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RESEARCH ARTICLE

Interactive effects of seawater acidification and elevated temperature on biomineralization and amino acid metabolism in the mussel *Mytilus edulis*

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ABSTRACT

Seawater acidification and warming resulting from anthropogenic production of carbon dioxide are increasing threats to marine ecosystems. Previous studies have documented the effects of either seawater acidification or warming on marine calcifiers; however, the combined effects of these stressors are poorly understood. In our study, we examined the interactive effects of elevated carbon dioxide partial pressure (P_{CO_2}) and temperature on biomineralization and amino acid content in an ecologically and economically important mussel, *Mytilus edulis*. Adult *M. edulis* were reared at different combinations of P_{CO_2} (pH 8.1 and 7.8) and temperature (19, 22 and 25°C) for 2 months. The results indicated that elevated P_{CO_2} significantly decreased the net calcification rate, the calcium content and the Ca/Mg ratio of the shells, induced the differential expression of biomineralization-related genes, modified shell ultrastructure and altered amino acid content, implying significant effects of seawater acidification on biomineralization and amino acid metabolism. Notably, elevated temperature enhanced the effects of seawater acidification on these parameters. The shell breaking force significantly decreased under elevated P_{CO_2}, but the effect was not exacerbated by elevated temperature. The results suggest that the interactive effects of seawater acidification and elevated temperature on mussels are likely to have ecological and functional implications. This study is therefore helpful for better understanding the underlying effects of changing marine environments on mussels and other marine calcifiers.

KEY WORDS: Marine calcifiers, Carbon dioxide, Seawater warming, Shell ultrastructure, Breaking force, Global climate change

INTRODUCTION

Seawater acidification and warming are both considered to be consequences of increasing anthropogenic CO_2 emissions (Doney et al., 2009). Uptake of CO_2 has decreased the ocean surface pH by 0.1 units since the Industrial Revolution. Global seawater surface pH is projected to drop a further 0.3–0.4 units by the end of this century, with seawater warming of 1–4°C (Bates et al., 2008; Doney et al., 2012). Seawater acidification and warming have negative impacts on the larval development, growth, reproduction, biomineralization, and physiological and biochemical performance of some marine organisms, especially marine calcifiers, by disturbing ion and acid–base equilibrium, energy budgets and calcium carbonate ($CaCO_3$) saturation state (Ceballos-Osuna et al., 2013; Gazeau et al., 2013; Paganini et al., 2014; Pimentel et al., 2014; Rosa et al., 2014; Schlegel et al., 2015; Stillman and Paganini, 2015; Turner et al., 2015; Wittmann and Portner, 2013; Yao and Somero, 2014).

Seawater acidification and warming usually act on marine calcifiers in an interactive manner. For example, field and laboratory investigations have demonstrated that seawater acidification exacerbates the negative effects of seawater warming on *Balanophyllia europaea* and *Patella caerulea* (Rodolfo-Metalpa et al., 2011). The size of larvae decreased and the number of abnormal larvae increased in the Sydney rock oyster *Saccostrea glomerata* exposed to seawater acidification, and these deleterious effects were greater at elevated temperature (Parker et al., 2009). Meanwhile, a meta-analysis has also highlighted a trend towards enhanced sensitivity of growth, biomineralization, survival and development to acidification when some marine calcifiers were concurrently exposed to elevated temperatures (Kroeker et al., 2013). Exposure to acidified seawater can reduce the tolerance of marine organisms to elevated temperature by influencing the metabolic system and, furthermore, elevated temperature may reduce their ability to regulate intracellular pH by influencing the permeability of cell membranes (Padilla-Gamiño et al., 2013; Pörtner, 2010). However, different calcifiers vary in their responses to the combined stressors. Elevated temperature negated the negative effects of ocean acidification on attachment and metamorphosis of *Crassostrea gigas* larvae (Ko et al., 2014). Seawater warming may ameliorate the negative impact of seawater acidification on corals (Kleypas and Yates, 2009; McNeil et al., 2004). Whether the combination of seawater acidification and elevated temperature affects shell growth, metabolic rate and periostracum production in the Mediterranean mussel, *Mytilus galloprovincialis*, is unclear (Gazeau et al., 2014). These results suggest that there are complex responses within different calcifiers to the combined climate stressors. Therefore, to fully understand the effects of seawater environment changes on marine calcifiers, it is necessary to further investigate the interactive impacts of seawater acidification and warming on these species.

Mytilus edulis is an ecologically and economically important mussel in coastal habitats globally, serving as a food resource for humans (FAO, 2010). It is the dominant marine calcifier in the rocky intertidal ecosystem, and has been used as an indicator species to study the effects of changes in the marine environment (Brenner et al., 2014). Increasing evidence has confirmed that growth (Gazeau et al., 2010), biomineralization (Melzner et al., 2011), shell mechanical properties (Gaylord et al., 2011; Mackenzie et al., 2014) and energy budget (Hüning et al., 2013; Michaelidis et al., 2005) in mussels are affected by seawater acidification. Furthermore, larval

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Received 2 August 2015; Accepted 10 September 2015

Journal of Experimental Biology (2015) 218, 3623-3631 doi:10.1242/jeb.126748

List of symbols and abbreviations

CA	carbonic anhydrase
CHI	chitinase
CHS	chitin synthetase
CNN	calponin-like protein
DIC	dissolved inorganic carbon
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NACR	nacrein-like protein
NCR	net calcification rate
P_{CO_2}	partial pressure of carbon dioxide
PLUC	perlucin-like protein
PWAP	perlwapin-like protein
RT-qPCR	real-time quantitative PCR
SEM	scanning electron microscopy
SILK	silk-like protein
TA	total alkalinity
XPS	X-ray photoelectron spectroscopy
Ω_{ara}	aragonite saturation state
Ω_{cal}	calcite saturation state

physiology (Vihtakari et al., 2013), shell growth (Hiebenthal et al., 2013), proteomics (Tomanek, 2014) and geographical distribution (Anestis et al., 2008) are affected by seawater warming. CO_2 stress alters the expression of *M. edulis* mantle genes, providing a molecular basis for the observed changes in physiology in response to ocean acidification (Hüning et al., 2013). Reduced pH causes significant decreases in hatching rate and shell growth of *M. edulis* larvae (Gazeau et al., 2010). It has also been documented that elevated temperature has a greater effect than ocean acidification on shell integrity in *M. edulis* when food availability is limited (Mackenzie et al., 2014). However, the interactive effects of seawater acidification and warming on *M. edulis* remain poorly understood.

