Elevated seawater temperature, not pCO₂, negatively affects post-spawning adult mussels (Mytilus edulis) under food limitation

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Pre-spawning blue mussels (Mytilus edulis) appear sensitive to elevated temperature and robust to elevated pCO₂; however, the effects of these stressors soon after investing energy into spawning remain unknown. Furthermore, while studies suggest that elevated pCO₂ affects the byssal attachment strength of Mytilus trossulus from southern latitudes, pCO₂ and temperature impacts on the byssus strength of other species at higher latitudes remain undocumented. In a 90 day laboratory experiment, we exposed post-spawning adult blue mussels (M. edulis) from Atlantic Canada to three pCO₂ levels (pCO₂ ~625, 1295 and 2440 μatm) at two different temperatures (16 °C and 22 °C) and assessed energetic reserves on Day 90, byssal attachment strength on Days 30 and 60, and condition index and mortality on Days 30, 60 and 90. Results indicated that glycogen content was negatively affected under elevated temperature, but protein, lipid, and overall energy content were unaffected. Reduced glycogen content under elevated temperature was associated with reduced condition index, reduced byssal thread attachment strength, and increased mortality; elevated pCO₂ had no effects. Overall, these results suggest that the glycogen reserves of post-spawning adult M. edulis are sensitive to elevated temperature, and can result in reduced health and byssal attachment strength, leading to increased mortality. These results are similar to those reported for pre-spawning mussels and suggest that post-spawning blue mussels are tolerant to elevated pCO₂ and sensitive to elevated temperature. In contrast to previous studies, however, elevated pCO₂ did not affect byssus strength, suggesting that negative effects of elevated pCO₂ on byssus strength are not universal.

Key words: global change biology, marine bivalves, nutritional quality, ocean acidification, ocean warming, shellfish health

Introduction

While it is well established that increased atmospheric CO₂ can result in planetary warming, it has only recently been recognized that the absorption of this excess CO₂ by the world’s oceans is altering their chemistry in a process known as ocean acidification (OA) (Doney et al., 2009). The oceans absorb ~25–30% of the excess CO₂ put into the atmosphere by human activity. As the oceans absorb increasing amounts of CO₂, water reacts with this CO₂, inevitably resulting in an increased concentration of hydrogen ions, or a decrease in oceanic pH, as well as other changes in
the marine carbonate system (Doney et al., 2009). Since the Industrial Revolution, oceanic pH has dropped by ~0.1 units and is expected to drop another 0.2–0.3 units by the end of this century (i.e. 2100 projection; Hoegh-Guldberg et al., 2014). This increase in seawater pCO2 and associated reduction in oceanic pH has since been linked to a number of biological effects including impacts on physiology, growth and development, calcification, survival (Kroeker et al., 2010, 2013) and animal behavior (Briffa et al., 2012; Clements and Hunt, 2015). Furthermore, these biological effects could potentially have broader implications for marine biodiversity and ecosystem structure and function (Fabry et al., 2008; Widdicombe and Spicer, 2008; Nagelkerken and Munday, 2016).

Testing for true OA effects on marine organisms can be difficult because it requires multi-generational exposure to OA conditions (McElhany, 2017). However, the effects of short-term OA exposure on marine biota have been well documented over the past two decades. It has also been well established that OA is unlikely to occur in isolation, and other global marine change stressors are likely to interact with OA to elicit biological effects (Breitburg et al., 2015; Baumann, 2016). Consequently, the biological effects of OA may be alleviated or exacerbated by concurrent exposure to other stressors such as warming (Kroeker et al., 2013; Harvey et al., 2013), hypoxia (Gobler and Baumann, 2016), and salinity changes (Dickinson et al., 2012; Freitas et al., 2017). Thus, studies assessing the cumulative impacts of both OA and ocean warming (OW) have become prominent, and suggest a wide range of effects that can vary between studies, and within and across study species (Kroeker et al., 2013; Harvey et al., 2013).

While studies assessing the effects of short-term warming and acidification on physiology and behavior have been increasing (Byrne, 2011; Kroeker et al., 2013; Harvey et al., 2013; Clements and Hunt, 2015), studies assessing the effects of these stressors on the energetics (and the associated functional outcomes) of commercially important marine species remain rare. Anacloto et al. (2014) assessed the effects of warming on the protein, glycogen, and fatty acid composition of native (Ruditapes decussatus) and invasive (Ruditapes philippinarum) clams in Portugal, reporting that warming had no effect on protein and glycogen content, but altered fatty acids. Valles-Regino et al. (2015) reported negative effects of elevated temperature on lipid content and complex effects of both temperature and pCO2 on fatty acid composition. In a more recent study, Tate et al. (2017) reported on the effects of elevated pCO2 and temperature on the energetic reserves of whelks, Dicathais orbita, reporting that warming resulted in reduced protein, glycogen and lipid content. Despite these reported findings, studies assessing the effects of global marine change stressors on the energetic reserves of marine invertebrates remain few and far between. Studies of this nature are much needed, however, particularly given that relating changes in energy reserves to functional outcomes is required to better understand the ways in which global marine change can affect marine species. Moreover, given the socioeconomic and ecological importance of coastal organisms in the northwest Atlantic region (Fisheries and Oceans Canada, 2017; Statistics Canada, 2016), it is imperative to understand how future seawater conditions might affect organisms in this region (Gledhill et al., 2015).

