Fig. 6B), while those in the absence of risk decreased antioxidant production by 20% below baseline (Fisher-Hayter: p = .47, Fig. 6A). Feeding history affected the antioxidant thermal response when risk was absent (Fig. 6A). Between 25.5 °C and 28.5 °C, fed and starved mussels in the no risk treatment showed an antagonistic response: at 25.5 °C, fed mussels decreased their antioxidant capacity by 16% from baseline and starved mussels increased antioxidant capacity by 22% (Fisher-Hayter: p = .09, LS Contrast: p = .0068, Fig. 6A). These responses switched at 28.5 °C: fed no risk mussels increased antioxidant capacity by 13% from baseline levels, significantly higher than at 25.5 °C (Fisher-Hayter: p = .18, Fig. 6A), and starved no risk mussels decreased antioxidant capacity by 7% from baseline from 25.5 °C (Fisher-Hayter: p = .64, Fig. 6A).

4. Discussion

Our study demonstrates that the short-term, coordinated response of *M. edulis* to the multiple stressors of predation risk, temperature and feeding history manifests differently in terms of physiological response, biochemical indicators of metabolic capacity, and antioxidant production. Heart rate was the most sensitive to acute changes in temperature and was initially affected by feeding history. The other biochemical indicators were less responsive to temperature and, over the course of 1 h, showed different responses to the experimental treatments: CS lagged behind the cardiac response, but after predator exposure displayed a thermal pattern that was similar to that for initial heart rate; cMDH showed no sensitivity to changes in temperature and was most influenced by feeding history and predation risk; and total antioxidant capacity displayed an interaction between all three factors (temperature, predation risk and feeding history). For some metrics, starved mussels in the absence of predation risk behaved similarly to fed mussels in the presence of risk, indicating that there might be energetic trade-offs underlying the short-term stress response of *M. edulis*.

4.1. Baseline—before predator addition

Of all the metrics analyzed in our study, heart rate was the only metric that had a baseline response to feeding history or temperature. As expected, heart rate followed a similar pattern to those described in other studies, increasing as temperature increased until it reached a breakpoint and began to decline (Bayne, 1976; Braby, 2006, Fig. 2).

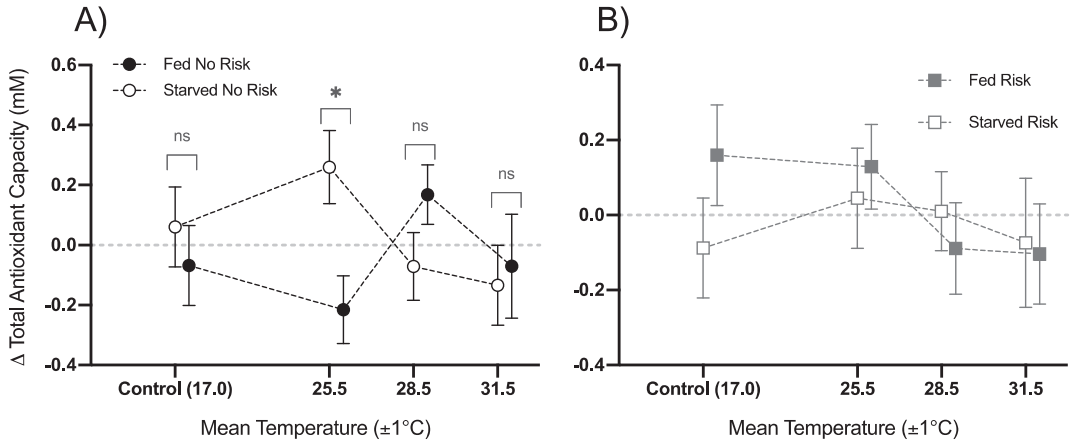

Fig. 6. Change in total antioxidant capacity in mussels depends on interaction between feeding history, temperature, and risk. Change in total antioxidant capacity (mM) across all temperatures (17 °C, 25.5 °C, 28.5 °C, and 31.5 °C ± 1) in adductor muscle from *Mytilus edulis* in A) the absence and B) presence of predation risk from the snail *Nucella lapillus* and maintained under fed (solid symbols) or starved (open symbols) conditions. Values are mean change from baseline (see methods) ± SE. The dashed lines help visualize interaction and the dotted line at zero indicates no change from baseline condition. The symbols in (A) denote which feeding history pairs (fed versus starved) are signficant for each temperature treatment based on LS Means contrasts; "ns" denotes no significant difference (p > .05) while "*" indicates significance at or below p = .002. Sample sizes for each treatment combination given in Appendix A Table 1.

