Ocean Warming, More than Acidification, Reduces Shell Strength in a Commercial Shellfish Species during Food Limitation

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Abstract
Ocean surface pH levels are predicted to fall by 0.3–0.4 pH units by the end of the century and are likely to coincide with an increase in sea surface temperature of 2–4 °C. The combined effect of ocean acidification and warming on the functional properties of bivalve shells is largely unknown and of growing concern as the shell provides protection from mechanical and environmental challenges. We examined the effects of near-future pH (ambient pH –0.4 pH units) and warming (ambient temperature +4 °C) on the shells of the commercially important bivalve, Mytilus edulis when fed for a limited period (4–6 h day−1). After six months exposure, warming, but not acidification, significantly reduced shell strength determined as reductions in the maximum load endured by the shells. However, acidification resulted in a reduction in shell flex before failure. Reductions in shell strength with warming could not be explained by alterations in morphology, or shell composition but were accompanied by reductions in shell surface area, and by a fall in whole-body condition index. It appears that warming has an indirect effect on shell strength by re-allocating energy from shell formation to support temperature-related increases in maintenance costs, especially as food supply was limited and the mussels were probably relying on internal energy reserves. The maintenance of shell strength despite seawater acidification suggests that biomineralisation processes are unaffected by the associated changes in CaCO3 saturation levels. We conclude that under near-future climate change conditions, ocean warming will pose a greater risk to shell integrity in M. edulis than ocean acidification when food availability is limited.


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Introduction
Ocean acidification (OA) has been reported to affect calcification processes in the shells of bivalve species by decreasing rates of calcium carbonate precipitation and/or increasing dissolution [1,2,3]. At the levels of pH reduction predicted for the end of the century, there are also many reports of the maintenance of calcification rates and shell growth despite reductions in pH and associated changes in CaCO3 saturation levels, especially in the blue mussel, Mytilus edulis (reviewed by Gazeau et al. [4]). Maintenance of shell growth, however, may compromise shell function as compensation for increases in shell dissolution under OA conditions can be energetically costly and compromise other homoeostatic functions [5] with more extensive corrosion of the shell when food supply is limited [2]. The resulting effects of OA on mechanical strength in bivalve shells is poorly understood with most studies focusing on early life stages and the development of shell formation [6,7,8]. These studies suggest that short to medium term exposure to decreases in pH of > = 0.4 pH units can thin and weaken the bivalve shell [9,10,11]. As OA is not occurring in isolation from other environmental factors, there is an increasing realisation that concomitant changes in the environment are much more relevant to the conditions experienced by marine calcifiers in their natural environment, such as those predicted to occur as a result of climate change [12–15]. For example, a decrease in ocean pH by 0.2 to 0.4 pH units by the end of the century under the IPCC IS92a CO2 emission scenario [16,17] is likely to coincide with an increase in mean sea surface temperature of 2–4 °C [16,18].

Temperature is a key environmental variable and has a significant effect on biomineralisation processes and growth in bivalve shells (reviewed by Gazeau et al. [4]). Generally, temperature has a positive linear effect on shell growth [19], and can also result in changes in mineral composition. For example, shells built of the aragonite polymorph of calcium carbonate are more
susceptible to dissolution at low temperatures [20], which may lead to a thinning of the shells [21], and could explain why shells in cold-water bivalves are characterised by an increase in the whole-shell calcite:aragonite ratio due to an increase in calcite, the less soluble but mechanically weaker polymorph of calcium carbonate [22]. More recently it has also been reported that temperature may also have an effect on shell microstructure by altering tablet width, thickness and angle spread of aragonite crystals in the nacreous structure of molluscan shells [23]. The functional significance of the observed changes, however, remains unclear [23]. In addition, the combined effects of OA and warming on the mechanical properties of the shells in adult bivalve molluscs over an extended exposure period (i.e. months) are largely unknown. Limited research on other taxa suggests that an elevation in temperature during OA is likely to have a negative synergistic effect on calcification processes with reductions in pH increasing the sensitivity of some species [13,14,24].

The effects of ocean warming and acidification on commercial shellfish species are of growing concern as molluscs and crustaceans comprise the majority of global seafood production [25]. Species such as the blue mussel Mytilus edulis represent an important regional and global resource with worldwide mussel fisheries estimated to be worth 390 million (US $US) [26]. M. edulis also provides a number of important ecosystem services including uptake and recycling of energy and nutrients (benthic-pelagic coupling), bioturbation and bioirrigation of marine sediments, sediment/shoreline stabilization and habitat formation [27,28]. The survival and health of M. edulis is dependent on its shell which performs a range of functions from providing primary defence against predators to delivering protection from physical environmental stressors. It also acts as a skeleton for muscle attachments and prevents sediment from collecting in the mantle cavity [29]. Additionally, the bivalve shell plays an important role in physiological homeostasis [30,31]. Therefore any reduction in shell thickness, mechanical strength or alterations in shell microstructure and composition could have a profound effect on survival, not only by reducing protection of the soft tissues from predators and anthropogenic activity, but by also influencing the ability of M. edulis to respond to environmental change.