Therefore, the aim of this study was to investigate the interactive effects of seawater acidification and warming on biomineralization and amino acid metabolism in the mussel *M. edulis*. To achieve this objective, adult *M. edulis* were maintained under the following pH and temperature conditions: pH 8.1 at 19, 22 and 25°C; pH 7.8 at 19, 22 and 25°C. After a period of 60 days, changes in calcification rate, calcium and magnesium content, shell ultrastructure, shell breaking force, gene expression and amino acid content in *M. edulis* were analysed by X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), mechanics testing machine, real-time quantitative PCR (RT-qPCR) and liquid chromatography–tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Animals and experimental design

Mytilus edulis Linnaeus 1758 were collected from the rocky intertidal zone in Jiaozhou Bay in Qingdao, China (38°47′29.94″N, 115°30′0.62″E). The average seawater conditions in the sampling area were 19.4±0.3°C, pH 8.10±0.04 and 31.0±0.5 PSU (practical salinity units). Prior to use, the *M. edulis* were maintained at 19.0°C, pH 8.10 and 33.0 PSU in glass aquaria filled with artificial seawater (Formula Grade A Reef Sea Salt, Formula, Japan).

Prior to experimentation, mussels of similar size (4–5 cm length) were assigned to 50 l automatic circulation tanks and acclimatized to experimental conditions through a gradual increase in seawater temperature from 19 to 25°C (1°C day^{-1}) and a gradual decrease in pH from 8.1 to 7.8 (0.5 units day^{-1}). The mussels were then maintained for 60 days under the following conditions: pH 8.1 and 33.0 PSU at 19.0°C (control), 22.0°C and 25.0°C; pH 7.8 and 33.0 PSU at 19.0°C, 22.0°C and 25.0°C. The treatments mimicked the near-future ocean scenarios for the year 2100 (Bates et al., 2008; Caldeira and Wickett, 2003) and the values fall within the seasonal pH and temperature fluctuations in this sea area. Each treatment was replicated in triplicate in three independent tanks and each tank contained 30 individuals. The mussels were fed twice daily with the microalgae *Isochrysis galbana* at a concentration of 1.5 mg l^{-1}.

For shell analysis, the shells were collected at the end of the treatment period, washed with deionized water, and immersed in 5% sodium hydroxide for 12 h to remove organic components on the inner surface. The shells were then washed thoroughly with deionized water, air dried and stored in a desiccator until required. Samples from three individuals randomly selected from each tank were pooled to obtain one biological replicate.

Seawater chemistry

Seawater was acidified by continuously pumping CO_2 into the tank and temperature was controlled by inserting a temperature controller into the bottom of the tank. pH (National Bureau of Standards scale) was monitored daily using a glass electrode on a digital pH controller (pH-201, IPA, Shenzhen, China) and temperature was measured daily using a temperature controller (D838-100, UP, Taiwan). Air was bubbled into the tanks to maintain oxygen levels. Salinity was measured using a Mini Salinometer (Model 2100, AGE Instruments Inc., Ottawa, ON, Canada). Total alkalinity (TA) was measured by Gran titration with a Kloehn digital syringe pump after filtering the seawater samples through a 0.45 µm Millipore filter. Total dissolved inorganic carbon (DIC) was determined using a DIC analyser (AS-C3, Apollo SciTech Inc., Bogart, GA, USA) after filtering the seawater samples. The CO_2 partial pressure (P_{CO_2}), carbonate concentration ([CO_3^{2-}]), bicarbonate concentration ([HCO_3^-]), aragonite saturation state (Ω_{ara}) and calcite saturation state (Ω_{cal}) were calculated with the carbonate chemistry calculation software CO_2SYS (Lewis et al., 1998) using pH, temperature, salinity, TA, DIC and dissociation constants K_1, K_2 and K_{SO_4} (Dickson, 1990; Dickson and Millero, 1987; Mehrbach et al., 1973).

Table 1. Seawater carbonate chemistry parameters under different P_{CO_2} and temperature conditions

	pH 8.1			pH 7.8		
Seawater parameters	19°C	22°C	25°C	19°C	22°C	25°C
pH	8.07±0.07	8.12±0.04	8.08±0.04	7.78±0.04	7.76±0.05	7.81±0.02
Temperature (°C)	19.77±0.82	21.94±0.33	25.05±0.42	18.70±0.50	22.70±0.55	24.70±0.41
Salinity (PSU)	33.67±0.94	33.24±0.40	32.94±0.49	33.48±0.57	33.15±0.55	33.03±1.25
TA (µmol l^{-1} kg^{-1})	2184.35±29.29	2180.42±20.38	2246.34±20.05	2303.00±18.43	2330.34±17.60	2209.52±18.68
DIC (µmol l^{-1} kg^{-1})	1930.85±16.30	1910±21.06	1939.48±30.01	2157.14±8.35	2214.05±10.99	2070.34±15.03
P_{CO_2} (µatm)	349.45±36.27	346.33±22.56	346.51±18.22	747.65±73.47	930.09±55.21	766.06±85.87
[CO_3^{2-}] (µmol l^{-1} kg^{-1})	178.93±13.94	191.19±10.02	218.23±15.33	117.56±7.73	101.38±15.39	112.63±13.77
[HCO_3^-] (µmol l^{-1} kg^{-1})	1740.35±46.64	1708.10±25.30	1711.34±34.01	2015.63±37.64	2083.13±30.89	1933.21±36.21
Ω_{ara}	2.79±0.22	4.65±0.11	5.34±0.34	1.85±0.12	1.80±0.10	1.81±0.21
Ω_{cal}	4.32±0.34	3.03±0.66	3.51±0.19	2.86±0.18	2.66±0.21	2.76±0.31

Partial pressure of carbon dioxide (P_{CO_2}), carbonate concentration [CO_3^{2-}], bicarbonate concentration [HCO_3^-], aragonite saturation state (Ω_{ara}) and calcite saturation state (Ω_{cal}) were calculated from temperature, pH value, salinity, total alkalinity (TA) and dissolved inorganic carbon (DIC) using CO_2SYS software (see Materials and methods). Experimental values correspond to means±s.d.