Before predators were added, mussels had significantly lower heart rates in the highest temperature treatment (31.5 °C), which is at or near the lethal limit for immersed *M. edulis* (Jones et al., 2009). Feeding history also affected heart rate as expected, but only before the addition of predator risk cues. At $t = 0$, starved mussels had 12% lower heart rates than fed mussels and tended to show a slight (7%) reduction in CS levels, which may be due to suppressed metabolic rates to account for diminished food supply (Widdows, 1973). For *M. edulis*, starved mussels commonly reduce metabolic rates at or near the standard metabolic rate (SMR) to conserve energy and maintain survival at a given temperature. Once food is supplied, starved mussels often increase their metabolic rate to an "active level" (Hochachka and Somero, 2002; Thompson and Bayne, 1972; Widdows, 1973), which allows them to allocate energy to reserves, growth and reproduction.

4.2. After predator addition

4.2.1. Cardiac responses

Even after the addition of predators, heart rate remained most sensitive to temperature. The highest increase in heart rate occurred in the 25.5 °C treatment, which is around the optimum temperature for this species in water (Bayne, 1976; Braby, 2006; Widdows, 1973) and falls within the wide range of breakpoint temperatures (22–31 °C) for mussels in this population (Appendix A Table 2). One hour after predators were added the effect of feeding history on heart rate was no longer evident, but mussel heart rate did tend to to increase in the presence of risk (Fig. 3). This pattern appears relatively constant across temperatures, and is consistent with patterns described by Rovero et al. (1999) who demonstrated that while mussels exposed to *Nucella* cues start to increase heart rates after 1 h, the most significant increases tend to come after exposures of 2 h or more. The delay in this reponse is likely due to the nervous decoupling of heart rate from respiration and ventiliation rates—indicative of whole organism metabolism—so changes in heart rate as a result of altered metabolic activity can take time to manifest (Bayne, 1976). For changes in heart rate to occur there must be a substantial change in metabolic rates (Bayne, 1976) which can be triggered by changes in environmental conditions, such as an influx of food (Thompson and Bayne, 1972; Widdows, 1973) or the addition of predator cues (Steiner and Van Buskirk, 2009). It is possible that in the presence of risk, mussel heart rates are starting to change as a result of a switch in metabolism to a "threat response" though this response is limited in other sessile species (Dodd et al., 2017).

4.2.2. Biochemical responses in energy metabolism

Our study found temperature as the main driver in aerobic capacity, as reflected by the production of citrate synthase activity, suggesting that predation risk is not likely a modulator of the cellular stress response within the 1-h timeframe of our study. It is not surprising to see aerobic capacity increase as the demand for ATP generation increases (Hochachka and Somero, 2002) given that mussels experience similar temperatures in the field (Appendix B Fig. 1). Total CS activity may decrease at particularly high temperatures (Fig. 3) because mussels may be producing other enzymes with similar properties that can help offset temperature-related reductions in catalytic efficiency (Clarke, 1998; Lesser and Kruse, 2004) that can reduce mussel performance. These results further support previous work, which found that gastropods and other mollusks under temperature stress increase metabolic enzymes production until heat denaturation occurs (Morley et al., 2009; Sokolova and Pörtner, 2001). Temperature may be the leading driver of metabolic depression as an energy-saving strategy to handle acute stress events (Bannister, 1974; Bjelde et al., 2015). In our study, 31.5 °C appears to be a highly stressful temperature when mussels may be metabolically shutting down, matching the thermal depression we saw in heart rates (Fig. 3).