The purpose of the current study was to examine the combined effects of near-future OA and warming on the morphology, shell composition and surface area of adult M. edulis shells, as well as to examine the resulting effects on their functional properties by determining mechanical strength and flexibility. Mussels were collected from a sub-tidal population not normally exposed to extreme pH fluctuations, and exposed to an elevation in seawater temperature (warming) and reduction in pH (acidification) to examine the influence of each factor on its own plus their interactive effects. The four treatments were: ambient pH at ambient temperature (ambient); ambient pH at ambient temperature +4°C (warming); reduced pH at ambient temperature (acidified); and reduced pH at ambient temperature +4°C (warming + acidified).

Seawater was pumped from the Menai Strait via an external settling tank to a central indoor sump tank (500 l) held in an air-conditioned (air temperature 12°C) aquarium. Seawater was triple-filtered and UV treated before delivery to each of four header tanks (150 l) representative of the four experimental treatments. The temperature of each header tank was kept constant through use of in-line heaters (Elecro 900 Evo Titanium Digital Aquarium Heater, Elecro Engineering Ltd., Hertfordshire, UK) or flow-through cooling units (Aqua Medic TITAN 2000, Aqua Medic Inc, Loveland, CO, USA). These circulation systems also ensured adequate mixing of the seawater. A reduction in pH was achieved by computer controlled addition of CO2 (g). A pH controller (Walchem Dual Input pH Series Controller, Walchem, Holliston, MA, USA) set to end-century pH conditions (pH 7.68) and pH of each high CO2 treatment regulated via solenoid valves. A handheld temperature-compensated HTC pH meter (Mettler Toledo SG2 SevenGO, MT Ltd., Leicester, UK) was used to measure pH and the pH controller was adjusted accordingly. The HTC pH meter was triple calibrated using Fisher Scientific buffer solutions (pH NIST = 4.01, pH NIST = 7.00, pH NIST = 10.01 at 25°C).

Seawater from each header tank was gravity-fed (flow rate ~300 ml minute⁻¹) to 20 smaller (1.5 l) mussel tanks with overflows running to waste. Eight individual mussels were haphazardly assigned to each tank per treatment. Mussels were acclimated to temperature treatments at an increase of 0.5°C day⁻¹. All tanks were cleaned three times per week and mussels were drip-fed concentrated algal feed (Instant Algae Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA; 40% Isochrysis sp., 15% Pavlova sp., 25% Tetraselmis sp., and 20% Thalassiosir weissglogii; 52% protein, 16.1% lipid, 22.0% carbohydrate and 9.9% ash) at a ration of 27 mg dry mass mussel⁻¹ day⁻¹ over a period of 4–6 hours day⁻¹.

Ethics Statement

No specific permits were required for the study, which complied with all relevant regulations. The species collected in this study is not endangered or protected.

Experimental Set-up

Adult M. edulis were exposed for six months in an aquarium-based CO2 system to represent present and near-future (i.e. 2100) pH (ambient pH and a decrease of 0.4 pH units) and temperature (ambient 12°C and an increase of 4°C). A factorial experimental design was applied in order to examine the influence of each factor on its own plus their interactive effects. The four treatments were: ambient pH at ambient temperature (ambient); ambient pH at ambient temperature +4°C (warming); reduced pH at ambient temperature (acidified); and reduced pH at ambient temperature +4°C (warming + acidified).

Materials and Methods

Animal Collection

Adult Mytilus edulis (L.) were collected subtidally from mussel beds in the Menai Strait, North Wales, UK using a mussel dredge (Deep Dock Ltd.) in May, 2011. Sub-surface (depth 3 m) seawater temperature in the Menai Strait for 2011–2012 as measured with a temperature data logger (Hobo Pendant Temperature Data Logger, Measurement Systems Ltd., Newbury, UK) is presented in Fig. 1. Mussels (640 in total with mean shell length = 50.51 ± 0.15 mm) were transported to aquaria at the School of Ocean Sciences, Bangor University where they were held in aerated seawater in large (200 litre) flow-through holding tanks (12.5 ± 0.26 SD °C, pH 0.01 ± 0.00 SD, Salinity 34 ± 1 SD psu, 12L:12D light regime) for 3 weeks prior to the start of the experiment. The mussels were drip-fed concentrated algal feed (Instant Algae Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA; 40% Isochrysis sp., 15% Pavlova sp., 25% Tetraselmis sp., and 20% Thalassiosir weissglogii; 52% protein, 16.1% lipid, 22.0% carbohydrate and 9.9% ash) at a ration of 27 mg dry mass mussel⁻¹ day⁻¹ over a period of 4–6 hours day⁻¹.
uals at the start of the experiment to represent baseline values, and
then from mussels from each treatment (n = 20) after six months
exposure to determine dry tissue mass by drying the total soft tissue
at 80°C for 72 h. The condition index (CI) of each mussel was
determined as:

$$CI = \frac{DW_f}{SL^3}$$

where $DW_f$ = mg total soft tissue dry weight, and $SL$ = shell length
(cm) after Clausen and Riisgård [32].

Seawater Parameters

For the analysis of total alkalinity and dissolved inorganic
carbon (DIC), seawater samples (60 ml) were taken in triplicate
every two weeks in air-tight glass-stoppered containers poisoned
with 0.02% mercuric chloride according to procedures outlined by
Dumousseaud et al. [33]. Temperature and salinity of each sample
were also recorded. All samples were analysed by the Carbonate
System Facility (LIMS) at National Oceanography Centre,
Southampton. Seawater samples (30 ml) were taken at the same
time from header mixing tanks, filtered (Whatman GFF 0.7 um)
and frozen (−20°C) for the determination of phosphate and
silicate concentrations at the Scottish Association for Marine
Sciences, Oban, Scotland, UK. Temperature, salinity and
carbonate parameters were entered into the CO2SYS model
[34] to determine the remaining seawater carbonate parameters
(i.e. pH, pCO$_2$, HCO$_3^-$, CO$_3^{2-}$, ΩAr, ΩCa cf Table 1 for
explanation) using the thermodynamic constants of Mehrbach
et al. [35] refitted by Dickson and Millero [36]. Table 1 outlines
the carbonate chemistry of each experimental treatment as
determined by the CO2SYS model. Additionally, seawater pH
and temperature of three randomly selected experimental tanks
were measured daily so as to ensure approximate pH and
temperature treatments were maintained over the course of the
experiment. Prior to seawater pH measurements, the electrode
was placed in seawater for approximately one hour to stabilise
the liquid junction potential of the electrode [9]. Weekly, nitrate levels
were monitored (API Liquid Nitrate Test Kits) and salinity was
measured with a refractometer (TMC V2 ATC) calibrated with
distilled water prior to use.

Shell Morphometrics

After six months of exposure, 18 mussels were removed from
each treatment group. Following the removal of all flesh, shells
were cleaned with de-ionised water and dried at room temperature
for 3 weeks. Shell weight was measured with an analytical
laboratory balance (Sartorius analytic), and shell length, width,
height and thickness (at umbo and distal margin of shell) as defined
by Seed [37] were measured with Vernier Callipers (Mitutoyo
Series 500, Mitutoyo UK Ltd, Hampshire, UK) to the nearest
0.01 mm.

Shell Strength

Shell strength was determined on the same shells used for the
morphometric measurements (n = 18 per treatment) by measuring
the maximum load that each shell valve could endure, and by
measuring shell extension properties. Individual load and exten-
sion values were calculated as the mean of the two valves for each
mussel. Maximum load refers to the highest point on the load-time
curve before failure (i.e. fracture) while extension refers to the
distance a shell will bend/flex before failure. A decrease in
extension may also indicate an increase in brittleness of the shells.
Maximum load and extension of individual shell valves were
determined using an Instron Universal Testing Machine (Model
2243; 5 kN load cell; 3-point fixture, High Wycombe, Bucks, UK)
with loading span of 50 mm and crosshead speed of 5 mm min$^{-1}$
(Illinois Tool Works Inc, IL, USA). All shell valves were placed on
the stage of the testing machine in an identical orientation (i.e.
shell length along horizontal axis, outer shell surface facing
upwards). Maximum loads were recorded at the central point of
the outer shell surface. Results were recorded and plotted using
Bluehill Software (v.2) (Illinois Tool Works Inc, IL, USA).

Shell Surface Analysis

A subsample (n = 3 per treatment) of shells from each treatment
was subsequently used to determine surface area, as well as to
further examine any structural anomalies to the surface of the

Figure 1. Sub-surface seawater temperature in the Menai Strait (Wales, UK) between October 2011 and October 2012. Temperature
was measured at 3 m below sea surface.
doi:10.1371/journal.pone.0086764.g001
Table 1. Seawater carbonate chemistry values for the four treatments.

