

Effects of extra feeding combined with ocean acidification and increased temperature on the carbon isotope values ($\delta^{13}\text{C}$) in the mussel shell

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ABSTRACT

Ocean acidification (OA) and global warming present future challenges for shell producing organisms such as mussels through reduction in the carbonate available to produce shells in these and other valuable aquaculture species. Molluscs control their shell growth through biomineralisation, but the response of the mechanisms behind biomineralisation to OA conditions are relatively unknown. It is unclear how much carbon is taken into the shell from the environment compared to the uptake through the food source. Shell production is energetically costly to molluscs and metabolic processes and energetic partitioning may affect their ability to perform the underlying mechanisms of biomineralisation under OA. It is possible that additional food consumption might alleviate some impacts caused by acidification. We assessed the ability of extra feeding to alter the impacts of OA and increased temperatures on adult *Mytilus edulis*. Carbon isotopes ($\delta^{13}\text{C}$) were used to examine the change in biomineralisation pathway in mussels. OA did not alter the $\delta^{13}\text{C}$ directly in separate analyses of the shell calcite and aragonite layers, mantle tissue and extrapallial fluid. However, ambient treatments with increased temperatures altered the mussel biomineralisation pathway in the shell calcite using CO_3^{2-} instead of HCO_3^- as the main source of carbon. The proportion of metabolic carbon uptake into the mussel shell calcite layer increased under OA, with additive effects when exposed to increased temperatures and extra feeding. The proportion of metabolic carbon uptake is higher (7%–11%) in the shell aragonite layer compared to calcite, under ambient treatments. OA initially reduced the metabolic carbon uptake into the shell aragonite, but after a period of 4-months with extra feeding, the mussels were able to adjust their metabolic carbon uptake to a level experienced under ambient treatments. This indicates that an abundance of food resources may enable changes in mussel biomineralisation pathways to compensate for any decrease in seawater inorganic carbon associated with OA. The impact of OA on phytoplankton varies from species to species, changing the structure of the community which could provide sufficient food resources to maintain metabolic carbon uptake for mussel shell growth. This study of $\delta^{13}\text{C}$ isotopic values has identified changes in biomineralisation pathway relating to the mussel metabolic carbon uptake from their food source, with varying results for the aragonite and calcite shell polymorphs. The implications of these findings suggest that some bivalve species with different shell composites may cope better under OA than others, demanding further study into species-specific biomineralisation pathways.

1. Introduction

Ocean acidification (OA) is caused by the absorption of anthropogenic carbon dioxide by the ocean with the ocean absorbing around 30% of the total anthropogenic carbon dioxide (Feely et al., 2004). The effect of OA includes, but is not limited to a decrease in: pH; carbonate ion concentration; and calcium carbonate mineral ion saturation state (Jiang et al., 2019). Such effects are expected to have negative impacts

on marine calcifiers, as the marine calcifiers utilise calcium carbonate from the environment to grow their shells and skeletons (Gazeau et al., 2007). The reduction in calcium and carbonate ions available in the environment may affect the calcification and growth of the shell, as marine calcifiers can build their shell using dissolved inorganic carbon (DIC) sourced as carbonate or hydrogen carbonate ions from the environment (Fitzer et al., 2019a). In molluscs calcium carbonate shells are constructed from environmental and/or metabolic sources of carbon

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(Fitzer et al., 2019; Marin et al., 2008). The molluscan shell geochemistry can therefore reflect that of the environment and is of great interest to determine biogeochemical cycling of carbon in estuarine environments (Gillikin et al., 2006). Molluscs can source CO₂ from seawater and use the highly conserved enzyme, carbonic anhydrase, to catalyse the formation of hydrogen carbonate for biomineralisation (Roleda et al., 2012). The production of mollusc shells through biomineralisation is reliant on sourcing carbon and where under OA environmental DIC may limit biomineralisation, respiratory CO₂ can provide an alternative source (Marin et al., 2008; Gillikin et al., 2006). Respiratory CO₂ enters the shell extrapallial fluid by diffusion from mantle tissue (Marin et al., 2008) and the amount of respired CO₂ entering the shell varies from species to species and in most marine calcifiers is estimated at <10% (Gillikin et al., 2006; Gillikin et al., 2007; McConnaughey et al., 1997). The respired CO₂ can be estimated using tissue carbon isotopes ($\delta^{13}\text{C}$; Gillikin et al., 2006). Determining the sources of carbon or carbon pathway in molluscan shell growth is key to understanding vulnerability of species under OA, where environmental carbon is limited (Fitzer et al., 2019a). Decreases in pH could also lead to direct erosion of the calcium carbonate structures (Harvey et al., 2018), which could weaken the shell or skeleton of the marine calcifier, reducing its protective function against predators or pathogens. In the mussel *Mytilus edulis* the thickness of the shell aragonite layer was significantly reduced in OA environments, suggesting a reduced shell strength problematic for predation (Fitzer et al., 2014, 2015a, 2015b). The breaking strength of *M. californianus* larvae cultured under increased pCO₂ conditions was reduced by 12–15% in comparison to the control group at the end of an 8 day experiment (Gaylord et al., 2011). Many studies have shown no significant difference in mussel survival rate in control and acidic conditions, although shell length and thickness have decreased (Gazeau et al., 2010; Thomsen et al., 2013).

It is suggested that OA will impact phytoplankton communities. The effect of OA on phytoplankton varies from species to species, and increases in temperature and dissolved carbon dioxide (pCO₂), can lead to an increase in the abundance of some species, changing the structure of the phytoplankton community (Collins et al., 2014; Dutkiewicz et al., 2015). Few studies have focused on changes in microalgal species as a food source in response to OA (Riebesell et al., 2000; Rossoll et al., 2012; Fitzer et al., 2019b). In microalgae it has been suggested that high pCO₂ cultures can decrease the proportion of long-chain polyunsaturated fatty acids relative to saturated fatty acids, for example in the diatom *Thalassiosira pseudonana* (Rossoll et al., 2012), and the coccolithophore *Emiliana huxleyi* (Riebesell et al., 2000). Increasing pCO₂ through OA experiments resulted in changes to the lipid production in *Isochrysis galbana*, but not in *Tetraselmis suecica*; both are important aquacultural feedstock algae (Fitzer et al., 2019b). Microalgae is the main food source of the edible mussel, and metabolic carbon (carbon derived from diet) can be used as a main source of carbon in carbonate used for shell or skeleton formation (Fitzer et al., 2019a). Increased food availability may counteract the effect of OA for marine calcifiers (Ramajo et al., 2016a). It is thought that any observed negative impact on calcification due to a decreasing environmental carbon caused by OA could be compensated by the increase in metabolic carbon uptake (Thomsen et al., 2013; Ramajo et al., 2016a, 2016b). It is even suggested that food availability plays a far bigger role than OA on growth (Thomsen et al., 2013). However, if there is a limitation in food abundance combined with OA, the negative impact is likely to be more than additive (Goethel et al., 2017).