In contrast to aerobic capacity, anaerobic capacity, measured here using cytosolic malate dehydrogenase (cMDH), showed a significant antagonistic interaction between feeding history and predation risk (Fig. 5), but no thermal response. cMDH functions to maintain redox balance in the cytosol by shuttling malate to the mitochondria (Livingstone, 1978; De Zwaan et al., 1982; Lockwood and Somero, 2011), where malate is used to produce succinate via the reverse (anaerobic) TCA cycle (de Zwaan et al., 1981). In the absence of risk, starved mussels tended to have higher total cMDH activity than fed mussels, while we found the opposite pattern in the presence of risk (Fig. 5). Previous studies have shown that increased production of cMDH, indicative of increased anaerobic activity, can occur when an organism is starved (Bayne, 1973). Although we did not measure mussel valve closure or gaping, filter-feeding bivalves tend to reduce gape in the absence of food (Cordeiro et al., 2015; Riisgård and Larsen, 2014), further reducing oxygen consumption (Famme, 1980) as a means to save energy during stressful periods of starvation (Riisgård and Larsen, 2014). The increase we saw in cMDH indicates that both starved mussels in the absence of risk and fed mussels in the presence of risk might be increasing anaerobic capacity; it is possible that starved mussels in the absence of risk were depressing aerobic metabolism to conserve energy (Albentosa et al., 2007; Bayne, 1973; Widdows, 1973; Widdows and Bayne, 1971), a behavioral modification that can result in higher anaerobic end-product (cMDH) levels (Vial et al., 1992). Fed mussels in the presence of risk also had increased cMDH levels and may have closed their valves as a predator avoidance strategy (Robson et al., 2010; Rovero et al., 1999). Although such avoidance behavior may help thwart predators, it often impacts the organism by reducing foraging ability (Schmitz and Trussell, 2016; Smee and Weissburg, 2006). Given this potential loss in consumption, a 'clamming up response' may only occur when predation seems imminent (i.e. predators actively feeding on conspecifics Smee and Weissburg, 2006) or when energetic conditions allow for this response; starved mussels in the presence of risk did not appear to increase anaerobic capacity and were likely energetically unable to take advantage of alternate metabolic pathways. These results suggest that there may be an energetic trade-off for mussels between keeping their valves open (gaping) to maintain aerobic respiration and adopting antipredator strategies that should decrease their risk of being consumed.

4.2.3. Cellular antioxidant defense mechanisms

Exposure to thermal stress has been shown to result in oxidative stress, increased production of reactive oxygen species (ROS), and damage to cellular components (Lesser, 2006), and previous studies have found an up-regulation of antioxidant defense mechanisms in anticipation of ROS generation from environmental stress (Ivanina and Sokolova, 2016). In this study, mussel antioxidant response to thermal stress was highly dependent on both feeding history and predation risk (Fig. 6). In the presence of risk, mussels did not up-regulate antioxidant production in response to thermal stress. A similar response has been shown in insect larvae, where exposure to predation risk suppressed antioxidant production despite an increase in respiration and up-regulation of heat shock proteins (Slos and Stoks, 2008). This suppression of antioxidant production has the potential to cause oxidative stress in the presence of higher concentrations of ROS, the by-product of higher metabolic rates needed to provide ATP during stressful events like the fight or flight response displayed in mobile species (Janssens and Stoks, 2013). Only at 25.5 °C did predation risk play a small role in increasing antioxidant capacity: fed mussels in the presence of risk produced ~10% more antioxidants relative to baseline while fed mussels in the absence of risk produced 16% fewer antioxidants relative to baseline. This result suggests that fed mussels had the energetic reserves necessary to both increase antioxidant production and potentially commit resources to the production of heat shock proteins (Lesser et al., 2010) or other anti-predator metabolic responses, which were not measured in our study.

Feeding history also had a strong impact on antioxidant production in *M. edulis*, but the effects depended on temperature. Total antioxidant

capacity was similar across treatment combinations at both the lowest and highest temperatures. However, at our mid-level temperature (25.5 °C), starved mussels produced more antioxidants than fed mussels. As temperature increased, however, fed mussels began to produce more antioxidants while starved mussels produced less. Somewhat counterintuitively, increased production of cMDH (as seen here among the starved, risk-free mussels) is often coupled with increased production of antioxidants (Fields et al., 2014; Yu et al., 2016), which are not typically associated with anaerobic metabolism (but see, Abele et al., 2007; Pannunzio and Storey, 1998). Unlike other anaerobic pathways, the reverse TCA cycle relies on the oxidation of malate to produce ATP via oxidative phosphorylation. One possibility is that this increase in antioxidant production observed among starved, risk-free mussels corresponds with higher cMDH activity and could result from a need to defend against oxidative damage resulting from both increased oxidation of malate and increased mitochondrial respiration (Ulrich and Marsh, 2006), It is possible, however, that mussels are upregulating production of antioxidants in anticipation for when conditions return to normal and aerobic respiration restarts (Ivanina and Sokolova, 2016). These results suggest that starved mussels are potentially able to upregulate antioxidant production at key thermal thresholds but may not have the energetic reserves to continue production as thermal stress increases. Similar to the suppression of antioxidant production in the presence of risk, there appear to be limits on how many stressors each mussel can respond to simultaneously.