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Table 2. Real-time quantitative PCR primer information

Gene	Accession no.	Gene description	Primer sequence
CHI	HE662827	Chitinase	CHI-F: ggatccaagcccttcacaca CHI-R: tcccaatccaaatcgaggcc
CHS	HE662847	Chitin synthase	CHS-F: tgtgctttgttgtcccggat CHS-R: tagtgcctggcttctgttgg
CNN	HE662833	Calponin-like protein	CNN-F: accccatctgagagtctccc CNN-R: tccgtatttgtgtgctgcct
CA	HE662842	Carbonic anhydrase	CA-F: tgacaccgacagctcaactc CA-R: gcctgtagacgcctgagatc
SILK	HE662819	Silk-like protein	SILK-F: ttcaggtggagcaggaggat SILK-R: acctccagcaccactaattgt
NACR	HE662840	Nacrein-like protein	NACR-F: cactcatcaccaccagaccc NACR-R: ccgatggaagctcaagtggt
PLUC	HE662824	Perlucin-like protein	PLUC-F: gaggagggcgattggagatg PLUC-R: acacggcctgtcattccatt
PWAP	HE662826	Perlwapin-like protein	PWAP-F: tcctgactcttcggcatctt PWAP-R: agacgatgtgcagcagtcac
18s rRNA	HE662843	18s ribosomal RNA	rRNA-F: ccggcgacgtatctttcaa rRNA-R: aggcatatcacgtaccatcgaa

The seawater chemistry parameters were stable throughout the experiments and are shown in Table 1.

Net calcification rate (NCR)

The TA before and after the treatments was used to calculate the NCR using the alkalinity anomaly technique (Gazeau et al., 2007), which is commonly used to calculate short-term calcification rates for marine calcifiers. The TA value in each tank was determined five times and the calcification rate was expressed as μmol $CaCO_3$ g^{-1} h^{-1}.

XPS

To analyse the calcium and magnesium content of the shells, samples from the nacreous layer (near the nacre–prism transition region) were cut into 7×7 mm pieces (the regions are given below). The calcium and magnesium content was determined using XPS (ESCALAB 250Xi, Thermo Scientific, Waltham, MA, USA) with monochromatic Al Ka radiation (1486.7 eV) and an angle of incidence of 45 deg. The binding energies were referenced to the adventitious C 1s peak at 285 eV and the accuracy of the binding energy measurements was 0.2 eV. High-resolution C 1s, Ca 2p, and Mg 1s spectra were obtained at a pass energy of 50 eV. The relative atomic concentration ratio of the elements on the shell surface was calculated by Advantage V4 (Thermo-VG Scientific, East Grinstead, West Sussex, UK) using the sensitivity factors and signal intensities of the elements. Two shell valves of each mussel were tested and three mussels in each tank were used to avoid individual error across mussels.

SEM

To observe the impact of elevated P_{CO_2} and temperature on the shell ultrastructure, the inner surface (near the nacre–prism transition region) of the shell samples was cut into small pieces (see above). The pieces were then mounted on stubs with carbon-based tape and coated with gold for 60 s. The nacreous and prismatic layers were observed and imaged by SEM (FEI Quanta 200, Eindhoven, The Netherlands) using an acceleration voltage of 15 kV. Shell samples from three individuals randomly selected from each tank were considered as one biological replicate.

Breaking force test

To analyse the strength of the shells, the left and right valves of each mussel in all the treatments were collected and washed with deionized seawater. The specific measuring method is shown below. The breaking force was measured individually (N=50) using a universal materials testing machine (Zwick Z010, Zwick Roell Group, Ulm, Germany). Compressive extension continued until the shell broke visibly in the middle. Shells that had existing small irregularities or cracks were discarded, and an additional sample was analysed. testXpert software (Zwick Roell Group) recorded the continuously applied force until the first failure of the shell occurred, and the first recorded breaking strength was considered the highest force that the shell could withstand. The breaking force was expressed in N. The length, height and width of the shells were measured prior to the breaking force test.

RT-qPCR

Ten mantle samples in 10 mussels were collected from each tank and pooled as a biological replicate. Three biological replicates were used for RT-qPCR analyses. The samples were washed with RNase-free water immediately. Total RNA extraction and cDNA synthesis of the mantle samples were carried out following the method of Hüning et al. (2013). Primers were designed using the online program Primer3 Version 4.0.0 (http://primer3.wi.mit.edu/). RT-qPCR was performed with 1 μl cDNA, 0.4 μmol l^{-1} of each primer and 2× SYBR Green Master Mix using SYBR® Premix Ex Taq™ II (Takara, Otsu, Japan) to obtain a total volume of 20 μl. All PCR reactions were run as follows: 1 cycle of 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 30 s; 1 cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The fluorescent products were detected using StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Eight biomineralization-related genes were analysed: the organic template protein-related genes chitinase (*CHI*) and chitin synthetase (*CHS*); the calcium-binding protein gene calponin-like protein (*CNN*); the CO_2 hydration reaction gene carbonic anhydrase (*CA*); and the shell matrix protein candidate genes silk-like protein (*SILK*), nacrein-like protein (*NACR*), perlucin-like protein (*PLUC*) and perlwapin-like protein (*PWAP*). The reference housekeeping gene 18s rRNA (Hüning et al., 2013) was used as the internal control, and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was

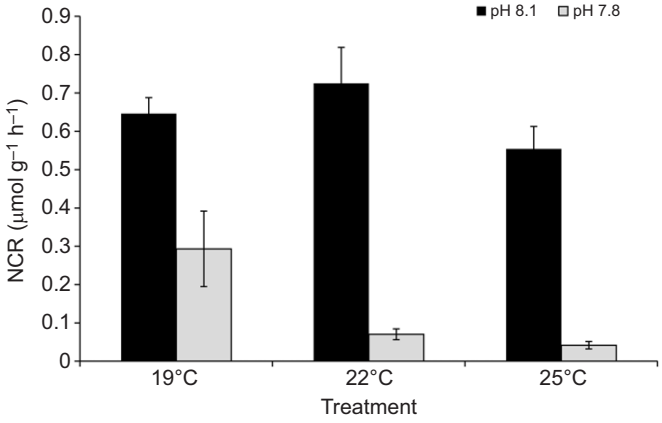

Fig. 1. Effects of elevated P_{CO_2} and temperature on *Mytilus edulis* net calcification rate (NCR).