M. edulis is a marine calcifier of great economic importance. In 2016, the volume cultured was 186,626 t globally with 77,735 t produced by wild fishery (FAO Fisheries and Aquaculture - Aquatic species, 2020). This will be the first study to test the impact of OA and/or increased temperatures combined with increased food availability on the carbon isotopic values in the *M. edulis* shell, to enable an understanding of how both environmental and metabolic carbon uptake affects biomineralisation in mussels. Here we use carbon isotopes ($\delta^{13}\text{C}$) to

examine biomineralisation pathways in both the shell calcite and aragonite layer under experimental OA and increased temperatures with increased food availability. We examine how the metabolic carbon uptake changes in the calcite layer under OA and increased temperatures, as a ratio of metabolic to environmental DIC, to better understand the mechanisms of shell formation under OA. We hypothesise that an increase in food availability under OA and warming will lead to an increase in metabolic carbon uptake altering the carbon available to the mussels for biomineralisation.

2. Materials and methods

2.1. Mussel collection and culture

Mussels (*M. edulis*) were supplied through Loch Fyne Oysters Ltd. and collected and delivered by Hebridean Mussels Ltd. from the Isle of Lewis. The mussels arrived on 21/09/2016. Mussels of 1.5 years old were placed in experimental tanks of 6 L and supplied with seawater at simulated seasonal temperature from Isle of Lewis (Fitzer et al., 2014, 2015a, 2015b, 2016). Six different conditions were created including: (i) ambient pCO₂ (400 $\mu\text{atm pCO}_2$), (ii) predicted OA at year 2100 (1000 $\mu\text{atm pCO}_2$), (iii) increased temperature combined with OA in year 2100 (1000 $\mu\text{atm pCO}_2 + 2^\circ\text{C}$), (iv) increased temperature without OA (400 $\mu\text{atm pCO}_2 + 2^\circ\text{C}$) and (v) extra feeding (EF) of 200% for ambient pCO₂ and (vi) OA (400 $\mu\text{atm pCO}_2 + \text{EF}$, 1000 $\mu\text{atm pCO}_2 + \text{EF}$).

In order to see the effect of extra feeding on mussel shell chemistry, extra feeding was provided to groups (400 $\mu\text{atm pCO}_2$ and 1000 $\mu\text{atm pCO}_2$), however, due to tank number limitation, set ups with extra feeding for combined OA and increased temperature were not possible.

Mussels were acclimated for 1 week, followed by 3 weeks of gradual acclimation to treatments, from 22/09/2016 to 17/10/2016. Before the start of the experiment the shells were stained prior to experimental culture using seawater containing the fluorescent dye calcein (150 mg L⁻¹ calcein C0875–25 g; Sigma-Aldrich, Sigma-Aldrich Company Ltd., Dorset, England, www.sigmaaldrich.com; Fitzer et al., 2014). Mussels were fed 5 mL of cultured microalgae (*Isochrysis galbana* (I.G.) and *Tetraselmis suecica* (T.S.) mixture) per tank every day for control feeding and 10 mL for extra feeding. The microalgae concentrations ranged from 7.69×10^6 cells mL⁻¹ to 9.58×10^6 cells mL⁻¹ for *I. galbana*, and from 2.88×10^5 cells mL⁻¹ to 9.88×10^5 cells mL⁻¹ for *T. suecica*.

The experiment consists of 12 experimentally treated sumps, each with four tanks containing 13 mussels in each, one replicate tank for each of the six conditions – a total of 156 mussels.

2.2. Environmental conditions

For detailed environmental set up of the experiment, please refer to previous experiments (Fitzer et al., 2014, 2015a). Experimental culture was conducted at 400 and 1000 $\mu\text{atm pCO}_2$, under seasonal temperatures and day length (light). The experimental conditions were achieved by addition of CO₂ mixed into air lines supplying all experimental tanks, gas concentrations were logged continuously using LI-COR® Li-820 CO₂ gas analysers, following established protocols (Fitzer et al., 2014, 2015a). Seawater salinity, temperature and dissolved oxygen (DO) were checked daily and recorded once a week (YSI Pro2030). Seawater samples were collected (once per month) to determine total alkalinity (AT) using standard semi-automated titration (Metrohm 848 Titrino plus), combining the spectrometric analysis using bromocresol indicator (Fitzer et al., 2014, 2015a) and dissolved inorganic carbon (DIC) using an Automated Infra Red Inorganic Carbon Analyser (AIRICA, Marianda instruments). Certified seawater references materials for oceanic CO₂ (Batch 156, Scripps Institution of Oceanography, University of California, San Diego) were used as standards to quantify the error of analysis (Measured $2186.39 \pm 4.56 \mu\text{molkg}^{-1}$, CRM value $2234.07 \pm 0.39 \mu\text{molkg}^{-1}$; Dickson et al. 2007). Seawater AT, DIC, salinity, temperature and pCO₂ are summarised in Table 1 and were used to calculate other

Table 1

Measured seawater $p\text{CO}_2$ (μatm), temperature ($^{\circ}\text{C}$), salinity (ppt) and seawater DIC ($\delta^{13}\text{C}$ VPDB) for each experimental treatments. All values expressed as means \pm standard deviation.

$p\text{CO}_2$ condition	Mean $p\text{CO}_2$ in μatm (n = 28)	Mean temperature in $^{\circ}\text{C}$ (n = 90)	Mean salinity in ppt (n = 32)	Mean Seawater DIC ($\delta^{13}\text{C}$ VPDB)
400 (tank1)	409 \pm 57	11.4 \pm 0.5	35.5 \pm 1.6	-1.98 \pm 0.38
400 (tank 2)	434 \pm 69	11.9 \pm 0.6	35.6 \pm 1.0	n/a
400 + 2 $^{\circ}\text{C}$ (tank1)	410 \pm 58	13.3 \pm 0.3	38.9 \pm 0.8	-2.61 \pm 0.76
400 + 2 $^{\circ}\text{C}$ (tank 2)	410 \pm 58	13.4 \pm 0.5	37.3 \pm 1.1	-2.45 \pm 0.22
400 + EF (tank1)	410 \pm 58	11.2 \pm 0.3	38.2 \pm 1.0	-1.13 \pm 0.55
400 + EF (tank 2)	410 \pm 58	11.4 \pm 0.4	37.4 \pm 1.1	-1.53 \pm 0.32
1000 (tank1)	1043 \pm 132	11.2 \pm 0.6	38.0 \pm 1.0	-4.09 \pm 0.44
1000 (tank 2)	984 \pm 80	11.2 \pm 0.5	37.0 \pm 1.1	-4.78 \pm 0.27
1000 + 2 $^{\circ}\text{C}$ (tank1)	984 \pm 80	13.5 \pm 0.3	38.1 \pm 1.1	-4.14 \pm 0.3
1000 + 2 $^{\circ}\text{C}$ (tank 2)	984 \pm 80	13.3 \pm 0.5	37.9 \pm 1.3	-4.91 \pm 0.78
1000 + EF (tank1)	1043 \pm 131	11.2 \pm 0.3	37.0 \pm 1.6	-4.73 \pm 0.52
1000 + EF (tank 2)	931 \pm 262	12.6 \pm 0.7	34.7 \pm 0.5	n/a

seawater parameters using CO2Sys (see supplementary Table S1).