<table>
<thead>
<tr>
<th>TRT</th>
<th>T (°C)</th>
<th>Sal (%)</th>
<th>pH_T</th>
<th>TA (µmol/kg)</th>
<th>DIC (µmol/kg)</th>
<th>pCO₂ (µatm)</th>
<th>HCO₃⁻ (µmol/kg)</th>
<th>CO₃²⁻ (µmol/kg)</th>
<th>Ωarag</th>
<th>Ωcalc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient</td>
<td>12.14 ± 0.48</td>
<td>33.58 ± 0.72</td>
<td>7.99 ± 0.05</td>
<td>2263.57 ± 15.70</td>
<td>2093.78 ± 12.69</td>
<td>465.82 ± 63.15</td>
<td>1959.02 ± 30.99</td>
<td>125.46 ± 12.67</td>
<td>1.91 ± 0.20</td>
<td>3.00 ± 0.31</td>
</tr>
<tr>
<td>Warming</td>
<td>15.84 ± 0.27</td>
<td>33.71 ± 0.69</td>
<td>7.95 ± 0.03</td>
<td>2263.75 ± 15.99</td>
<td>2087.47 ± 13.67</td>
<td>516.77 ± 40.94</td>
<td>1942.52 ± 18.28</td>
<td>131.45 ± 8.00</td>
<td>2.02 ± 0.12</td>
<td>3.15 ± 0.19</td>
</tr>
<tr>
<td>Acidified</td>
<td>12.18 ± 0.48</td>
<td>33.63 ± 0.71</td>
<td>7.65 ± 0.06</td>
<td>2264.69 ± 16.97</td>
<td>2213.62 ± 19.08</td>
<td>1087.69 ± 169.87</td>
<td>2113.12 ± 21.27</td>
<td>63.35 ± 9.57</td>
<td>0.97 ± 0.14</td>
<td>1.52 ± 0.23</td>
</tr>
<tr>
<td>Warming+Acidified</td>
<td>16.11 ± 0.28</td>
<td>33.54 ± 0.72</td>
<td>7.63 ± 0.05</td>
<td>2265.32 ± 16.50</td>
<td>2205.95 ± 15.69</td>
<td>1161.35 ± 136.86</td>
<td>2101.03 ± 17.23</td>
<td>69.07 ± 7.37</td>
<td>1.06 ± 0.11</td>
<td>1.66 ± 0.17</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of bimonthly measures taken over the six months exposure period (n = 12 for each treatment). Mussels were either exposed to: ambient pH and ambient temperature (ambient); ambient pH and elevated temperature (warming); reduced pH and ambient temperature (acidified); or reduced pH and elevated temperature (warming+acidified). TRT represents treatment, T = temperature, Sal = Salinity, pH_T = pH (total scale), TA = total alkalinity, DIC = dissolved inorganic carbon, pCO₂ = CO₂ partial pressure, HCO₃⁻ = bicarbonate, CO₃²⁻ = carbonate, Ωarag = aragonite saturation state, and Ωcalc = calcite saturation state. Measured values are: temperature, salinity, TA, and DIC. Calculated values are pH, pCO₂, HCO₃⁻, CO₃²⁻, Ωarag and Ωcalc.

doi:10.1371/journal.pone.0086764.t001

shells. These additional examinations were carried out to determine whether ocean acidification and/or warming are capable of dissolving the surface of the shells causing surface abrasions and pitting. In order to determine the surface area of a shell valve, mussel shells were dried and then broken into smaller fragments of approximately 5 mm length. To ensure representative results of the whole shell at least three subsamples consisting of a number of shell fragments (of 0.1–0.3 g total mass) per shell were analysed to determine mean values. Analysis was performed on a Gemini Surface Area Analyser (Micromerites Instrument Corporation, Georgia, USA) using liquid nitrogen as the coolant. Surface area was determined by nitrogen adsorption on to the surface of the shell fragments. The amount of gas adsorbed at a given pressure allowed for calculation of the surface area utilising the Brunauer, Emmett and Teller (B.E.T.) theory [38].

Shell Composition

X-ray diffraction was used to determine the mixture of calcium carbonate polymorphs (calcite and aragonite) in each shell. Powder samples of five shells per treatment were analysed using a Bruker AXS D8 Advance X-ray Diffractometer equipped with a superspeed PSD:Vantec-1 detector and CuKα X-ray source. Data was obtained in the 2-theta range 20 to 60°, with a step size of 0.016° and time of 0.538 seconds per step. X-ray diffraction patterns were background corrected using AXS software (Bruker Corporation, Germany). The procedure reported by Kontoyannis and Vagenas [39] was employed for quantitative analysis. This method uses a calibration line constructed from the intensities of the (104) reflection peak for calcite, (C), 2-theta 29.4° and the (221) reflection peak at 2-theta 46° for aragonite, (A). Linear regression of peak intensity, (I), ratios against molar fraction, (X), ratios allowed Kontoyannis and Vagenas [39] to derive the relationship shown in the following equation, which was used to calculate the calcite(aragonite ratios of the shells studied.