In Atlantic Canada, the blue mussel (Mytilus edulis) is an important socioeconomic and ecological resource (Fisheries and Oceans Canada, 2003). While a number of studies report that blue mussels are robust to elevated pCO2 and sensitive to increases in seawater temperature, these studies typically focus on pre-spawning individuals. Given the substantial amount of energy required in spawning (Bayne et al., 1983b), however, the responses of these bivalves to environmental stressors may differ between pre- and post-spawning individuals. Furthermore, while a single study suggests that the byssal attachment strength of mussels (M. trossulus) is negatively impacted by elevated pCO2 (O’Donnell et al., 2013), studies testing for such effects in other species at more northern latitudes are lacking. As such, the aim of this study was to test for the interactive effects of elevated pCO2 and elevated temperature on the energetics, byssal attachment strength, and survival of post-spawning adult blue mussels (M. edulis) from Atlantic Canada.

**Methods**

**Animal collection and husbandry**

Mussels (M. edulis) ranging in size from 29.1 to 44.6 mm (60 mussel subsample of experimental animals = 37.9 mm) were collected from a M. edulis farming operation St. Peter’s Bay, Prince Edward Island (PEI), Canada (46° 25’ 55.524”N, 62° 40’ 54.732”W) on 21 June 2016; species identities were confirmed using shell characteristics based on the methods of Mallet and Carver (1995). We collected more mussels than the actual number needed for our experiments to ensure that adequate samples sizes were achieved for each metric. At time of collection, the water temperature was 15°C and salinity was 29.4. Upon collection, mussels were immediately placed in a cooler with indirect contact with ice and were transferred to the St. Andrew’s Biological station (SABS) in St. Andrews, New Brunswick, Canada on the same day (~5 h transit time). Upon arrival at SABS, the mussels were placed in a flow through holding system (with filtered natural seawater from the bay adjacent to SABS) at a water temperature of 14°C and a salinity of 30.8 where they were acclimated to these conditions for four weeks prior to the initiation of the experiment on 27 July 2016. One day after arrival at SABS, a single spawning event was observed, providing us with a unique chance to test for effects of elevated pCO2 and temperature on post-spawning individuals; while gonad condition was not directly measured, the 5-week acclimation period and subsequent experimental period fall within the timeframe in which tissue mass is depressed due to spawning (Mallet and Carver, 1995).
During this acclimation period, mussels were fed daily with 75 ml (5% body weight) of Instant Algae® Marine Microalgae Concentrates Shellfish Diet 1800 (Reed Mariculture Inc., USA; 40% Isochrysis sp., 15% Pavlova sp., 25% Tetraselmis sp. and 20% Thalassiosir weissglogii; ~52% protein, 16% lipid, 22% carbohydrate and 10% ash) over the course of 8 h (each day). While this is not an ad libitum feeding regime and restricts food availability, it is unlikely that mussels completely starved under this feeding regime (Bayne et al., 1983a).

**Experimental setup and design**

The experimental setup consisted of two header tanks delivering filtered natural seawater from St. Andrews Bay to nine individual cylindrical black polyethylene tanks (~58 cm high x 42 cm diameter, ~70 l of seawater; 18 replicate tanks in total). In each tank, the mussels sat atop a hollow plastic insert with a lattice-style surface (i.e. criss-crossed plastic bars) that raised the mussels ~20 cm above the bottom of each tank. Water flowed into each replicate tank near the top of the tank and exited the tank via an outflow pipe; the intake of the outflow pipe was located at the bottom of each tank and the spout was located near the top to ensure continuous mixing (water flow ~1 l min⁻¹; Fig. 1). In each header tank, water temperature was maintained at either 16°C or 22°C, and was then delivered to each of the nine replicate tanks for each treatment, which sat in a water bath maintained at the same experimental temperature to help stabilize the temperature in each replicate tank (Fig. 1). The nine replicate tanks for each temperature were then randomly assigned to three pCO₂ treatments via CO₂ injection: 625 μatm (ambient control; unmanipulated seawater), −1295 μatm and −2440 μatm. Experimental treatments were chosen based on conservative near-future projections (pH 7.60; ΔpH −0.2 units based on RCP8.5 projections; Hoegh-Guldberg et al., 2014) and an extreme scenario representative of the extreme-high pCO₂ conditions expected under future variability (pH 7.30; ΔpH −0.3 units based on RCP8.5 projections plus an additional 0.2 unit decrease expected due to natural variability). While the average projected increase in pCO₂ is important to consider, it is equally as important to consider the extreme values that would be observed when pCO₂ variability is taken into account, particularly given the tremendous variability already observed in nearshore coastal systems and that such extreme conditions can persist for long periods of time (Hoffmann et al., 2011; Reum et al., 2014; Wallace et al., 2014).