2.3. Shell, extrapallial fluid and tissue sample collection

Samples were collected at the end of acclimation after 1 month and again at the end of the 4 months of experimental treatment.

Samples of mantle tissue, extrapallial fluid and shell carbonate samples were taken from mussels (Fig. 1). The mantle tissue has been shown to identify metabolic contributions in molluscs similarly to other tissues in both adult and juvenile molluscs (Beirne et al., 2012). The adductor muscle was cut using a scalpel blade, the two valves were opened, and a needle was inserted between the mantle and the shell into the extrapallial space. Extrapallial fluid was extracted using a syringe; approximately 500 μL collected per adult mussel valve (Fig. 1). Once extracted the extrapallial fluid was stored on ice and frozen prior to freeze-drying and homogenising into a powder for carbon isotopes analyses. Mantle tissue and extrapallial fluid samples were collected and stored frozen in Eppendorfs, freeze-dried and then homogenised into a powder using pestle and mortar. Powdered mantle tissue and extrapallial fluid (0.7–1.0 mg) was weighed into tin capsules and analysed for $\delta^{13}\text{C}$ analysis via continuous flow isotope ratio mass spectroscopy (Elementar vario-Pyrocube, Elemental Analyser interfaced with a Thermo Fisher Scientific, Delta Plus XP, Mass Spectrometer).

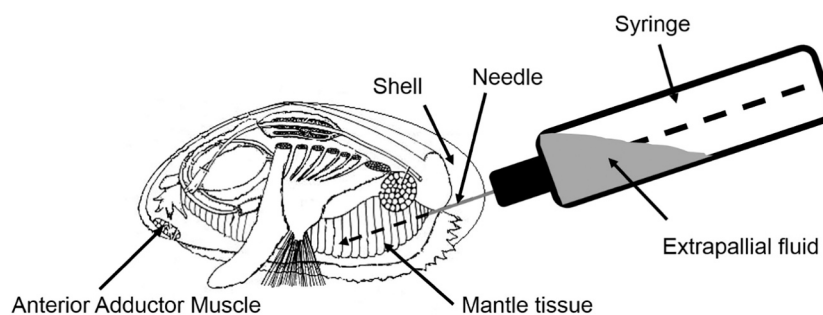


Fig. 1. Extraction of extrapallial fluid using a syringe and needle. The needle is injected between the mantle tissue and the shell, within the extrapallial space, at the edge of the valve towards the internal hinge. The needle is guided along the shell avoiding puncture of the mantle tissue. The fluid is extracted whilst tilting the valve to ensure that all seawater is removed prior to extrapallial fluid extraction.

For calcite and aragonite sample preparation, all the remaining organic tissue was removed from the shell. Then shells were rinsed in water and left to air dry at room temperature. The shells were stored in a dark room at room temperature. For inorganic DIC analyses, the shells were micro-milled to produce shell calcite and aragonite powder. At the start of the experiment mussels were stained with the fluorescent dye calcein (150 $\mu\text{g}\text{L}^{-1}$) following the methods of Fitzer et al. (2014) to determine the new shell growth during experimental OA culture using fluorescent microscopy and enable sectioning of the shell for micro-milling (Fitzer et al., 2014). 2–4 mg of powder was prepared and plasma ashed (Emitech K1050X) overnight to remove any trace of organic tissue, following methods of Fitzer et al. (2019a).

2.4. Carbon isotopes analysis protocol

Carbon isotopes ($\delta^{13}\text{C}$) analyses for organic and inorganic carbon samples were performed at the Scottish Universities Environment Research Centre (SUERC).

For organic carbon $\delta^{13}\text{C}$ determination, shell powder samples were analysed by continuous flow isotope ratio mass spectrometry (CF-IRMS) using an Elementar vario-Pyrocube Elemental Analyser interfaced with a Thermo Fisher Scientific Delta Plus XP Mass Spectrometer. Instrumental error for analysis of standards USGS40 has a standard deviation of 0.08‰ (n = 4).

Shell powder samples were prepared in triplicate (one sample per individual mussel) and were analysed using a VG Optima interfaced with an Analytical Precision AP 2003 continuous flow mass spectrometry automated carbonate system (Waldron et al., 2014) giving results as VPDB $\delta^{13}\text{C}$ (Vienna Pee Dee Belemnite) Marine Carbonate Standard obtained from a Cretaceous marine fossil, *Belemnitella americana*. Instrumental error of measurements on international standards give a standard deviation of 0.06‰ (n = 2) for NBS18 (carbonatite) and IAEA-CO8 (calcite).

For all organic and inorganic carbon isotope analyses at least 3 mussels were sampled at both 1 month and 4 months experimental acclimation. However, for the organic samples initially some of the samples were too large, saturating the detectors, and were repeated using a smaller size. For the inorganic shell carbonates, a few samples were below the detection levels not providing sufficient CO_2 peaks for detection. This problem occurred in only 2 mussels for shell calcite and aragonite, and in 3 mussels in the case of mantle and extrapallial fluid. The relevant n-values are provided for all figures.

Dissolved inorganic carbon was measured in triplicate for seawater collected in 12 ml screw capped glass vials (Exetainer®, Labco, Lampeter, UK; Waldron et al., 2014). Samples were analysed and reported as $\delta^{13}\text{C}$ VPDB using a VG Optima interfaced with an Analytical Precision (AP) isotope ratio mass spectrometer gas preparation bench (Waldron et al., 2014; Scottish Universities Environment Research Centre, SUERC).

2.5. Data analysis

A comparison of the seawater, shell, extrapallial fluid and mantle tissue across different cultures was made using a general linear model (GLM, Nelder & Wedderburn, 1972) with $p\text{CO}_2$, temperature increase, extra feeding as fixed factor and seawater dissolved inorganic carbon (DIC) and average salinity as covariance. The full GLM was used to test whether different conditions had an effect on the $\delta^{13}\text{C}$ within the mussels (see supplementary Tables S4–S11).

In addition, a one-sample t -test was used to test the carbon isotope data of the mussels to see if there was a change in biomineralisation pathway (Fitzer et al., 2019a), by setting the null hypothesis as $H_0: \mu = -1$ and alternative hypothesis as $H_1: \mu < -1$. The rejection of H_0 suggests a change in biomineralisation pathway.

All statistical analyses were done using Minitab V18 (Minitab, Inc. www.minitab.com).

Analysis of proportion of metabolic and environmental carbon uptake in the mussel calcite and aragonite layer is done with reference to the Lu et al. (2018). Carbon uptake via environmental route depends on the seawater DIC. The relative proportion of $\delta^{13}\text{C}$ contributed by metabolic carbon and seawater DIC can be calculated from the equation below which has been derived from McConnaughey et al. (1997).

$$\% \text{CM} = \frac{(\delta^{13}\text{C}_{\text{Cal}} - \epsilon_{\text{cal-b}} - \delta^{13}\text{C}_{\text{DIC}})}{(\delta^{13}\text{C}_{\text{Mantle}} - \delta^{13}\text{C}_{\text{DIC}})} \times 100$$

$\epsilon_{\text{cal-b}}$ and $\delta^{13}\text{C}_{\text{Cal}}$ were replaced by $\epsilon_{\text{arag-b}}$ and $\delta^{13}\text{C}_{\text{Arag}}$, respectively, when calculating the proportion in aragonite layer.