\[ \frac{I_{04}^{calc}}{I_{46}^{arag}} = 3.157 \times \frac{X_c}{X_A} \]  

Data Analysis

Data was tested for normality and homogeneity of variances (Levene’s Test). If necessary, data was log-transformed to meet assumptions. The significance of the effects of temperature, or the effects of pH, or any interactions between the two factors on the parameters measured were tested by two-way ANOVA. Significant findings were followed by LSD post-hoc tests. All F statistics and p values given in parenthesis in the text represent the results of the respective ANOVAs, and all p values given on their own represent the outcome of subsequent post-hoc tests. Data are presented as means ± SE unless otherwise stated. Statistical analyses were performed using SPSS software (SPSS 14, SPSS INC, Chicago, IL, USA).

Results

Condition Index (CI)

After six months exposure to the treatments, mean CI of adult mussels was significantly affected by temperature (F = 11.869, p = 0.001) but not by pH (F = 0.014, p = 0.906) (Fig. 2). Interaction between the two factors was marginally significant (F = 3.644, p = 0.008). Warming at 4°C resulted in a highly significant reduction in CI from 4.1 ± 0.19 to 2.9 ± 0.17 in mussels held at ambient pH (p<0.001). After six months, CI was significantly lower than the baseline value of 6.44 ± 0.25 (n = 20) for all treatments (p<0.001).

Shell Morphometrics

After six months exposure temperature had a significant effect on shell thickness (at distal edge), but had no effect on total shell dry weight, length, width, height, and thickness at the umbo (Tables 2 and 3). Mussels held at decreased pH had significantly thicker shells at the distal edge at elevated compared with ambient temperatures (p = 0.008). Seawater pH had no effect of any of the morphological parameters but there was a significant interaction between pH and temperature with regards to shell height (Table 3). Mussels held at ambient pH and elevated temperature (warming) had significantly shorter shell heights than those at elevated temperature and decreased pH (warming+acidified) (p = 0.007) and those at ambient pH and ambient temperature (ambient) (p = 0.018) (Table 2).

Shell Strength

Two-way ANOVA showed that temperature, but not pH, had a significant effect on the maximum load endured by the mussel shells (F = 31.624, p<0.001 and F = 1.131, p = 0.289, respectively), although there was a near-significant interaction between the two
factors (F = 3.590, p = 0.060). Shells from mussels held in ambient conditions (ambient) had significantly higher maximum load values than shells from all other treatments (warming, acidified, warming + acidified) (p < 0.001, p = 0.040 and p < 0.001, respectively). Likewise, those mussels kept at ambient temperature and reduced pH (acidified) had significantly higher maximum loads than those kept at higher temperature (warming + acidified; warming (p = 0.010 and p = 0.002) (Fig. 3A).

Seawater pH had a significant effect on shell extension values (F = 12.649, p = 0.001), but not temperature (F = 1.113, p = 0.293), and there was a significant interaction between the two factors (F = 6.439, p = 0.012). Shell extension values were significantly lower in mussels held at elevated temperature and decreased pH (acidified) when compared with mussels in all other treatments (ambient, warming, acidified) (p = 0.001, p < 0.001 and p = 0.013, respectively) (Fig. 3B).

Shell Surface Analysis
Changes in surface areas of the shells under the various treatments are given in Fig. 4. Two-way ANOVA showed a significant effect of temperature on shell surface area (F = 10.355, p = 0.003) but neither pH nor the interaction of pH and temperature were significant (F = 2.045, p = 0.162 and F = 0.582, p = 0.451, respectively). The largest mean surface area of the shells at 0.478 ± 0.085 m² g⁻¹ was observed in control mussels kept under ambient conditions. The smallest surface areas were obtained in shells from mussels exposed to the combined effects of elevated temperature and low pH (warming + acidified) but these differences were only significant when compared with mussels held at ambient conditions (p = 0.002). At ambient pH, elevated temperature decreased shell surface area by 50%, which was a significant change (p = 0.008).

Shell Composition
The signal-to-noise ratio on the X-ray diffraction patterns indicated that all samples contained a significant amount of amorphous material, however, it was not possible to quantify this component. Calculations were not carried out for samples where the signal-to-noise ratio was very low, i.e. signal strength was not significantly greater than the background noise. Calcite:aragonite molar fractions are given in Table 2 where n = 4, except for under acidified conditions where only two values were obtained. Two-

Table 2. Shell morphometrics and shell composition.