Eighty-five mussels were placed into each replicate tank on 27 July 2016 (total n = 1530 animals). The temperature was initially set at the same temperature of the holding tank (14°C) and was gradually increased by 1–2°C per day until the desired experimental temperature (i.e. 16°C or 22°C) was achieved (this 1–2°C per day increase was representative of field conditions in this area). On 2 August 2016, CO₂ injection to obtain desired seawater carbonate chemistry was initiated. Animals were drip fed a 5% body weight ration of Shellfish Diet 1800 (see previous section in Methods) over the course of 8–10 h each day; food availability was adjusted at each sampling period (i.e. Days 30 and 60) to account for reduced competition.

We sampled a total of 20 mussels per tank on Days 30, 60 and 90 of the experiment. On all three sampling days, a total of 10 mussels from each tank were sampled for condition index (see Methods section Condition index). On Days 30 and 60, an additional 10 mussels were sampled for byssal attachment strength (see Methods section Byssal attachment strength) and 10 additional mussels were sampled on Day 90 for energy reserve analysis (see Methods section Energetics). We also recorded mortality for each tank on all three sampling days.

**Seawater carbonate chemistry**

Seawater carbonate chemistry was maintained via CO₂ injection (with the exception of the ambient treatment in which seawater was not injected with CO₂ using the ‘pond’ setting on an IKS Aquastar system. Individual pH probes calibrated with potassium phosphate buffers (2-point calibration; pH 4 and 7) connected to the IKS system were placed in each experimental replicate tank (i.e. six tanks per temperature treatment; three replicates each of 1295 and 2440 μatm). A pH setpoint for each probe (i.e. each experimental tank) was established in the IKS system and a pH reading was taken by the system every 300 s. For a given tank, if the system detected a pH value >0.05 units above the pH setpoint, a solenoid valve connected to a CO₂ cylinder (with regulator) was triggered and a 30 s burst of CO₂ was delivered to the tank; two sets of flow meters controlled the flow of CO₂ to ensure that the burst of CO₂ did not result in overshooting of the pH setpoint. Once the 30 s burst was delivered, the solenoid was shut off. If the subsequent pH reading was still >0.05 pH units above the setpoint, a second 30 s CO₂ burst was delivered; otherwise, the solenoid remained closed and the system continued to monitor pH. Mixing and continual replenishment of seawater ensured oxygen saturation (see next paragraph).

Water samples for carbonate system parameters were taken fortnightly and samples were collected based on the protocols of Dickson et al. (2007). For each water sample, salinity, total alkalinity (Aₜ) and total inorganic carbon (TIC) were measured directly (Table 1). Salinity was measured using a standard benchtop conductivity meter, while Aₜ was measured using open cell potentiometric titration with full-curve fit Gran end-point determination (Metrohm Titrando with an 800 Dosino dosing module controlled by Tiamo 1.3 software) as per Standard Operating Procedure 3b in Dickson et al. (2007). Titrations were conducted using a custom built, fully automated sample handling system. TIC analyses were also conducted according to the guidelines of Dickson et al. (2007) (Standard Operating Procedure 2). In short, TIC analyses were conducted using a SOMMA sample processing instrument in which a calibrated volume of
Carbonate system parameters for each treatment during the 90 day experiment

| Treatment         | Measured          | Calculated          |
|-------------------|-------------------|---------------------|
|                   | Temp (°C) | Salinity (psu) | TIC (μmol kg⁻¹) | A₇ (μmol kg⁻¹) | pH_total | pCO₂ (μatm) | Ω_calcite | Ω_aragonite |
| 625 μatm/16°C     | 16.00 ± 0.00     | 31.79 ± 0.35          | 2017.26 ± 7.96 | 2154.87 ± 12.58 | 7.90 ± 0.02 | 565.64 ± 28.62 | 2.62 ± 0.12 | 1.67 ± 0.08 |
| 625 μatm/22°C     | 21.61 ± 0.07     | 31.75 ± 0.31          | 2011.25 ± 8.55 | 2155.85 ± 13.66 | 7.83 ± 0.02 | 685.46 ± 26.35 | 2.79 ± 0.11 | 1.81 ± 0.07 |
| 1295 μatm/16°C    | 16.00 ± 0.00     | 31.70 ± 0.30          | 2104.56 ± 40.52 | 2155.1 ± 13.54 | 7.63 ± 0.13 | 1160.05 ± 382.17 | 1.54 ± 0.43 | 0.99 ± 0.27 |
| 1295 μatm/22°C    | 21.60 ± 0.05     | 31.71 ± 0.31          | 2101.46 ± 44.04 | 2156.64 ± 12.94 | 7.56 ± 0.15 | 1429.38 ± 580.89 | 1.68 ± 0.54 | 1.09 ± 0.35 |
| 2440 μatm/16°C    | 16.00 ± 0.00     | 31.77 ± 0.37          | 2207.67 ± 58.60 | 2157.32 ± 12.02 | 7.30 ± 0.16 | 2619.63 ± 1063.18 | 0.77 ± 0.24 | 0.49 ± 0.16 |
| 2440 μatm/22°C    | 21.61 ± 0.05     | 31.81 ± 0.23          | 2172.04 ± 19.09 | 2155.56 ± 12.79 | 7.33 ± 0.06 | 2259.28 ± 563.97 | 0.98 ± 0.13 | 0.64 ± 0.08 |