$\% \text{C}_M$ is the percentage of metabolic carbon in the calcite or aragonite layer. $\delta^{13}\text{C}_{\text{Cal}}$ and $\delta^{13}\text{C}_{\text{Arag}}$ are the $\delta^{13}\text{C}$ in the calcite and aragonite layer respectively. $\delta^{13}\text{C}_{\text{DIC}}$ is the $\delta^{13}\text{C}$ in the seawater and $\delta^{13}\text{C}_{\text{Mantle}}$ is the $\delta^{13}\text{C}$ in the mantle tissue of the mussel. $\epsilon_{\text{cal-b}}$ is the bicarbonate enrichment factor (Romanek et al., 1992) between calcite and HCO_3^- which is calculated to be 1.0 and $\epsilon_{\text{arag-b}}$ is the bicarbonate enrichment factor between aragonite and HCO_3^- calculated to be 2.7 by Romanek et al. (1992).

3. Results

3.1. Water chemistry in experiment

A summary of the water and carbon chemistry is shown in Table 1. The $p\text{CO}_2$ of all treatments showed some degree of deviation from the targeted $p\text{CO}_2$ for ambient (400 μatm) and acidified (1000 μatm), however the difference in $p\text{CO}_2$ between the ambient and acidified set up was sufficient to observe the effects of an acidified environment on the mussel. The seawater DIC showed a significant lower mean value in acidified compared with ambient treatments, which have been attributed to acidification ($F_{x,y} = 75.39$, $x = 1$, $y = 10$, $p < 0.001$). The lower (more negative) $\delta^{13}\text{C}$ values in seawater DIC, was to be expected, known as the ^{13}C -Suess Effect (Beirne et al., 2012), is caused by the addition of isotopically “light” CO_2 from fossil fuels to the atmospheric reservoir.

3.2. Shell chemistry

The mussel shell aragonite and calcite layers were analysed to determine $\delta^{13}\text{C}$ values (Table 2). The calcite $\delta^{13}\text{C}$ values appeared less negative in acidified treatments compared to ambient treatments (Fig. 2). However, this was found to be insignificant when comparing the GLM model for increasing parameters of $p\text{CO}_2$ ($p = 0.290$), temperature ($p = 0.414$), extra feeding ($p = 0.586$), seawater DIC ($p = 0.357$) and average salinity ($p = 0.392$). No significant differences in $\delta^{13}\text{C}$ values were observed between treatments in the calcite shell layer. In comparison, the aragonite $\delta^{13}\text{C}$ values appeared less negative in ambient conditions with the exception of the ambient plus extra feeding, but the most negative value was obtained from the acidified plus extra

Table 2

$\delta^{13}\text{C}$ values in different mineral layers for mussel shells, under different experimental conditions. $p\text{CO}_2$ (μatm) and mineral type (aragonite or calcite) measured by $\delta^{13}\text{C}$ VPDB. All values expressed as means \pm standard deviation.

$p\text{CO}_2$ condition	Mineral	Mean 1 month $\delta^{13}\text{C}$ VPDB (‰)	%COV	Mean 4 months $\delta^{13}\text{C}$ VPDB (‰)	%COV
400	Aragonite	-1.11 ± 0.15 (n = 3)	-13.26	-0.43 ± 0.36 (n = 3)	-83.19
400 + 2	Aragonite	-0.85 ± 0.86 (n = 3)	-101.08	-0.48 ± 0.55 (n = 3)	-115.83
400 + EF	Aragonite	-0.88 ± 0.69 (n = 3)	-78.74	-0.89 ± 0.49 (n = 3)	-55.40
1000	Aragonite	-1.29 ± 0.26 (n = 3)	-20.46	-0.70 ± 0.59 (n = 3)	-84.60
1000 + 2	Aragonite	-0.94 ± 0.59 (n = 3)	-63.21	-1.29 ± 0.22 (n = 2)	-17.24
1000 + EF	Aragonite	-1.29 ± 0.26 (n = 3)	-20.46	-0.09 ± 0.48 (n = 3)	-541.68
400	Calcite	-0.93 ± 0.44 (n = 3)	-47.40	-0.87 ± 0.16 (n = 3)	-18.13
400 + 2	Calcite	-0.66 ± 0.26 (n = 3)	-39.74	-1.22 ± 0.08 (n = 3)	-6.33
400 + EF	Calcite	-1.30 ± 0.52 (n = 2)	-40.12	-0.66 ± 0.01 (n = 2)	-1.33
1000	Calcite	-1.08 ± 0.13 (n = 3)	-12.26	-0.66 ± 0.28 (n = 3)	-42.12
1000 + 2	Calcite	-1.01 ± 0.28 (n = 3)	-27.94	-0.50 ± 1.09 (n = 3)	-217.63
1000 + EF	Calcite	-0.99 ± 0.27 (n = 3)	-27.98	-0.48 ± 0.36 (n = 2)	-75.71

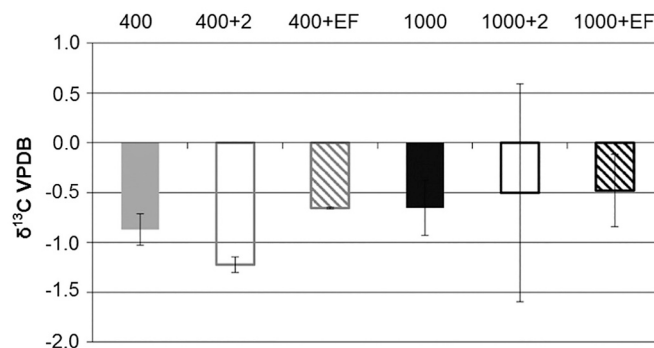


Fig. 2. Mean calcite $\delta^{13}\text{C}$ VPDB after 4 months experimental acclimation to treatments: 400 (μatm $p\text{CO}_2$) refers to ambient condition (grey bars), 1000 (μatm $p\text{CO}_2$) refers to acidified condition (black bars), +2 ($^\circ\text{C}$) refers to increased temperature (unfilled bars) and +EF refers to extra feeding (diagonally patterned bars). Error bars represent standard deviation from the mean. (n = 3, except for treatment 400 + EF, where n = 2).

feeding treatments (Fig. 3). However, these differences were not significant (increasing temperature ($p = 0.086$), extra feeding ($p = 0.596$) and salinity ($p = 0.252$)), although parameters $p\text{CO}_2$ ($p = 0.058$) and seawater DIC ($p = 0.059$) had close to significant p -values.