<table>
<thead>
<tr>
<th>RESPONSE</th>
<th>UNITS</th>
<th>TREATMENT</th>
<th>Ambient</th>
<th>Warming</th>
<th>Acidified</th>
<th>Warming+Acidified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shell Dry Weight</td>
<td>g</td>
<td>3.18(± 0.17)</td>
<td>2.97(± 0.12)</td>
<td>3.07(± 0.15)</td>
<td>3.09(± 0.16)</td>
<td></td>
</tr>
<tr>
<td>Shell Length</td>
<td>mm</td>
<td>49.32(± 0.85)</td>
<td>49.71(± 0.55)</td>
<td>49.43(± 0.62)</td>
<td>50.00(± 0.87)</td>
<td></td>
</tr>
<tr>
<td>Shell Width</td>
<td>mm</td>
<td>23.44(± 0.34)</td>
<td>23.55(± 0.18)</td>
<td>23.55(± 0.22)</td>
<td>23.95(± 0.37)</td>
<td></td>
</tr>
<tr>
<td>Shell Height</td>
<td>mm</td>
<td>9.30(± 0.20)</td>
<td>8.87(± 0.12)</td>
<td>9.17(± 0.17)</td>
<td>9.35(± 0.22)</td>
<td></td>
</tr>
<tr>
<td>Shell Thickness_U</td>
<td>mm</td>
<td>1.40(± 0.06)</td>
<td>1.35(± 0.06)</td>
<td>1.35(± 0.05)</td>
<td>1.35(± 0.05)</td>
<td></td>
</tr>
<tr>
<td>Shell Thickness_DE</td>
<td>mm</td>
<td>1.10(± 0.04)</td>
<td>1.13(± 0.05)</td>
<td>1.06(± 0.03)</td>
<td>1.17(± 0.04)</td>
<td></td>
</tr>
<tr>
<td>Calcite:Aragonite</td>
<td></td>
<td>2.11(± 0.48)</td>
<td>1.7(± 0.15)</td>
<td>3.2, 2.9</td>
<td>2.2(± 0.39)</td>
<td></td>
</tr>
</tbody>
</table>

Mean values ± SE for shell dry weight, shell length, shell width, shell height and shell thickness (umbo and distal edge), and molar fraction of calcite:aragonite in M. edulis following a six month exposure period to four pH-temperature treatments: ambient pH and ambient temperature (ambient); ambient pH and elevated temperature (warming); reduced pH and ambient temperature (acidified); or reduced pH and elevated temperature (warming + acidified). N = 18 per treatment for the morphometric determinations, and N = 4 for shell composition, with the exception of the acidified treatment when only 2 values were recorded and both of these are given. ML: maximum load, E: extension, MF: molar fraction, U: umbo, DE: distal edge.

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Discussion

Data from the maximum loading measurements demonstrated that warming by 4°C for six months had a greater effect on shell strength in *M. edulis* when food was limiting, than a reduction in seawater pH of 0.4 pH units. Consequently, mussels held at 16°C had weaker shells than mussels held at 12°C, regardless of pH level. Shell strength in *M. edulis* has been attributed to material composition and overall shell thickness, size and shape [20]. As the morphology of the shells was generally unaffected by all treatments, the changes in shell strength with temperature observed here cannot be attributed to changes in biometrics. The reduction in shell height during warming at ambient pH was one exception, as was the increase in shell thickness. The decline in shell height during warming at ambient pH was observed here cannot be attributed to changes in biometrics. Shell height is not normally associated with shell strength, and although shell height has been shown to be an effective predictor of strength in *M. edulis* [40], the increase in thickness noted here had no effect on overall strength, and may not be representative of shell thickness in other parts of the shell margin. In addition, preliminary determinations of shell composition using X-ray diffraction of the same mussel shells used for the loading experiments, indicated that the calcite:aragonite molar fraction was unaffected by six months exposure to either warming or acidification. Consequently changes in material composition are unlikely to have been involved, although the possibility that warming affected shell strength via shifts in CaCO₃ polymorph composition requires more sophisticated investigation. Instead, the present study was able to demonstrate that reductions in shell strength with warming were accompanied by decreases in shell surface area and a fall in whole-body CI.

The reductions in shell surface area with warming recorded here represent surface area reductions of both the outer and inner nacreous layer of the shell fragments. Bivalve shell nacre possesses a deformation mechanism that permits it to exhibit inelastic strain when a force is exerted upon the shell. Inelastic strain allows the nacreous material to redistribute stress around strain concentration sites thereby improving the resistance of the shell to fracture. The level of force at which the inelastic deformation occurs is controlled by mineral asperities on the surface of the aragonite tablets which dictate sliding resistance of neighbouring laminae [41]. In the present study the reduction in shell surface area of the mussels maintained in the warming and acidified condition corresponded with a reduction in shell flexibility. A decreased surface area to volume ratio suggests more extensive shell corrosion and reduced microstructural complexity (e.g. decreased occurrence of asperities, decreased number of laminae, reduced tablet structure) and results in a reduction in the shell's ability to redistribute stress, thereby reducing material maximum load and extension capabilities.