Data are means ± SD of fortnightly water samples.

To ensure that bi-weekly sampling adequately captured the day-to-day variability in seawater pH, daily pH measurements were obtained using a YSI for the first month of the experiment (i.e. August); bi-weekly sampling was adequate in describing the daily mean and variability for each treatment (mean ± SD pH values for daily sampling were 7.78 ± 0.04, 7.52 ± 0.13 and 7.34 ± 0.15, compared to 7.86 ± 0.04, 7.60 ± 0.14 and 7.34 ± 0.16 for fortnightly sampling). During this 1 month period, dissolved oxygen was also measured using the YSI to confirm that oxygen remained saturated and did not differ between pCO₂ treatments; mean ± SD oxygen saturation for 625, 1295 and 2440 μatm, respectively, were 87.1 ± 7.7%, 86.0 ± 6.9% and 86.2 ± 6.0%.

**Condition index**

Condition index was assessed on Days 30, 60 and 90 of the experiment. On each of the three sampling days, 10 mussels from each replicate tank were collected and frozen for later analysis. Shell height, length and width (as defined by Galtsoff, 1964) were measured with digital calipers. The mussels were then opened and tissue was separated from the shell. Both tissue and shell were then dried at 70°C for a minimum of 48 h, after which the dry tissue and shell mass were weighed to the nearest 0.0001 g. The tissue was then
ashed at 500°C for 6 h and weighed again. Condition index was then calculated using the equation of Scott and Lawrence (1982)

\[
CI = \frac{(T_{dry} - T_{ash}) \times 100}{S_{dry}}
\]

where CI is condition index, \(T_{dry}\) is the dry tissue mass in grams, \(T_{ash}\) is the ash tissue weight in grams, and \(S_{dry}\) is the dry shell weight in grams.

**Byssal attachment strength**

The attachment strength of mussel byssal threads was measured for 10 mussels from each replicate tank on Days 30 and 60 of the experiment. Unforeseen technical issues with our force gauge prevented measurements on Day 90, and logistical issues during sampling on Day 60 prevented two tanks (one 16°C/ambient pCO₂ tank, and one 22°C/1295 µatm pCO₂ tank) from being sampled, resulting in a lower sample size. Byssal attachment strength (in Newtons) for mussels attached to the tank was measured using a digital force gauge (PCE-FM50, PCE Instruments) equipped with a battery clip (attached to the force gauge via a rope), which facilitated a secure hold of individual mussels without causing shell damage (Babarro and Comeau, 2014). To capture maximum force required to remove each individual mussel, the force gauge was zeroed prior to removing each mussel from the tank. Using the force gauge, mussels were manually pulled upward until they released from the tank substrate and the force on the force gauge was recorded.

**Energetic reserves**

On Day 90 of the experiment, 10 mussels from each tank were collected and immediately stored at -80°C for later dissection and analysis. On the day of dissection, animals were thawed and individual morphometrics (length, width and total weight) were recorded. The shell and flesh were then separated and weighed after the careful removal of excess water with absorbent paper. The flesh was then ground in a glass cylinder using a variable speed homogenizer (Tissue Tearor model 398, BioSpec Products); the addition of 0.5 ml distilled water was necessary to promote flesh homogenization. The homogenate of each individual was finally divided into two sub-samples, one for lipid content analysis, and another for protein and glycogen content analyses.

Lipid content, expressed in mg of lipids per gram of dry tissue, was performed on the first subsample of tissue homogenate according to the method of Bligh and Dyner (1959). This extraction method uses a mixture of dichloromethane and methanol. The quantity of lipids subsequently retained in the dichloromethane fraction (lower phase) is then transferred to a pre-weighed 2 ml amber, evaporated to dryness under nitrogen, and then re-weighed to determine the amount of total lipids.