For shell carbonates, it has been reported that, $\delta^{13}\text{C}$ values of less than -1‰ indicates that CO_3^{2-} is the source of carbon, whereas $\delta^{13}\text{C}$ values between -1‰ and $+1\text{‰}$ indicates HCO_3^- as a source of carbon (Fitzer et al., 2019a; Grossman, 1984; Rohling and Cooke, 2003). A one sample t -test was used to test for a change in biomineralisation pathway in both aragonite and calcite shell samples, where the null hypothesis was -1‰ and alternative hypothesis was $< -1\text{‰}$ ($+/-0.06\text{‰}$). For aragonite, there were no significant observed differences in $\delta^{13}\text{C}$ values (1 sample t -test, $p > 0.05$, n = 16, see supplementary Table S2). For calcite, $\delta^{13}\text{C}$ values were significantly lower under ambient $p\text{CO}_2$ with increased warming treatments compared to ambient treatments ($p = 0.019$), which suggests a change in source of carbon uptake from HCO_3^-

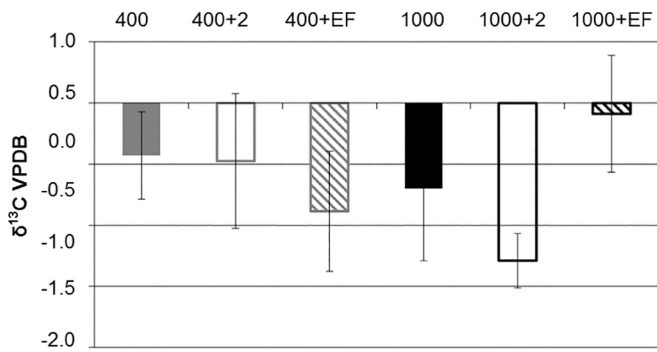


Fig. 3. Mean aragonite $\delta^{13}\text{C}$ VPDB after 4 months experimental acclimation to treatments 400 ($\mu\text{atm } p\text{CO}_2$) refers to ambient condition (grey bars), 1000 ($\mu\text{atm } p\text{CO}_2$) refers to acidified condition (black bars), +2 ($^{\circ}\text{C}$) refers to increased temperature (unfilled bars) and + EF refers to extra feeding (diagonally patterned bars). Error bars represent standard deviation from the mean. ($n = 3$, except for treatment 1000 + 2, where $n = 2$).

to CO_3^{2-} by the mussel. There was no other observed significant impact of all treatments on the $\delta^{13}\text{C}$ values in the mussel shell calcite layer (see supplementary Table S4, S5).

3.3. Mantle tissue and extrapallial fluid

Both mantle tissue (GLM, $p > 0.05$, $n = 25$, see Supplementary

Table S6) and extrapallial fluid (GLM, $p > 0.05$, $n = 23$, see Supplementary Table S7) did not show any significant differences in $\delta^{13}\text{C}$ values between the different conditions.

3.4. Metabolic carbon proportions in the shell calcite and aragonite

The proportion of metabolic carbon in the shell calcite grown under 400 $\mu\text{atm } p\text{CO}_2$ treatments collected after 1 month's acclimation remained similar (<1%) to the mussel shell calcite analysed after 4 months. The proportion of metabolic carbon uptake into the shell calcite has been observed to increase in the mussels exposed to acidified treatments (Fig. 4). The proportion of metabolic carbon is reduced when comparing between OA treatments at 1 month and 4 months acclimation (from 29% to 19%) including for those OA treatments in combination with increased temperatures (from 29% to 21%) and extra feeding (from 52% to 24%; Fig. 4). When comparing the proportion of metabolic carbon uptake in the shell calcite between the ambient and OA treatments at the end of the experiment, the metabolic carbon uptake increases from 1% to 19% respectively (Fig. 4). Under increased temperatures, the proportion of metabolic carbon uptake into mussel shell calcite under 400 $\mu\text{atm } p\text{CO}_2$ and 1000 $\mu\text{atm } p\text{CO}_2$ are 2% and 21% respectively by the end of the 4 months acclimation. When provided with extra feeding, the proportion of metabolic carbon uptake into the mussel shell calcite under 400 $\mu\text{atm } p\text{CO}_2$ is 1% and under 1000 $\mu\text{atm } p\text{CO}_2$ is 24% (Fig. 4).

The shell aragonite proportions of the metabolic compared to seawater DIC carbon uptake can be seen in Fig. 4. The proportion of

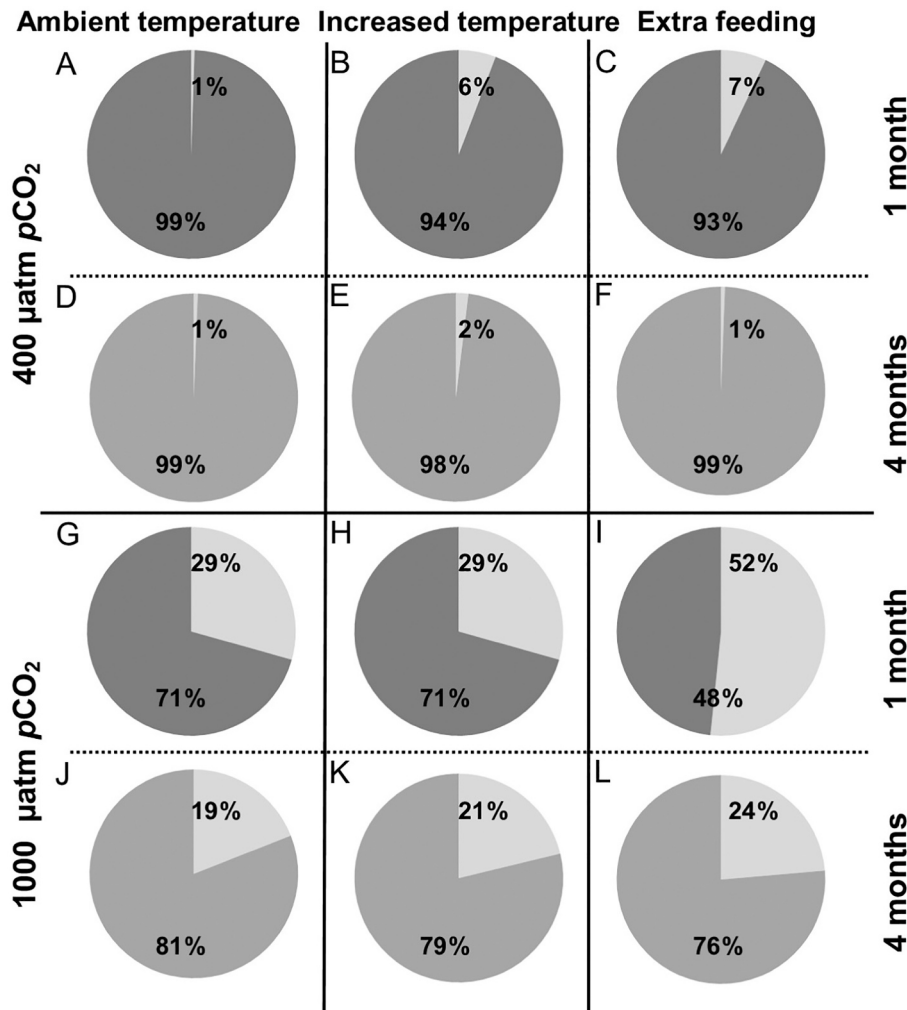


Fig. 4. Proportions of metabolic carbon (Lighter pie chart segment) to environmental carbon (darker pie chart segment) as seawater dissolved inorganic carbon ($\delta^{13}\text{C}$) in the calcite layer of the mussel shells grown under different experimental treatments. Treatments include 400 μatm (3A-F) referring to ambient treatments, 1000 μatm (3G-L) referring to acidified treatments in combination with ambient temperature, extra feeding and increased temperature treatments. Light grey charts represent 1 month experimental acclimation and dark grey charts represent 4 months experimental acclimation. ($n = 3$ for all shell calcite samples with the exception of treatment 1 month 400 + EF and 1000 + EF, and 4 months 400 + EF, where $n = 2$. For mantle tissue, $n = 4$ for all treatments).