The maintenance of shell integrity is a dynamic process that is under biological control and takes place in a small compartment, the extrapallial cavity [2,4]. Shell corrosion is reported to occur on a daily basis in mussels inhabiting the intertidal zone because they are unable to regulate increases in extracellular pCO₂, which occur at the mantle shell interface during emersion [2,42]. The resulting extracellular acidosis increases shell dissolution but shell integrity is maintained by continuous shell formation [2]. Shell formation and repair, however, are energetically expensive because of the costs associated with the accumulation, transportation and precipitation of calcium carbonate [43,44,45], as well as the costs of the processes involved in the formation of the biologically active, organic matrix [43]. Consequently, shell corrosion in *M. edulis* is related to energy budgets, even under conditions of ambient CO₂, because shell corrosion is more extensive when food, and therefore energy supply, is limiting both in the laboratory and in the field [2,46]. In the current study, mussels received 27 mg dry mass mussel⁻¹ day⁻¹ of algae but were not fed continuously, instead having their entire daily ration delivered over an approximate time span of 4–6 hours. As a result all mussels, even those held in the control treatment of ambient temperature and ambient pH, experienced a decrease in CI which generally indicates a state of lowered “health” [47] and illustrates that the mussels were receiving insufficient food. Moreover, none of the mussels showed any growth over the six month period, regardless of treatment. If restricted access to algae prevented growth and reduced condition in the mussels then exposure to warming could have further increased energy demands at a time when energy supply was limited. Similar temperature-related decreases in bivalve CI have been demonstrated in *M. edulis* and *Arctica islandica* [48,49], and also in the abalone, *Haliotis iris,* where decreases in shell strength induced by bifacial porosis and endobiont infestations were correlated with reduced CI [50]. The decline in CI observed here suggests that energy reserves were depleted and tissues reabsorbed which typically occurs in bivalves in response to stressful conditions in order to support maintenance energy requirements [51,52].

| Table 3. Two-way ANOVA results comparing the effect of pH and temperature on shell morphometrics (n = 18). |
| Source of variation | df | SS | MS | F-ratio | P-value |
| Dry Weight | | | | | |
| pH | 1 | 0.002 | 0.002 | 0.046 | 0.939 |
| Temperature | 1 | 0.361 | 0.361 | 0.862 | 0.355 |
| pH*Temperature | 1 | 0.474 | 0.474 | 1.130 | 0.290 |
| Length | | | | | |
| pH | 1 | 1.383 | 1.383 | 0.128 | 0.721 |
| Temperature | 1 | 8.119 | 8.119 | 0.753 | 0.387 |
| pH*Temperature | 1 | 0.281 | 0.281 | 0.026 | 0.872 |
| Width | | | | | |
| pH | 1 | 2.341 | 2.341 | 1.604 | 0.207 |
| Temperature | 1 | 2.298 | 2.298 | 1.575 | 0.212 |
| pH*Temperature | 1 | 0.767 | 0.767 | 0.525 | 0.470 |
| Height | | | | | |
| pH | 1 | 1.124 | 1.124 | 1.970 | 0.163 |
| Temperature | 1 | 0.515 | 0.515 | 0.903 | 0.344 |
| pH*Temperature | 1 | 3.320 | 3.320 | 5.821 | 0.017* |
| Thickness (umbone) | | | | | |
| pH | 1 | 0.023 | 0.023 | 0.449 | 0.504 |
| Temperature | 1 | 0.022 | 0.022 | 0.437 | 0.510 |
| pH*Temperature | 1 | 0.019 | 0.019 | 0.374 | 0.542 |
| Thickness (edge) | | | | | |
| pH | 1 | 0.000 | 0.000 | 0.007 | 0.933 |
| Temperature | 1 | 0.165 | 6.282 | 6.282 | 0.013* |
| pH*Temperature | 1 | 0.048 | 1.827 | 1.827 | 0.179 |