From the second subsample of homogenate, protein and glycogen content were assayed by spectrophotometry using a microplate reader (Molecular Device, SpectraMax 190, USA). For the protein assay, we used the assay principle based on the colorimetric method of Bradford (1976) and modified this method for microplate analysis. This consisted of a simple and precise method for determining the concentration of solubilized proteins by adding an acidic dye (Protein assay Dye Reagent Concentrate, Biorad) to the protein solution and incubating for at least 5 min at 595 nm. The microplate reading was then carried out after incubation. The total glycogen assay was carried out using a commercial assay kit (Glucose assay kit, Sigma-Aldrich, USA) according to the manufacturer’s protocol and follows a suitable protocol for microplate analysis (Carr and Neff, 1984). This method evaluates the free glucose as well as the glucose released by the action of amyloglucosidase on the glycogen, which thus gives the total amount of glucose. Subtraction of total glucose and free glucose corresponds to the amount of glycogen in the individual. These values were compared to a standard curve containing known concentrations of glucose for verification.

Finally, using the lipid, protein and glycogen content, we also calculated overall energy content, allowing for the determination of energy contained in the various organic compounds that comprise an organism. Total energy content was calculated according to the conversion factors of Gnaiger (1983) using the equation

\[
E (kJ g^{-1}) = \frac{(39.5 \times C_{lip}) + (24.0 \times C_{prot}) + (17.5 \times C_{glyc})}{1000}
\]

where \(E\) is the total energy content in kJ per gram of dry tissue, \(C_{lip}\) is the lipid content in mg per gram of dry tissue, \(C_{prot}\) is the protein content in mg per gram of dry tissue and \(C_{glyc}\) is the glycogen content in mg per gram of dry tissue.

**Statistical analysis**

All statistical analyses were conducted using R version 3.3.0 (R Core Development Team, 2016) with a significance threshold of \(\alpha = 0.05\); data are reported as means ± 1 SEM. All percentage data were arcsin transformed prior to analysis according to Sokal and Rohlf (1995). For each factor including time as a predictor variable (i.e. all factors except for energetic reserve data), we built four linear mixed models. Each model tested for the independent and interactive effects of temperature (fixed factor with two levels), pCO₂ (fixed factor with three levels), and time (fixed factor with three levels), and included tank as a random factor. To account for a lack of sphericity and control for potential temporal autocorrelation, each of the four models contained a defined correlation structure: (1) no correlation structure, (2) compound symmetry correlation structure, (3) compound symmetry correlation structure with heterogenous variances and (4) first-order autoregressive correlation structure. We then
used AIC/log likelihood comparisons to determine which model best fit the data; the best model was defined as the model with the lowest AIC value. That model was then selected to test for the effects of the three fixed factors, controlling for the random effect of tank. The best model for all factors was always the model with no defined correlation structure (lowest AIC value), with the exception of lipid content, which was best fit with a first-order autoregressive correlation structure. Where applicable, pairwise differences were detected using Tukey HSD post hoc test. For energetic reserves, we tested for effects of temperature and pCO2 with linear mixed effects models incorporating tank as a random variable to control for any random effects of tank. Where it was applicable, Tukey HSD post hoc tests were conducted to determine pairwise differences between groups. Assumptions of normality and homoscedasticity were assessed visually using Q–Q plots and residual-fitted plots, respectively; data did not violate statistical assumptions.

Results

Mortality

Mortality was not affected by seawater pCO2 over the duration of the experiment, but increased over time depending on temperature. Linear mixed effects revealed a significant temperature × time interaction on mussel mortality (Table 2). In the low temperature treatment, mortality ranged from 0% to 4.70% and there was no significant difference in mortality between the three dates assessed (Fig. 2). However, in the elevated temperature treatment, mortality increased significantly over time, with mortality on each of the three days being higher than the previous assessment day (Fig. 2). In the elevated temperature treatment, mortality was 1.31 ± 0.69%, 5.62 ± 1.14% and 16.47 ± 0.98% on Days 30, 60 and 90, respectively. Tukey HSD also revealed that mortality was significantly higher in the elevated temperature treatment when compared to the control temperature on Days 30 (1.31% vs. 0.92%) and 90 (14.00% vs. 3.44%), but not Day 60 (5.62% vs. 2.61%) (difference on Day 60 was marginally non-significant; Fig. 2).

Condition index

Linear mixed effects revealed a significant interactive effect of temperature and time on mussel condition index, but no effect of seawater pCO2 or any other interactions (Table 2). Tukey HSD showed that mussel condition index decreased over time and under elevated temperature, but that differences between the two temperature treatments were only apparent at Days 60 and 90, and not Day 30 (Fig. 3a). Furthermore, at both temperatures, condition index was significantly reduced at Days 60 and 90 when compared to Day 30, while Days 60 and 90 were statistically similar (Fig. 3a).