metabolic carbon uptake in the shell aragonite decreased in all treatments between 1 month and 4 months acclimation, with the exception of shells grown under 400 $\mu\text{atm } p\text{CO}_2$ plus increasing temperature which increased from 2% to 4% between 1 and 4 months experimental acclimation (Fig. 5). Under OA (1000 $\mu\text{atm } p\text{CO}_2$), following 1 months experimental acclimation, there was a 4% increase in the proportion of metabolic carbon uptake into the shell aragonite (from 11% to 15%), with a combined increase in temperature this proportion increased by 15% (from 2% to 17%) and with extra feeding this proportion of metabolic carbon uptake into the shell aragonite increased from 18% to 34% (Fig. 5). However, after 4 months experimental acclimation to OA and OA with extra feeding (EF), the proportion of metabolic carbon uptake into the aragonite became more similar to that of the ambient treatments (Fig. 4) at 7% and 13% respectively. In contrast to the shell calcite, by the end of the 4 months acclimation, the proportions of metabolic carbon uptake into the shell aragonite is very similar across the all experimental treatments compared to the seawater DIC, with the exception of mussels grown under increased temperature treatments (Fig. 4).

4. Discussion

4.1. Ocean acidification and $\delta^{13}\text{C}$ isotopic values

OA has been shown to have a significant effect on the growth of *M. edulis* shells including reductions in growth, internal shell corrosion, changes in shape, aragonite thickness, and shell thickness (Melzner

et al., 2011; Thomsen et al., 2013; Fitzer et al., 2014, 2015a). However, few OA studies have focused on shell $\delta^{13}\text{C}$ isotope values to determine the impact on mussel shell biomineralisation pathways. Carbon pathway is commonly determined in molluscan shell geochemistry using carbon isotopes ($\delta^{13}\text{C}$) in the shell carbonate of interest to determine biogeochemical cycling of carbon in estuarine environments as proxy reconstructions (Beirne et al., 2012; Gillikin et al., 2006). $\delta^{13}\text{C}$ in bivalve shells has been previously linked with the environmental carbon uptake (Fitzer et al., 2019a) to understand how coastal acidification could impact the biomineralisation pathway in the oyster *S. glomerata*. In the case of *S. glomerata*, the wild oysters sourced carbon as CO_3^{2-} from the seawater, resulting in a reduction in shell growth where coastal acidification reduced the bioavailable environmental DIC (Fitzer et al., 2019a). In this study we examined whether additional feeding could alleviate any potentially negative impact of OA and increased temperatures, as it has been suggested that increased food availability may provide a counteracting effect on OA for marine calcifiers (Ramajo et al., 2016a). OA and/or increased temperatures were not observed to significantly change the $\delta^{13}\text{C}$ isotopic values in either the aragonite or calcite layers of the adult mussel shell. The observed similarities in shell $\delta^{13}\text{C}$, in the adult mussels under varying OA treatments, may be due to a decrease in mussel shell $\delta^{13}\text{C}$ value with age. This has been observed in scallops and explained as incorporation of an increasing proportion of metabolic carbon during ontogeny, rather than an environmental control over shell growth (Lorrain et al., 2004). The effect of OA and increased temperature may be reduced, as environmental carbon is no longer the main source of carbon for the adult mussel (Lorrain et al.,

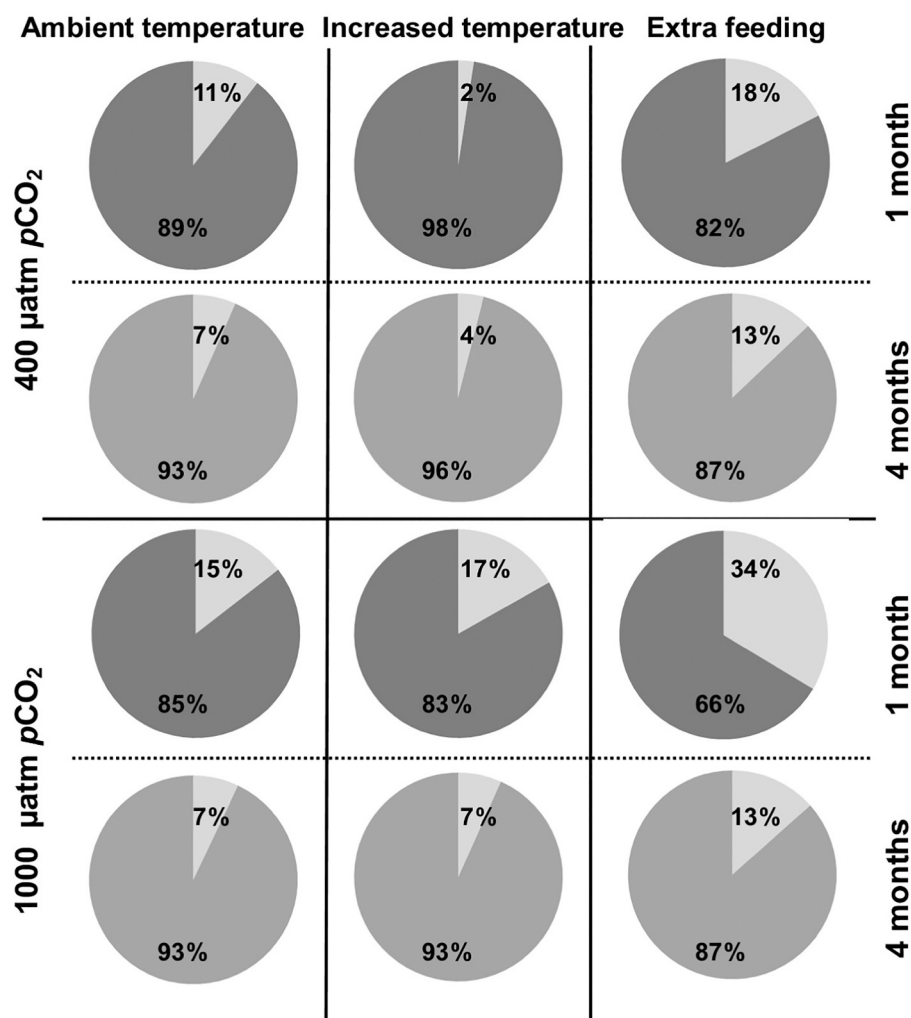


Fig. 5. Proportions of metabolic carbon (Lighter pie chart segment) to environmental carbon (darker pie chart segment) as seawater dissolved inorganic carbon ($\delta^{13}\text{C}$) in the aragonite layer of the mussel shells grown under different experimental treatments. Treatments include 400 μatm (4A-F) referring to ambient treatments, 1000 μatm (4G-L) referring to acidified treatments in combination with ambient temperature, extra feeding and increased temperature treatments. Light grey charts represent 1 month experimental acclimation and dark grey charts represent 4 months experimental acclimation. (n = 3 for all shell aragonite samples with the exception of treatment 4 months 1000 + 2, where n = 2. For mantle tissue, n = 4 for all treatments).