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Table 3. Two-way ANOVA results showing the effects of P_{CO_2} and temperature on *M. edulis* NCR, Ca/Mg and breaking force

Parameter	Source of variation	d.f.	MS	*F*	*P*
NCR	pH	1	1.055	2744.904	<0.001
	Temperature	2	0.046	119.981	<0.001
	pH×temperature	2	0.032	82.414	<0.001
Ca/Mg	pH	1	78.277	1521.219	<0.001
	Temperature	2	6.96	135.261	<0.001
	pH×temperature	2	0.935	18.161	<0.001
Breaking force	pH	1	13,228.053	345.684	<0.001
	Temperature	2	1102.188	28.803	<0.001
	pH×temperature	2	37.811	0.988	0.401

NCR, net calcification rate; Ca/Mg, calcium/magnesium ratio.

used to analyse the relative fold changes of the genes. Detailed information on the genes, accession numbers and primers is given in Table 2.

LC-MS/MS

To determine the amino acid content in *M. edulis*, a standard solution was prepared by dissolving amino acid standards in acetonitrile. All the standards and other regents were obtained from Sigma-Aldrich (St Louis, MO, USA; ≥99.99% purity). The stock standard mixture (10 µg ml^{-1}) was prepared by diluting the standard solutions, from which the calibration standards with a range 5–2500 ng ml^{-1} were prepared by serially diluting the mixture with acetonitrile.

The entire edible parts from 10 mussels in each tank were collected and pooled together to obtain one biological replicate. A homogeneous 1.0 g sample was hydrolysed by 6 mol l^{-1} HCl at 110°C for 14 h. The hydrolysate was cooled to room temperature and HCl was added to obtain a total volume of 10 ml; 1 ml of the hydrolysate was freeze-dried, redissolved in 2.0 ml of 0.1 mol l^{-1} HCl, and stored at 4°C until analysis. Before determination, the sample was mixed for 3 min and centrifuged at 10,000 ***g*** for 5 min at room temperature. The amino acid content was determined with a 1290 analytical HPLC system connected to a 6460 triple quadrupole mass spectrometer (LC-MS/MS, Agilent Technologies, Santa Clara, CA, USA). A 10 µl sample was injected through an auto-sampler. The separation was collected with a C_{18} column (Perkin-Elmer; 4.6×250 mm, 5 µm). The mobile phase was composed of (A) acetonitrile and (B) acetic acid sodium acetate buffer (0.03 mol l^{-1} sodium acetate, 0.15% triethylamine, pH 5.2). The column temperature was 40°C and the determination wavelength was 360 nm. The equation obtained from the standard solutions was used for the sample analysis. The results are presented as mg amino acid g^{-1} mantle (±s.d., *N*=3). Eighteen amino acids were analysed: alanine and β-alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), isoleucine (Ile), lysine (Lys), methionine (Met), leucine (Leu), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val).

Statistical analysis

All statistical analyses were carried out in SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). The individual and interactive effects of elevated P_{CO_2} and temperature on NCR, calcium and magnesium content, gene expression, shell breaking force and amino acid content were assessed by one-way and two-way ANOVA, respectively. NCR, SEM, breaking force and gene expression were visualized in SigmaPlot version 12.5 (Systat Software, San Jose, CA, USA) and calcium and magnesium content was visualized in Origin version 7.5 (Originlab, Northampton, MA, USA). All of the results are presented as means±s.d.

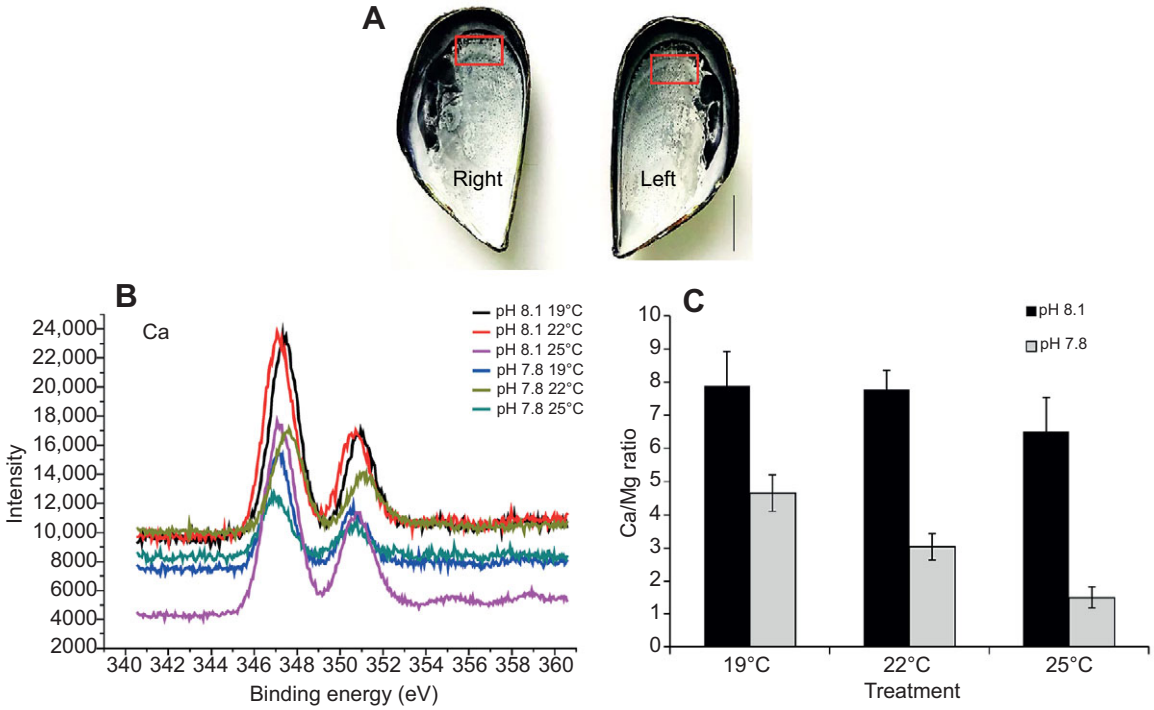

Fig. 2. Effects of elevated P_{CO_2} and temperature on the calcium content and the ratio of calcium to magnesium (Ca/Mg) in the *M. edulis* nacreous layer. (A) The left and right shell valves of *M. edulis*. The red box indicates the sampling site. (B) Calcium content in the different treatments. The survey spectra record the presence of calcium on the shell surface and the peak areas indicate the relative calcium content. (C) Ca/Mg ratio in different treatments.