When the individual constituents of condition index (i.e. dry tissue mass and dry shell mass) were analyzed separately, linear mixed effects modeling revealed a significant independent effect of temperature, as well as a significant interaction of pCO2 × time on mussel tissue mass (Table 2). With respect to temperature, tissue mass was significantly lower under 22°C (0.13 ± 0.003 g) than under 16°C (0.15 ± 0.003 g) (Fig. 3b). The significant pCO2 × time effect was driven by a significantly higher tissue mass under 1295 µatm on Day 30, compared to all other pCO2 × time treatments (Fig. 3c); in general, tissue mass significantly decreased over time. In contrast, neither elevated pCO2 nor elevated temperature (or any interactions) had significant effects on dry shell mass, although there was a significant effect of time whereby shell mass decreased over time (Fig. 3d; Table 2).

Byssal attachment strength

Linear mixed effects revealed significant independent effects of temperature and time on byssal attachment strength, but no effect of seawater pCO2 or any interactions (Table 2). At Day 30, the force required to remove mussels from the substrate was 5.49 ± 0.30 Newtons, compared to 3.70 ± 0.34 Newtons on Day 60. Regardless of time or pCO2, byssal attachment strength was significantly lower under elevated temperature (22°C) when compared to the control temperature (16°C), with a force of 5.56 ± 0.35 and 3.73 ± 0.28 Newtons required to remove mussels at 16°C and 22°C, respectively.

Energetic reserves

Linear mixed effects revealed a significant independent effect of temperature on glycogen content, but not protein, lipid or total energy content (Table 2). Elevated temperature resulted in significantly lower glycogen content when compared to control temperature (Fig. 4a), while protein content, lipid content and overall energy content remained similar under both temperatures (Fig. 4b–d). Under elevated temperature, average protein content was 9.92 ± 3.43 mg g−1 compared to 11.47 ± 2.60 mg g−1 under control temperatures, while glycogen content was 22.39 ± 0.86 mg g−1 and 18.08 ± 0.91 mg g−1 under control and elevated temperatures, respectively. Under elevated and control temperatures, respectively, lipid content was 118.98 ± 5.82 mg g−1 and 115.24 ± 3.43 mg g−1, and total energy content was calculated at 7.29 ± 0.25 kJ g−1 and 7.57 ± 0.16 kJ g−1. There were no significant independent or interactive effects of seawater pCO2 on any measures of energetics (Table 2).

Discussion

Our results suggest that while elevated seawater pCO2 has no effect on the energetics of blue mussels, elevated temperature does, and these effects can translate into negative functional consequences in the form of reduced byssal attachment strength and survivability. Our results suggest that elevated temperature can exert negative effects on the carbohydrate (i.e. glycogen) content of adult blue mussels. In contrast, however, lipids were statistically similar between high and low temperatures, displaying a slight increase under
Table 2: Results of linear mixed effects model analysis (with defined correlation structure) testing for independent and interactive effects of temperature, pCO₂, and time (where applicable) on mussel survival, condition index, tissue mass, shell mass, byssal attachment strength, and energetics. All models included tank as a random variable. Effects were deemed statistically significant when P-value is <0.05.

| Source of error | numDF | denDF | F-value | P-value |
|-----------------|-------|-------|---------|---------|
| **Mortality**   |       |       |         |         |
| Intercept       | 1     | 24    | 41103.3 | <0.0001 |
| Temperature     | 1     | 12    | 31.8    | 0.0001  |
| pCO₂            | 2     | 12    | 0.3     | 0.7329  |
| Time            | 2     | 24    | 180.8   | <0.0001 |
| Temperature × pCO₂ | 2 | 12   | 1.0     | 0.3951  |
| Temperature × Time | 2 | 24   | 83.1    | <0.0001 |
| pCO₂ × Time     | 4     | 24    | 0.9     | 0.4669  |
| Temperature × pCO₂ × Time | 4 | 24 | 0.9 | 0.4997 |
| **Condition index** | | | | |
| Intercept       | 1     | 508   | 4888.0  | <0.0001 |
| Temperature     | 1     | 12    | 35.5    | 0.0001  |
| pCO₂            | 2     | 12    | 0.3     | 0.7745  |
| Time            | 2     | 508   | 95.9    | <0.0001 |
| Temperature × pCO₂ | 2 | 12   | 0.1     | 0.8896  |
| Temperature × Time | 2 | 508 | 7.6 | 0.0005 |
| pCO₂ × Time     | 4     | 508   | 2.1     | 0.0818  |
| Temperature × pCO₂ × Time | 4 | 508 | 2.2 | 0.0716 |
| **Dry tissue mass** | | | | |
| Intercept       | 1     | 508   | 7305.0  | <0.0001 |
| Temperature     | 1     | 12    | 49.3    | <0.0001 |
| pCO₂            | 2     | 12    | 7.8     | 0.0068  |
| Time            | 2     | 508   | 86.5    | <0.0001 |
| Temperature × pCO₂ | 2 | 12   | 2.3     | 0.1396  |
| Temperature × Time | 2 | 508 | 2.2 | 0.1151 |
| pCO₂ × Time     | 4     | 508   | 7.6     | <0.0001 |
| Temperature × pCO₂ × Time | 4 | 508 | 1.2 | 0.3016 |
| **Dry shell mass** | | | | |
| Intercept       | 1     | 508   | 11814.9 | <0.0001 |
| Temperature     | 1     | 12    | 0.1     | 0.7235  |
| pCO₂            | 2     | 12    | 1.8     | 0.2007  |
| Time            | 2     | 508   | 11.1    | <0.0001 |
| Temperature × pCO₂ | 2 | 12   | 2.8     | 0.1038  |
| Temperature × Time | 2 | 508 | 0.3 | 0.7770 |
| pCO₂ × Time     | 4     | 508   | 0.6     | 0.6834  |
| Temperature × pCO₂ × Time | 4 | 508 | 0.9 | 0.4730 |