Significant differences in bold and presented by an asterisk (p<0.05). doi:10.1371/journal.pone.0086764.t003
Studies on the effects of OA on the growth or calcification rates of shells from M. edulis have demonstrated that exposure to the pH levels predicted for 2100 has no effect. Maintenance of shell formation and growth in M. edulis despite changes in seawater carbonate chemistry associated with OA has been attributed to a number of factors, such as protection against shell dissolution by the protective organic layer (periostracum), and the mineralogy of the shell [4]. In addition, the ability of M. edulis to compensate for the possible dissolution effects of reduced pH on their shells is related to their occupation of habitats characterised by fluctuating pH levels and the capacity of mussels to maintain control rates of shell growth as long as food is in ample supply [46]. The lack of any pH effect on shell morphometrics or shell strength in terms of maximum load capabilities in the present study may also be explained in terms of the cellular model for biomineralisation which maintains that shell formation in molluscs involves the formation of CaCO3 crystals in haemocytes which are deposited at the mineralisation front [53,54]. The involvement of intracellular crystal formation negates the requirement for CaCO3 supersaturation in the extracellular compartment where the shell is formed [53,55], although Roleda et al. [55] also argue that HCO3− and metabolic CO2 are more important to calcification processes than CO32− and hence CaCO3 saturation levels. Collectively these studies demonstrate that reductions in seawater pH do not have a

Figure 3. Effects of warming and/or acidification on shell strength. A) Maximum load endured until fracture and B) extension or distance a shell will bend/flex before failure of shells from M. edulis held for six months under: ambient temperature and ambient pH (ambient); ambient temperature and reduced pH (acidified); elevated temperature and ambient pH (warming); and elevated temperature and reduced pH (warming+acidified). Values given as means ± SE (n = 18 per treatment). Acidified treatments shown in dark grey. Significant differences indicated by different lowercase letters (p < 0.05).

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Figure 4. Effects of warming and/or acidification on shell surface area. Shell surface area was determined in at least three subsamples consisting of a number of shell fragments (of 0.1–0.3 g total mass) per shell from M. edulis held for six months at either: ambient temperature and ambient pH (ambient); ambient temperature and reduced pH (acidified); elevated temperature and ambient pH (warming); or elevated temperature and reduced pH (warming+acidified). Values given as means ± SE (n = 3). Acidified treatments shown in dark grey. Significant differences indicated by different lowercase letters (p < 0.05).

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direct effect on calcification processes in mussel shells, and this may help to explain the maintenance of shell strength in the present study when mussels were exposed to reduced pH for six months. However, the impact of lowered pH to extension properties of the shell which paralleled temperature effects on shell strength [4] and suggests that ocean acidification does pose some threat to the condition of the shell. Such decreases may indicate an increased brittleness of the shell brought on by acidified conditions. However the actual mechanisms behind such changes in brittleness require further investigation.

The reduction in shell strength observed here in M. edulis is likely to influence survival given the critical role of the shells in prey selection. Predator species have demonstrated an aptitude for testing shell strength and using spot weaknesses to gain access to their food source [56]. Nagarajan et al. [21] also demonstrated that predators tend to attack weaker, more thinly-shelled mussels. Therefore, any weakening of shells under future conditions of ocean warming and acidification suggests that M. edulis may become increasingly vulnerable to predation. Although Mytilus have demonstrated an ability to increase shell thickness and mass, and consequently strength, in high predation sites [57], such compensatory adjustments may not be possible under future warming conditions because of the allocation of energy for temperature-related increases in maintenance requirements. The energy required for compensatory adjustments in shell thickness and mass may be further restricted by any associated declines in food supply that may occur as a result of warming. The observed reductions in soft tissue mass with temperature are concerning because this implies reduced aerobic scope of M. edulis and a decline in reserves for ecologically important activities such as growth, fecundity and annual recruitment [31].

In conclusion, it appears that under near-future climate change conditions when food supply is limited, ocean warming will pose a greater risk to shell integrity in the commercial species M. edulis, than OA. Whereas food limitation reduced body condition in all mussels, warming by 4 °C had a detrimental effect on shell strength which was associated with a reduction in shell surface area. It appears that warming has an indirect effect on shell strength via the re-allocation of energy resources away from costly biomineralisation processes in order to support higher maintenance costs, which may be exacerbated when daily food availability is limited [2,50]. Seawater acidification had no effect on shell strength but did affect shell flexibility suggesting that shell integrity was mostly maintained in M. edulis shells despite reductions in CaCO3 saturation. When food is in short supply, M. edulis may be at greater risk in its natural environment under future climate change conditions as the shells may not offer sufficient protection of the soft tissues from predators and the rigors of industrial harvesting.

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Author Contributions

Conceived and designed the experiments: CLM GAO NMW SKM. Performed the experiments: CLM GAO SFC RJB. Analyzed the data: CLM SFC RJB. Contributed reagents/materials/analysis tools: GAO SFC RJB. Wrote the paper: CLM NMW. Maintenance of the animals: CLM NMW.

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