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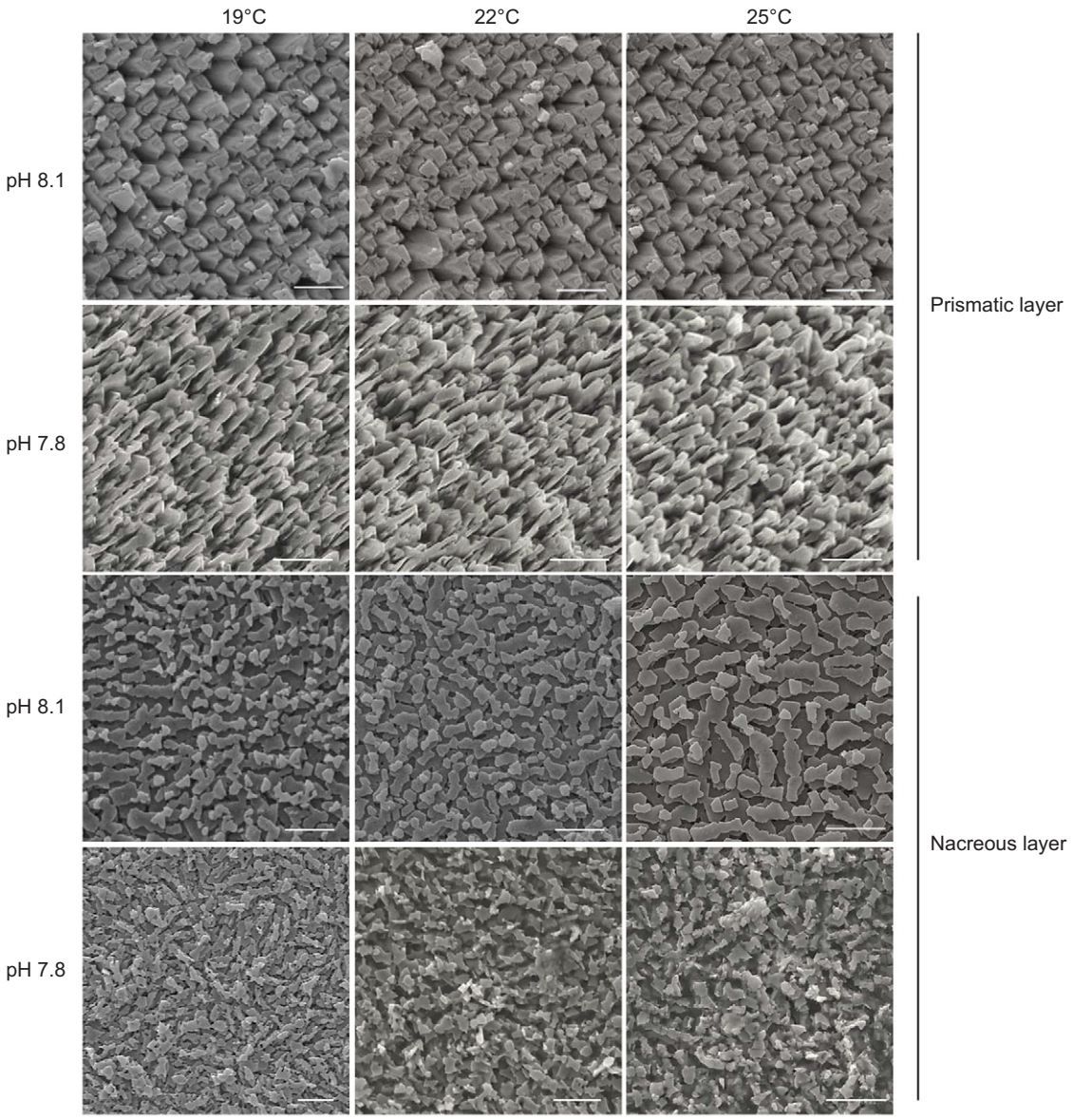

Fig. 3. Effects of elevated P_{CO_2} and temperature on *M. edulis* shell microstructure. Scale bars, 5 μm.

RESULTS

NCR

As shown in Fig. 1, a reduction in pH from 8.1 to 7.8 significantly decreased the NCR by 55.38% at 19°C, 90.28% at 22°C and 92.73% at 25°C (one-way ANOVA, $P<0.05$). The NCR significantly decreased when the temperature was elevated from 19 to 25°C at pH 7.8, while no significant impact was found at pH 8.1 (one-way ANOVA, $P<0.05$). There was a significant interaction between pH and temperature, and elevated temperature enhanced the negative effect of reduced pH on the NCR (two-way ANOVA, $P<0.001$; Table 3).

Calcium and magnesium content

As shown in Fig. 2B, the calcium content on the shell surface decreased significantly with an increase in temperature from 19 to 25°C at pH 7.8, while no significant differences were found at pH 8.1 (one-way ANOVA, $P<0.05$). The elevated temperature exacerbated the effect of P_{CO_2} on calcium content. The same results were also obtained for the ratio of calcium and magnesium (Ca/Mg; Fig. 2C). With a reduction in pH from 8.1 to 7.8, the Ca/Mg ratio decreased by 40.91%, 61.05% and 76.92% at 19, 22 and 25°C, respectively. Elevation of temperature from 19 to 22 and 25°C at pH 7.8 decreased the Ca/Mg ratio by 34.84% and 67.74%, respectively, compared with that at pH 8.1. Moreover, there was a significant interaction between pH and temperature, and elevated temperature enhanced the negative effect of reduced pH on the Ca/Mg ratio (two-way ANOVA, $P<0.001$; Table 3).