(Continued)
elevated temperature. This slight increase in lipid content appears to have been enough to offset the effects of elevated temperature on total energy content, as temperature had no effect on the overall energy content. Although overall energy content was unaffected, mussel condition index and byssal attachment strength were both negatively affected under elevated temperature. With respect to condition index, we observed a decrease in dry tissue weight under elevated temperature, but no difference in dry shell weight – a likely result of increased mussel metabolism at higher temperatures. This reduction in tissue mass as a result of elevated temperature was associated with increased mussel mortality, as mussels in elevated temperature treatments experienced significantly higher mortality than those under control temperatures.

**Effects on condition index**

The reduced condition index and carbohydrate content under the higher temperature treatment is consistent with previous studies assessing the effects of elevated pCO2 and temperature on the energetics of marine molluscs. For example, Tate *et al.* (2017) found that elevated temperature reduced both protein and glycogen content in predatory whelks, *D. orbita*. Reductions in condition index in adult
M. edulis have also been reported in previous studies in relation to elevated temperature and pCO$_2$, with elevated temperature having a more profound effect than pCO$_2$ (e.g. Mackenzie et al., 2014); a likely consequence of increased metabolism. Ultimately, our results are consistent with previous literature on the energetic content and health of marine molluscs under conditions of marine global change, and provide further evidence that warming, more so than acidification, can exert negative effects on adult blue mussels.

**Effects on energetics**

We observed significant decreases in glycogen, as well as condition index in response to elevated temperature. Additionally, a significant pCO$_2$ × time effect on tissue mass was detected, whereby tissue mass was significantly higher at 1295 μatm than at the ambient and extreme pCO$_2$ levels on Day 30 only. While it is possible that the initial exposure to 1295 μatm resulted in a temporary increase in tissue production, the
biological relevance of this result seems negligible given that the highest pCO2 treatment elicited no effect on tissue mass at Day 30, and no differences were detected on any other days; furthermore, this spike appeared to be driven by a few individuals with high tissue mass values for this treatment, suggesting that this was not a biologically significant increase. Physiologically, glycogen is reported to maintain animal condition under periods of environmental stress (Bayne et al., 1976; Patrick et al., 2006). Thus, the maintenance of biological structures and animal condition under elevated pCO2 and temperature can be directly linked to glycogen content. Interestingly, the decrease in condition index was solely attributable to tissue mass rather than shell mass. The maintenance of a calcium carbonate shell is energetically demanding due to the energetic costs of acquiring, transporting, and precipitating calcium carbonate (Palmer, 1983, 1992; Ries, 2011). Thus, because shell mass was maintained under both elevated pCO2 and elevated temperature and only glycogen was reduced by elevated temperature, it appears that, under food limitation, the mussels in our experiment allocated their carbohydrate reserves into maintaining shell at the cost of tissue maintenance, negatively affecting condition index and ultimately increasing mortality. Interestingly, this finding is consistent with anecdotal reports from mussel growers in PEI during ‘bad years’ (i.e. growers often report good shell quality but low meat yield in warm years).