4.3. Metabolic trade-offs

The complex responses of mussels to short-term changes in temperature, feeding history, and predation risk may be due to trade-offs in their ability to respond to environmental stress and enact predator defenses based on available energetic reserves. Mussel heartrates were initially depressed in starved individuals and increased as temperatures warmed from 15 °C to 25.5 °C, but declined at both 28.5 °C and 31.5 °C. In contrast, mussel cMDH activity was not responsive to temperature, but did show an antagonistic relationship between mussels in the presence and absence of risk based on feeding history. Looking at these differences across metabolic pathways, we begin to see how multiple stress response pathways may interact to produce a complex pattern of antioxidant production where fed and starved mussels respond more dynamically to temperature when risk is absent, but show a more limited response when risk is present. Although we did not measure heat shock proteins in this study, numerous studies have shown that mussels and other invertebrates produce these metabolically costly proteins in response to thermal stress (Jones et al., 2010; Lesser et al., 2010; Tomanek and Zuzow, 2010), predation risk (Slos and Stoks, 2008) and other environmental stressors (Yu et al., 2016), and similar patterns may occur here that could have lead to energetic deficits among mussels at stressful temperatures exposed to risk and food limitation. When food is readily available, mussels are able to maintain higher metabolic costs (ATP-generation) while enabling antioxidant enzyme activity over short and long time scales (Coe and Fox, 1942; Dahlhoff and Stillman, 2002; Dowd et al., 2013; Ji et al., 2018; Widdows, 1978). In the long term, these energetic deficits could compound to produce declines in fitness associated not only with oxidative damage (Janssens and Stoks, 2013), but decreased growth and fecundity (Lesser et al., 2010). Thus, evolving a set of adaptive physiological responses, such as increasing metabolic rates (both aerobic and anaerobic) and allocation of resources to support emergency functions, may be advantageous to organisms that need to avoid predators (Hawlena and Schmitz, 2010b).

4.4. Overall conclusions

This study looked at the short-term stress response that is likely to occur in pulses during daily low-tides or initial exposure to marine heatwaves. Our results highlight the complex effects of multiple stressors, and indicate that the combination of abiotic and biotic stressors creates non-additive responses that can differ across multiple pathways and timeframes of exposure even over very short timescales. At the whole organism level, heart rate responds rapidly to acute changes in thermal stress, and, to a lesser degree, the threat of predation. On the other hand, the indicators of cellular metabolism and stress display a delayed, yet relatively fast, response to balancing metabolic concerns with predation risk; it is only at the cellular level where we see the differential effect of feeding history on the metabolic response to risk as well as the stress response across temperatures. Even under short-term periods of acute stress, an organism may not be able mount a coordinated response to all stressors simultaneously. These results provide critical insight into the physiological strategies that enable coastal organisms to withstand a broad range of environments and highlight specific conditions where both cellular damage and energetic debts might occur. Short-term responses to environmental stress can deplete energetic reserves and scale across an individual's life-span, leading to changes in individual fitness that can impact how resilient populations and communities are to environmental change.

Declaration of competing interest

None.

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Author contributions

GT, BH, and MZ conceptualized the study; JT and SD designed the experimental set-up; JT and SD performed field collections; JT, KH, SD and MZ conducted laboratory experiments; JT optimized heart rate methods and analysis; KH optimized enzyme methods and analysis; JT and KH analyzed the data; JT and KH drafted the manuscript; GT; BH; MZ; SD; JT and KH reviewed and edited the manuscript.

Submission declaration

We declare that this manuscript has not been published previously nor is it under consideration for publication in any other journal. This publication is approved by all authors listed.

Appendix A–C. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seares.2020.101891.

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