2004). It might therefore be expected that in *M. edulis* juveniles the shell $\delta^{13}\text{C}$ values may be changed more by OA and warming, corresponding to observations of reduction in the shell aragonite thickness with increasing $p\text{CO}_2$ (Fitzer et al., 2014) and this warrants further investigation.

4.2. Shell aragonite and calcite $\delta^{13}\text{C}$ isotopic values

The calcite layer is the outer layer of a mussel shell and is considered more important in terms of protective function against predation and mechanical damage. OA and/or increased temperature has previously been shown to impact the fracture toughness of the shell calcite layer in *M. edulis* (Fitzer et al., 2015b). In this study, no significant change in shell calcite $\delta^{13}\text{C}$ was found across all OA treatments including extra feeding. However the size, weight and sexual maturation of the mussel has not been taken into account here, and so extra feeding could potentially lead to tissue growth or even sexual maturation as mussel sexual maturation is age and growth rate dependent (Sprung, 1983), rather than a higher growth rate in the shell. Seawater $\delta^{13}\text{C}$ DIC values are significantly lower in seawater under OA compared to ambient treatments, suggesting that under OA less DIC is available for mussel environmental carbon uptake. The shell $\delta^{13}\text{C}$ data suggest that there is sufficient carbon available in all treatments for the mussel to continue to utilise environmental carbon uptake for shell growth regardless of the $p\text{CO}_2$ and temperature. Although this may occur at the expense of the chosen biomineralisation pathway (CO_3^{2-} versus HCO_3^-) limiting shell growth, as seen in *S. glomerata* under coastal acidification (Fitzer et al., 2019a). The sub-lethal effects of OA treatments were not studied here, and so it is not known if the mussels have been able to sacrifice somatic growth to maintain shell growth for protection, as survival and reproduction are always priority. The zebra mussel, *Dreissena polymorpha* has been found to sacrifice somatic growth for reproduction when exposed to near lethal temperatures (Hoddle, 2011). *M. edulis* was also found to have a reduced immune response in terms of suppressed phagocytosis after 32 days of exposure to acidified conditions (Bibby et al., 2008).

4.3. Mantle and extrapallial fluid $\delta^{13}\text{C}$ isotopic values

Mussel extrapallial fluid and mantle tissue $\delta^{13}\text{C}$ values did not differ across all experimental treatments, which differs from the findings in oysters *S. glomerata* where lower $\delta^{13}\text{C}$ values were observed in both the extrapallial fluid and tissues grown under a lower seawater DIC (acidified condition; Fitzer et al., 2019a, 2019b). This suggests that making generalisations about biomineralisation pathways, across bivalve species, may not be possible and that it is important to investigate species-specific responses to determine the most vulnerable bivalve species under OA.

4.4. Change in biomineralisation pathway

The carbon isotope values from the mussel shells in this study have been used as a proxy for biomineralisation pathway where a $\delta^{13}\text{C}$ value of less than -1‰ indicates that CO_3^{2-} is the source of carbon, whereas between -1‰ and $+1\text{‰}$ indicates HCO_3^- as a source of carbon (Fitzer et al., 2019a; Grossman, 1984; Rohling and Cooke, 2003). Ambient $p\text{CO}_2$ with increased warming was the only treatment to result in a change in carbon source from HCO_3^- to CO_3^{2-} in the formation of the mussel calcite layer. This could be due to an increase in CO_3^{2-} in warmer environments, where the $p\text{CO}_2$ concentration is lower leading to more abundant CO_3^{2-} in the environment (Melzner et al., 2011). CO_3^{2-} is suggested to be more suitable for inorganic precipitation of CaCO_3 than HCO_3^- (Lu et al., 2018), and in the case of this study has been observed as the carbonate source of choice under increased temperatures. The influence of OA on the changing carbonate source is still not clear from the data of this study, therefore further research is required to confirm the effect of environmental change on biomineralisation pathway in mussels (Bach,

2015; Lu et al., 2018).

4.5. Metabolic carbon uptake

In this study, we applied $\delta^{13}\text{C}$ isotopes to determine the ratio of metabolic to environmental carbon incorporated in calcite and aragonite shell layers under OA, and/or increased temperatures and extra feeding. Environmental carbon as seawater DIC is found to be the main source of carbon in *M. edulis* shells under ambient $p\text{CO}_2$. OA and increased temperatures were found to increase the proportion of metabolic carbon uptake in *M. edulis* calcite, but seawater DIC still remains the main source of shell carbon similar to the findings of (Lu et al., 2018). Increasing temperature is suggested to play a more significant role in reducing mussel shell strength than OA due to a re-allocation of energy from biomineralisation to maintenance cost (Mackenzie et al., 2014), which supports the findings of this study. This study is the first to examine the longer-term effects of OA with increased temperature and extra feeding on mussel metabolic carbon uptake, studying a 4 month period of experimental acclimation, a longer duration of experimental culture in comparison to previously reported studies of 5 weeks (Lu et al., 2018). It is important to consider the separate carbon uptake pathways for both shell calcite and aragonite due to different mineral polymorphs having different bicarbonate enrichment factors as calculated by Romanek et al. (1992). Lu et al. (2018) has examined metabolic and environmental carbon uptake in the shell calcite of *M. edulis*, but was unable to determine this for aragonite due to the shorter culture period and insufficient aragonite growth in the shells. In our study the proportion of metabolic carbon uptake varied over the period of experimental acclimation, depending on the culture conditions i.e. whether mussels were grown under OA, increased temperature and when extra feeding was provided. In the mussel shell calcite, the proportion of metabolic uptake, under ambient $p\text{CO}_2$ (400 μatm) treatments, reached a maximum during the 1-month acclimation period and plateaued through the 4-month experimental acclimation. In contrast, we saw a decrease in the proportion of metabolic carbon uptake into the shell calcite under all OA (1000 μatm) treatments, for OA alone from 29% to 19%, for OA plus increased temperatures from 29% to 21% and for OA plus extra feeding from 52% to 24% between 1 month and 4 months experimental acclimation. This suggests that although the metabolic proportion is increased at the start of the experiment after 1 month acclimation, this proportion of metabolic carbon uptake into the shell cannot be maintained long-term. In the shell aragonite, after 4 months under OA, the proportion of metabolic carbon uptake is still much higher compared to ambient treatments (1%–19%) and is even higher under OA plus increased temperatures (21%) and OA plus extra feeding (24%). The data of this study provides evidence in favour of extra feeding to mussels as a means to continue shell growth through the up-regulation of metabolic carbon uptake which can be maintained for a period of at least 4 months. The impact of OA on phytoplankton varies from species to species, increases in temperature and dissolved carbon dioxide ($p\text{CO}_2$) can lead to an increase in the abundance of some species, changing the structure of the phytoplankton community (Collins et al., 2014; Dutkiewicz et al., 2015). This could provide sufficient food resources to mussels under OA and increased temperatures to continue a prolonged switch to metabolic carbon uptake for shell growth. However, further research is needed to understand if this change, towards increased utilisation of metabolic carbon pathways for biomineralisation, can be maintained in the longer-term allowing for adaption, and whether the phytoplankton species would provide sufficient resources in a future changing environment.