The content of energetic reserves in marine bivalves are known to be affected by environmental conditions and vary seasonally (e.g. Newell and Bayne, 1980; Beninger, 1984). In our experiment, exposure to elevated temperatures reduced the carbohydrate (i.e. glycogen) content of adult M. edulis, which was associated with reduced condition index, byssal attachment strength and increased mortality. However, condition index and survival are not the only functional endpoints that can be affected by changes in energy reserves. Reduced carbohydrate content can also impact other critical biological processes such as reproduction (Racotta et al., 1998; Marshall et al., 1999; Rodríguez-Astudillo et al., 2005; Moussa et al., 2014). In contrast to glycogen, however, we did not detect any effect of pCO2 or temperature on the protein or lipid content of M. edulis. This is in contrast
to previous studies on molluscs reporting reduced protein and lipid concentrations under elevated temperatures (e.g. Valles-Regino et al., 2013; Tate et al., 2017). Lipids have long been known to play important roles in the formation of gametes (Walne, 1970; Holland, 1978; Taylor and Venn, 1979; Beninger, 1984) and as an energy reserve to be used in times of nutritional stress (Walne, 1970; Beukema and DeBruin, 1977; Beninger and Lucas, 1984). Interestingly, while carbohydrate reserves were depleted under elevated temperature (likely for shell maintenance), lipids slightly increased and maintained the overall energy reserve at a constant level under elevated temperature. As such, it appears that the mussels did not utilize their lipid reserves under periods of temperature stress, even though other energy reserves were reduced. While unclear, it may have been that, despite reduced health and increased mortality, the depletion of glycogen alone was insufficient to warrant the activation of lipid reserves, as such activation would theoretically reduce reproductive success. Thus, there may be some evolutionary threshold of energy depletion that is required to initiate the activation of lipid reserves. Given the contrasting results observed between this and other studies, such a threshold is likely species specific among marine bivalves. This aspect of energetic reserves under global change stressors warrants future research and the ecological consequences of such effects warrant attention, particularly given that ecological energy flows could become weighted more heavily toward bacterial decay (and thus eutrophication) in systems where critical filter feeders show energetic impairment.

**Effects on byssal thread attachment strength**

In our experiment, elevated temperature, but not elevated pCO₂, resulted in reduced byssal thread attachment strength. These proteinaceous byssal threads are used by mytilid mussels to attach to hard substrates, thus facilitating proper biological functioning (e.g. feeding and respiration) and protection from predators, as well as influencing population and ecosystem structure. The effects of temperature on byssus strength observed herein are in line with the results of previous studies (Lachance et al., 2008; Seguin-Heine et al., 2014). In contrast, however, our results are contrary to previous studies reporting significant negative effects of elevated pCO₂ on the byssal attachment strength of mussels, *Mytilus trossolus*, from California (O’Donnell et al., 2013). It is important to note, however, that O’Donnell et al. (2013) employed a much lower temperature (10°C) than we employed in this study (low of 16°C), and that the byssus of *M. edulis* reared at lower temperatures (i.e., below 16°C) may be affected by elevated pCO₂. Thus, at 16°C it appears that elevated pCO₂ does not affect byssal attachment strength in *M. edulis*, suggesting that the negative effects of OA on byssus strength are not universal.

Like energetic reserves, byssal attachment strength can vary seasonally and is related to external environmental conditions (Carrington, 2002; Carrington et al., 2009, 2015). With respect to byssal attachment strength, temperature and pCO₂ are suggested to affect two different aspects of byssus production, with elevated temperature reducing the production and strength of threads, and elevated pCO₂ reducing thread maturity and the adhesive capability of the adhesive plaque (Carrington et al., 2016). Given that we observed a negative effect of elevated temperature, but not elevated pCO₂, on byssal attachment strength, it appears that adult blue mussels are able to maintain thread maturity and their adhesive plaque under elevated pCO₂, but not thread strength under elevated temperature.

While our results suggest that elevated temperature can inflict negative effects on the energy reserves, body condition, byssal attachment strength, and survival of adult blue mussels, it is important to recognize that food supply was limited in our experiment. However, given the ration of food provided (5% of mussel body weight), it is unlikely that mussels were starving in our experiment (Bayne et al., 1983a). Food availability has been reported to alter the allocation of metabolic energy (Pan et al., 2015) and can affect the sensitivity of calcifying marine organisms to global marine change stressors (e.g. Thomsen et al., 2013; Ramajo et al., 2016a, b; Büscher et al., 2017). Thus, the responses observed here are not applicable to mussels reared under different food regimes and the effects of marine global change on the food availability of marine bivalves should be carefully considered when predicting species’ responses to future global change.

**Conclusions**

Ultimately, our results confirm a high degree of pCO₂ tolerance in adult *M. edulis*, but suggest that elevated temperature can impact these mussels such that condition index and byssal thread attachment strength are reduced, and mortality is increased, under high temperatures, likely resulting from temperature effects on mussel energetics (glycogen content). Consequently, ocean warming is likely to impact northwestern Atlantic mussels accordingly. Potential effects on mussel populations and associated economic implications require more research, particularly given the importance of mussels to local benthic ecosystems and commercial fisheries. Furthermore, the ecological consequences of such effects warrant attention, particularly given that ecological energy flows could become weighted more heavily toward bacterial decay in systems where critical filter feeders show energetic impairment.

**Supplementary material**

Supplementary material is available at Conservation Physiology online.

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