Shell ultrastructure

As shown in Fig. 3, the micrographs obtained from SEM revealed that normal calcite and aragonite crystals on the prismatic and nacreous layers in *M. edulis* reared at pH 8.1 and 19°C have uniform structure orientations. At pH 8.1, elevated temperature (22 and 25°C) appeared to have no substantial impact on the ultrastructure, while the calcite and aragonite crystals appeared to be disorientated at pH 7.8 (at all temperature levels). This disorganization of calcite

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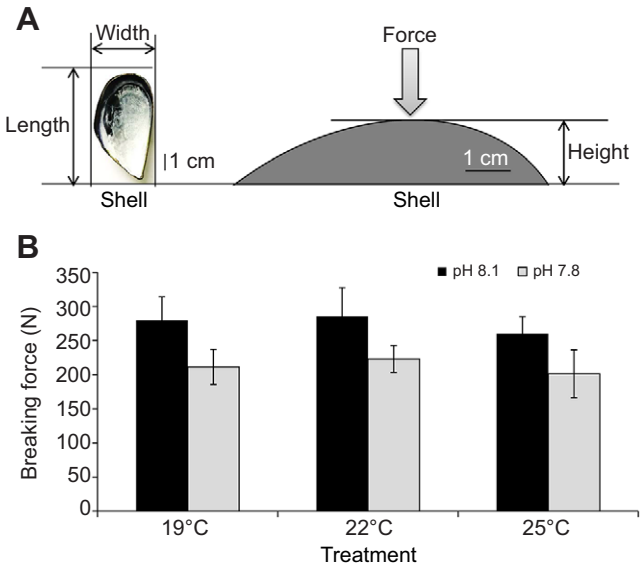

Fig. 4. Effects of elevated P_{CO_2} and temperature on *M. edulis* shell breaking force. (A) Measurement methods for shell morphology and breaking force. (B) Shell breaking force of *M. edulis* exposed to different treatments.

and aragonite crystals appeared to be enhanced as temperature increased.

Shell breaking force

No significant differences in shell length, width and height were observed in any of the treatments (one-way ANOVA, $P<0.05$). When pH was decreased from 8.1 to 7.8, the breaking force of the shells significantly decreased by 24.37% at 19°C, 21.75% at 22°C and 22.69% at 25°C (one-way ANOVA, $P<0.05$; Fig. 4B). There were no significant differences in breaking force observed (one-way ANOVA, $P<0.05$) under elevated temperature within each pH level. Statistical analysis revealed that there was no significant interaction between pH and temperature [two-way ANOVA, $P=0.401$; Table 3], and that pH is the determinant for mechanical shell properties in *M. edulis*.

Expression of biomineralization-related genes

As shown in Fig. 5, *CHI* and *CNN* expression levels increased significantly with a reduction in pH from 8.1 to 7.8 at 19°C (one-way ANOVA, $P<0.05$). At 22°C, *CHI* expression levels increased significantly with a reduction in pH from 8.1 to 7.8 (one-way ANOVA, $P<0.05$), while *CHS*, *CA*, *NACR*, *SILK*, *PWAP* and *PLUC* expression levels were insignificantly depressed (one-way ANOVA, $P>0.05$). At 25°C, the expression levels of all the genes decreased significantly with a reduction in pH from 8.1 to 7.8 (one-way ANOVA, $P<0.05$). At pH 8.1 and 25°C, no notable changes were observed for the expression levels of *CHS*, *CA*, *NACR*, *SILK*, *PWAP* and *PLUC* compared with those at pH 8.1 and 19°C. However, at pH 7.8, expression levels of the eight genes exhibited a decreasing trend with an increase in temperature from 22 to 25°C.

Amino acid content

As shown in Table 4, exposure to pH 7.8 (for all the temperature treatments) led to significant increases in the content of Ala, Arg, Asn, Leu, Met, Phe, Pro, Ser, Glu, Gly, Ile, Thr, Tyr and Val compared with that at pH 8.1 (one-way ANOVA, $P<0.05$), while no significant changes were found for Asp and Gly. The content of all amino acids exhibited an increasing trend with an increase in temperature from 22 to 25°C at pH 7.8, with the exception of Asp and Gly. There were no significant changes in content of any amino acids at pH 8.1 with an increase in temperature from 19 to 22°C; however, the content of all the amino acids increased when the temperature was elevated from 19 to 25°C.

DISCUSSION

When exposed to acidified seawater, marine calcifiers often allocate more energy to essential physiological processes, such as acid–base homeostasis, which will negatively affect the growth, reproduction and defence of these species (Kroeker et al., 2014; Wood et al., 2008). The disturbance of ion and acid–base regulation indirectly inhibits biomineralization by influencing energy and ion supplies for $CaCO_3$ deposition processes. The interaction of elevated temperature and seawater acidification is likely to be highly dependent on the thermal window of marine organisms (Pörtner and Farrell, 2008). For *M. edulis* in our experiments, the highest seawater temperature at the sampling sites was 24–25°C for a duration of no more than 1 month. Elevated temperature may enhance the negative effects of seawater acidification when the level exceeds the thermal window. Firstly, elevated temperature can alter cell membrane permeability, which may further disrupt acid–base homeostasis caused by seawater acidification (Padilla-Gamiño et al., 2013; Pörtner, 2010). Secondly, elevated temperature can influence the activities of proteins involved in acid–base regulation and defence reaction (Anthony et al., 2008; O'Donnell et al., 2009). Finally, elevated temperature can lead to metabolic disturbance