Irrespective of the length of experimental acclimation it is evident that OA increases the proportion of metabolic carbon uptake into the shell calcite from 1% to 19%, and when combined with increased temperatures or extra feeding the proportion of metabolic carbon uptake increases further from $<7\%$ to 21%–29% and 24%–52% respectively. These observations suggest an additive effect of OA plus increased

temperatures on mussel biomineralisation as shown by a higher proportion of metabolic carbon uptake compared to mussels grown under OA treatment alone, in agreement with the findings of Lu et al. (2018). Grossman and Ku (1986) also observed a significant dependence on temperature in respect to mollusc shell oxygen isotopes, but with little evidence to support carbon isotope fractionation as a function of seawater temperature (Grossman and Ku, 1986). An increase in the proportion of metabolic carbon uptake into the shell calcite under extra feeding suggests that mussels can compensate for a decrease in seawater DIC by increasing carbon derived from diet. Which may explain the previous findings where extra feeding has been shown to alleviate the negative impacts on mussel shell growth caused by OA (Thomsen et al., 2013; Ramajo et al., 2016b).

The aragonite and calcite layers in the mussel shell show a different response to OA, increased temperatures and extra feeding - in terms of metabolic carbon uptake. Under ambient (400 $\mu\text{atm } p\text{CO}_2$) treatments the proportion of metabolic carbon uptake is higher in aragonite compared to calcite at 7%–11%, regardless of the time of experimental acclimation, this concurs with the hypothesised respiratory gas exchange model estimates of 10% for aquatic molluscs, calculated using adult and juvenile adductor muscles (Beirne et al., 2012). The metabolic carbon uptake into the aragonite increases to 13%–18% with extra feeding. Interestingly the addition of increased warming reduces the proportion of metabolic carbon uptake into the aragonite to between 2% and 4%, the opposite of our findings for the shell calcite. It is suggested that under increased temperature, there is a decrease in overall $\delta^{13}\text{C}$ values in the biogenic aragonite of foraminifera *Hoeglundina elegans* and other ancient mollusc fossils, attributed to a decrease in metabolic carbon uptake in the aragonite layer (Grossman and Ku, 1986). OA increased the proportion of metabolic carbon uptake into the shell aragonite to between 11% and 15% similarly to the metabolic carbon uptake in the shell calcite from ambient treatments at the start of the 1 month acclimation period. It is important to note that in this study there is compensatory difference in the metabolic carbon uptake response to OA from 1 month to 4 months experimental acclimation with all OA treatments returning to ambient levels. For OA in addition to extra feeding at 1-month experimental acclimation the proportion of metabolic carbon uptake into the aragonite shell layer is increased to 34% from that of ambient treatments with extra feeding alone at 18%. Prolonged experimental acclimation of 4 months enables the mussel to adjust the proportion of metabolic carbon uptake into the aragonite back to the level experienced under ambient treatments with extra feeding at 13%. This suggests that mussels can alleviate the impact of OA on shell aragonite growth over prolonged experimental exposure when provided with additional food resources. This may therefore be able to alleviate any negative impacts on biomineralisation and therefore growth and survival.

The energetic cost of metabolic carbon uptake in shell formation must be considered. Studies have estimated the energy cost of calcification and production of the organic matrix, where calcification of CaCO_3 costs only 1–2 J/mg as it is a relatively simple process of accumulating, transporting, and precipitating (Palmer, 1992). The formation of the organic matrix is done by synthesis which is much more costly due to its complexity and it is estimated that the organic matrix only accounts for 1.5%–5% of the shell, but the energy required ranged from 22% to 50% (Palmer, 1992). This could be of major concern for mussels, where an increase in the proportion of metabolic carbon uptake for shell formation is taken from the organic matrix due to the lack of seawater DIC for calcification (under future OA). The increased energetic costs of shell formation may lead to reduced growth rate if energy is insufficient, for example tissue weight loss maybe a consequence of organic matrix formation when no feeding is provided (Palmer, 1983). The feed mixture is therefore important to distinguish the true impact of OA and warming on shell biomineralisation. In this study the microalgae used is perhaps not an accurate representation of a mussel's diet in nature, as mussels not only feed on phytoplankton, but also animal material (Davenport

et al., 2000). The effect of OA and increased warming on zooplankton should therefore be considered in future experiments, as animal material forms a major part of the mussel's diet.

The differences in metabolic carbon uptake - between the aragonite and calcite layer - under OA and extra feeding, suggest that different environmental factors govern the ability of mussels to increase their metabolic carbon uptake and therefore biomineralisation pathway. This is an important finding for other bivalve species with different calcium carbonate composites, particularly where shells are completely formed of aragonite or calcite. Aragonitic species are potentially more vulnerable to OA as aragonite is 50% more soluble than calcite, and it has been suggested that some species which produce aragonite only shells, have recorded no net shell calcification under OA (Gazeau et al., 2013), where we find a reduction in metabolic carbon uptake into the aragonite.

5. Conclusion

In conclusion, this study has found OA to increase the metabolic carbon uptake in the calcite layer of *M. edulis* shells. This appears to be an additive effect of both increased temperatures and extra feeding on the metabolic uptake of carbon to shell calcite in *M. edulis*, suggesting a change in the biomineralisation pathway, however it is unclear whether this increase in metabolic uptake of carbon can be maintained. An understanding of the future response of food sources is key to determining whether long-term upregulated metabolic carbon uptake can be maintained, as phytoplankton communities can vary in their response at a species-specific level, under OA. The metabolic carbon uptake of the aragonite layer of *M. edulis* shells differed from that of the calcite layer, being affected by increased temperatures. In mussels the energetic cost of metabolic carbon uptake is much higher than for seawater DIC, suggesting that although the mussels are able to continue carbon uptake through metabolic biomineralisation pathways this may be at the expense of other physiological pathways with consequences for mussel survival and growth. This study has used $\delta^{13}\text{C}$ values to identify changes in biomineralisation pathway relating to mussel metabolic carbon uptake from their food source, with different results observed in aragonite and calcite mineral polymorphs. The implications of these findings suggest that some bivalve species with different shell composites may cope better with biomineralisation under OA than others, demanding further study into species-specific pathways.

Author contribution

Conceptualization, funding acquisition, investigation, project administration and supervision of Tin Hang Lee (THL) was done by Dr Susan Fitzer (SCF). Data curation was done by SCF and Dr Rona McGill. Formal analysis and writing of the original draft was done by Tin Hang Lee (THL) under the supervision of Dr Susan Fitzer and Dr Rona McGill.

Declaration of Competing Interest

There are no conflicts of interest for this submission.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jembe.2021.151562>.

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