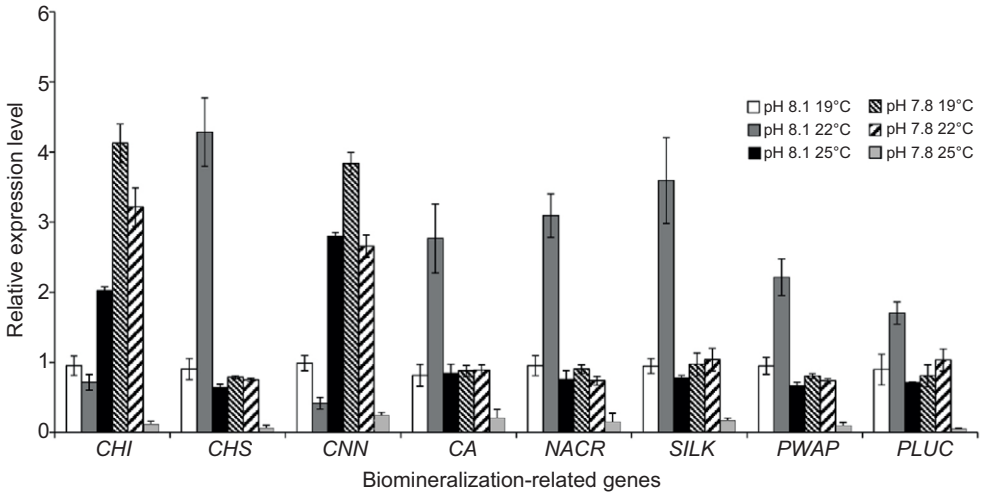

Fig. 5. Effects of elevated P_{CO_2} and temperature on the expression of biomineralization-related genes in *M. edulis*. *CHI*, chitinase; *CHS*, chitin synthase; *CNN*, calponin-like protein; *CA*, carbonic anhydrase; *SILK*, silk-like protein; *NACR*, nacrein-like protein; *PWAP*, perlwapin-like protein; *PLUC*, perlucin-like protein.

Journal of Experimental Biology (2015) 218, 3623-3631 doi:10.1242/jeb.126748

Table 4. Effect of elevated P_{CO_2} and temperature on amino acid content in *M. edulis*

	Amino acid content (mg g^{-1})					
	pH 8.1			pH 7.8		
	19°C	22°C	25°C	19°C	22°C	25°C
Ala	59.36±3.04	61.68±11.75	70.24±7.72	74.17±12.51	90.07±8.45	107.6±18.06
Arg	52.53±2.11	56.24±3.25	86.24±3.77	108.72±9.37	124.62±15.31	142.17±9.56
Asn	7.72±1.43	7.44±1.10	92.44±2.47	14.92±1.71	30.82±1.64	48.37±7.40
Asp	134.44±14.86	134.08±6.27	154.48±15.30	136.56±13.27	132.46±19.21	140.02±12.57
Cys	4.04±0.072	4.03±0.42	4.74±0.42	1.82±0.12	2.41±1.06	15.27±2.35
Gln	3.58±0.12	3.33±0.06	3.46±0.16	0.45±0.02	2.71±2.96	33.91±2.46
Leu	8.92±0.37	8.45±1.10	15.38±2.06	13.93±1.29	29.83±1.23	47.38±1.41
Lys	2.55±0.19	2.47±0.10	4.57±0.78	0.51±0.09	1.85±1.92	4.96±3.40
Met	12.98±2.00	11.80±1.03	17.08±2.12	20.28±2.17	36.18±4.11	53.74±5.33
Phe	16.49±1.30	15.80±2.56	30.19±4.41	34.29±1.29	50.19±7.22	67.74±5.84
Pro	24.26±2.98	25.39±3.35	38.25±4.85	57.87±1.26	73.77±6.20	91.32±9.65
Ser	56.69±2.51	54.79±4.11	79.56±8.05	73.28±4.56	89.18±1.50	106.73±10.42
Glu	117.68±12.41	115.24±10.91	152.35±15.98	153.73±8.97	169.63±14.91	187.17±17.21
Gly	70.43±9.39	71.06±7.25	71.29±10.11	73.54±19.58	70.44±25.52	74.99±13.56
Ile	11.95±3.60	13.92±1.04	33.20±5.00	16.40±1.99	32.30±7.93	49.85±3.35
Thr	22.30±4.94	21.72±1.61	59.05±8.27	42.20±5.10	58.10±2.04	75.66±5.92
Tyr	27.98±2.10	29.78±1.74	49.24±7.30	62.26±2.55	78.16±6.49	95.72±3.04
Val	7.41±1.46	7.78±1.23	24.17±5.31	17.26±2.11	33.16±8.05	50.71±2.54

Ala, alanine and β-alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; Ile, isoleucine; Lys, lysine; Met, methionine; Leu, leucine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

(O'Connor et al., 2009), which may affect the energy tradeoff in *M. edulis*. These responses have been demonstrated in many marine calcifiers, suggesting that the accumulation effects are universal in marine ecosystems (O'Donnell et al., 2009; Pörtner, 2010).

There is always an interactive effect of seawater acidification and elevated temperature on calcification; however, the degree of interaction changes from species to species. The echinoderms and shelled molluscs are vulnerable to seawater acidification and warming because their calcified tissues are formed from highly soluble crystals (Dupont et al., 2010). Consistent with the result for Pacific oyster *C. gigas* (Clark et al., 2013), elevated temperature enhanced the effect of seawater acidification on NCR in *M. edulis*. In addition to NCR, elevated temperature also enhanced the reduction in Ca/Mg ratio caused by seawater acidification. Magnesium can also be incorporated into crystals during biomineralization. Ca/Mg ratio usually changes synchronously with the marine environment and has been used widely as a parameter to estimate the effects of temperature (Raddatz et al., 2013) and pH (Chan et al., 2012) on calcified tissues. The calcium content and Ca/Mg ratio on the shell surface decreased under the stress of seawater acidification and warming, indicating a decreasing trend in $CaCO_3$ deposition in *M. edulis*. Our results therefore provide new evidence that the interaction of these stressors inhibits biomineralization in mussels (Kroeker et al., 2013).

The direct consequence of decreased biomineralization ability is a reduction in shell growth. Mussel shell contains two calcium carbonate crystals: aragonite and calcite. The ultrastructure of calcite and aragonite was apparently altered in *M. edulis* exposed to seawater acidification. Seawater acidification can inhibit aragonite growth in the larval stage and induce the mussels to produce stiffer and harder calcite crystal; meanwhile, calcite growth occurs at a cost to the structural integrity, resulting in more brittle shells (Fitzer et al., 2014a,b; Melzner et al., 2011). Consequently, a significant decrease in shell breaking force was found in adult *M. edulis* in our study. Similar reductions in shell breaking force in the juvenile oyster *Crassostrea virginica* (Dickinson et al., 2012) and the mussel *Mytilus californianus* (Gaylord et al., 2011) have also been reported. However, there was no significant decrease in the shell breaking force in adult *Pinctada fucata* exposed to similar pH conditions (Welladsen et al., 2010), implying the effects of seawater acidification on shell properties may be species specific in marine calcifiers. Hiebenthal et al. (2013) showed that, independently of temperature, P_{CO_2} (308–1655 µatm) had no significant effect on the shell breaking force of Western Baltic Sea *M. edulis*. This contrast with our result may be caused by local adaptation of the different populations of *M. edulis* used in the experiments. The aquaria system (e.g. artificial or natural seawater, flow-through or seawater changes system) may also have influenced the experimental conditions for the mussels, hence the results. Moreover, elevated temperature did not enhance the negative effects of seawater acidification on the shell breaking force in *M. edulis*, indicating that the mussel shells are not more susceptible to seawater acidification in higher temperatures.

The gene expression data revealed the underlying mechanism involved in the biomineralization changes. Our results indicate that *CHI* expression was up-regulated at pH 7.8 at both 19 and 22°C, while no significant changes were detected in *CHS* expression. CHI and CHS function as enzymes to catalyse the degradation and synthesis of chitin in the *M. edulis* mantle (Hüning et al., 2013; Suzuki et al., 2007). Chitin is an important component of the organic framework in shells (Weiss and Schönitzer, 2006), which can directly bind shell matrix proteins, provide sites for nucleation, and control the location and orientation of mineral phases (Ehrlich, 2010). Therefore, the up-regulation of *CHI* expression suggests an increase in chitin degradation, thus causing the disruption of chitin homeostasis and influencing biomineralization in *M. edulis*. Moreover, previous studies suggested that calponin-like protein, a calcium-binding protein, functions as an inhibitor of calcification in *M. edulis* (Funabara et al., 2001; Hüning et al., 2013). The up-regulation of *CNN* at pH 7.8 at 19 and 22°C suggests that the calcification process might be depressed in mussels exposed to seawater acidification. Additionally, no significant changes in either *CA* or shell matrix protein (*SILK*, *NACR*, *PLUC* and *PWAP*) gene expression were detected at pH 7.8 at 19 and 22°C, implying that the carbonate homeostasis and matrix protein regulation might not be significantly influenced. These results are consistent with a previous study on the expression of matrix protein genes in the mantle of *M. edulis* exposed to seawater acidification (Hüning et al., 2013). From

the above information, we speculate that elevated P_{CO_2} (pH 7.8) mainly affects the organic framework formation and biomineralization ability in *M. edulis*. Furthermore, the expression levels of all of the genes decreased at pH 7.8 at 25°C, suggesting that the interaction of elevated P_{CO_2} and temperature might have exceeded the physiological tolerance limits of *M. edulis*. The gene expression changes are consistent with physiological changes in the mussels exposed to seawater acidification and elevated temperature in our study.

Marine organisms usually experience higher energy demands under environmental stress, so changes in the concentration of metabolic parameters (e.g. amino acids) can be used as biomarkers to assess the organism's physiological status (Vargas-Chacoff et al., 2009). Increases in Ala, Pro, Leu and Tyr content were found in *M. edulis* reared at pH 6.5 (Ellis et al., 2014), which is consistent with the changes at pH 7.8 in this study. Asp content decreased at pH 6.5 (Ellis et al., 2014), but the Asp, Gly, Gln and Lys content did not change significantly at pH 7.8 in this study. We propose that Asp metabolism can be stimulated by elevated P_{CO_2}, but in a level-dependent manner, and exposure to higher CO_2 concentrations (pH 6.5) may have triggered the action. Our results also suggest that the interaction of seawater acidification and elevated temperature led to further depression of amino acid metabolism. Exposure to high temperatures that exceed optimum growth conditions can result in metabolic depression in the mussels *Modiolus barbatus* (Anestis et al., 2008) and *M. galloprovincialis* (Anestis et al., 2010). In our study, long-term temperature exposure cumulatively increased the inhibitory effects of seawater acidification on amino acid metabolism in *M. edulis*. Furthermore, metabolism of Ala, Phe and Tyr is involved in energy regulation, biomineralization and byssus production in marine calcifiers, respectively (Ellis et al., 2014; Evans et al., 2013; Reddy and Yang, 2015; Yu et al., 2014). Free amino acids are critical for regulating cellular osmotic equilibrium in calcifiers (Zurburg and De Zwaan, 1981). Therefore, the depression of amino acid metabolism in *M. edulis* may negatively affect energy metabolism, biomineralization and osmoregulation. An investigation of the effects of elevated P_{CO_2} on physiological energetics and biomineralization in different types of mussels supports this hypothesis (Wang et al., 2015).

If the seawater conditions we examined in this study represent future conditions, the interactive effects of elevated P_{CO_2} and temperature may have important ecological and functional implications. On the one hand, the mechanical properties of the shell are critical in protecting the shelled molluscs from damage caused by predation and environmental changes. Changes in *M. edulis* shells are likely to influence predator–prey dynamics in coastal ecosystems (Kroeker et al., 2014). On the other hand, changes in amino acids will alter the nutritional component of the edible parts, which might also influence predator-feeding behaviour at higher trophic levels in the marine food chain.

Our results revealed that elevated temperature enhances the negative effects of seawater acidification on *M. edulis* biomineralization and metabolism on the Yellow Sea coast. The interactive effects of these stressors on mussels may influence coastal ecosystems and have significant ecological and functional implications. These results highlight the importance of examining how stressors will interact, so that accurate predictions of the underlying effects can be made. Therefore, our study will be helpful in understanding the effects of further changes in marine environments on both mussels and other calcifiers.

Acknowledgements
The authors would like to thank Jun Wang for helping with the sample collection. We also thank the editors and reviewers for their helpful comments on the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
S.L. carried out the molecular laboratory work, participated in the design of the study and drafted the manuscript; C.L. carried out the shell analysis. S.L., J.H. and Y.L. participated in data analysis; S.L. and C.L. carried out the statistical analysis; G.Z. collected samples; L.X. and R.Z. coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

Funding
This work was supported by National Basic Research Program of China [grant 2010CB126405], China Postdoctoral Science Foundation Funded Project [grant 2014M550748] and National Natural Science Foundation of China [grants 31372508 and 31372502].

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