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Ocean acidification alters the burrowing behaviour, Ca^{2+}/Mg^{2+}-ATPase activity, metabolism, and gene expression of a bivalve species, *Sinonovacula constricta*

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ABSTRACT: Although the impacts of ocean acidification on fertilization, embryonic development, calcification, immune response, and behaviour have been well studied in a variety of marine organisms, the physiological and molecular mechanism manifesting acidification stress on behavioural response remains poorly understood. Therefore, the impacts of future ocean acidification scenarios (pH at 7.8, 7.6, and 7.4) on the burrowing behaviour, Ca^{2+}/Mg^{2+}-ATPase activity, metabolism, and expression of energy-producing-related genes of the razor clam *Sinonovacula constricta* were investigated in the present study. The results showed that elevated CO_2 partial pressure (pCO_2) (pH at 7.6 and 7.4) led to a significant reduction in the digging depth of the razor clam. In addition, exposure to pCO_2-acidified seawater depressed the metabolism and activity of Ca^{2+}/Mg^{2+}-ATPase, which may partially contribute to the reduced digging depth detected. Furthermore, the expression of energy-producing-related genes was generally induced by exposure to acidified seawater and could be accounted for by an increased energy demand under acidification stress. The results obtained suggest ocean acidification may exert a behavioural impact through altering physiological condition in the razor clam.

KEY WORDS: Ocean acidification · Burrowing behaviour · Metabolism · Gene expression

INTRODUCTION

Since the Industrial Revolution, anthropogenic activities such as fossil fuel utilization, cement production, and biomass burning have increased atmospheric CO_2 partial pressure (pCO_2) dramatically (Gattuso et al. 1998, Widdicombe & Spicer 2008). A large amount (about one-fourth to one-third) of anthropogenic CO_2 in the atmosphere is taken up by the ocean (Sabine et al. 2004), which increases the hydrogen ion concentration in surface seawater through the hydrolysis of CO_2, a process commonly termed ocean acidification (Caldeira & Wickett 2003, Orr et al. 2005, Raven et al. 2005). Ocean acidification is projected to impact marine organisms from the open sea to estuaries and coastal areas (Feely et al. 2009, 2010, Zhao et al. 2017a). To date, ocean acidification has been reported to affect a wide range of marine organisms, such as marine plankton (Porzio et al. 2011, Riebesell et al. 2000), invertebrates (Crim et al. 2011, Waldbusser et al. 2011, Yu et al. 2011), and fish (Munday et al. 2009, 2010, Dixson et al. 2010). It has been shown that ocean acidification not only hampers fertilization success (Kurihara & Shirayama 2004, Havenhand et al. 2008, Shi et al. 2017) and embryonic development (Talmage & Gobler 2010, Crim et al. 2011, Moulin et al. 2011) of free-spawning invertebrates but also leads to a series of

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physiological consequences such as inhibiting calcification of marine calcifiers (Riebesell et al. 2000, Gazeau et al. 2007, Waldbusser et al. 2011, Zhao et al. 2017b), hampering metabolism (Lannig et al. 2010, Zhao et al. 2017b), and depressing the immune responses of marine invertebrates (Bibby et al. 2008, Liu et al. 2016, Su et al. 2017). In addition, ocean acidification may decrease harvests and fishery revenues from shellfish, their predators, and coral reef habitats. It has been predicted that the harvest of shellfish will decrease 6 to 25% accompanied by a 0.1 to 0.2 unit pH decrease over 50 yr (2010 to 2060) (Cooley & Doney 2009). Taking the potentially broad and far-reaching impacts of this phenomenon into account, ocean acidification is therefore envisioned as a threat to marine ecosystems, especially those in coastal regions (Fabry et al. 2008, Widdicombe & Spicer 2008, Porzio et al. 2011).

To date, a series of ocean acidification-induced behavioural changes such as impaired predator discrimination (Munday et al. 2009, Dixson et al. 2010, Roggatz et al. 2016), reduced burrowing behaviour (Green et al. 2013, Clements & Hunt 2015, Clements et al. 2016, 2017), and depressed feeding (Fernández-Reiriz et al. 2011, 2012, Navarro et al. 2013, Vargas et al. 2013, 2015) have been reported in various marine invertebrates. Several studies have shown that the burrowing behaviour of bottom-dwelling bivalves could be sensitive to ocean acidification. For instance, the burrowing behaviour during early post-settlement of the bivalve species *Mercenaria mercenaria* was significantly reduced in acidified sediment (Green et al. 2013). Similarly, it has been shown that the burrowing behaviour of juvenile *Mya arenaria* can be negatively affected by sediment acidification (Clements et al. 2017).

To illustrate the mechanisms underlying ocean acidification-induced behavioural changes, one recent effort in the field is to establish links between physiological and molecular responses and the behavioural changes observed. For instance, a study conducted on the shore crab *Carcinus maenas* showed that ocean acidification changed the charge, structure, and function of important peptide signaling molecules (glycyl-L-histidyl-L-lysine, glycyl-glycyl-L-arginine, and L-leucyl-L-arginine), which led to alteration in egg ventilation behaviour (Roggatz et al. 2016). In addition, some studies suggest that the disruption of gamma-aminobutyric acid (GABA) type A receptor function might offer an explanation by which ocean acidification altered the predator-escaping and burrowing behaviour of marine mollusks (Watson et al. 2014, Clements et al. 2017). Moreover, ocean acidification-induced metabolism change has also been suggested to be one of the reasons causing behavioural alterations of marine organisms (Pörtner et al. 2004, Roggatz et al. 2016). However, few efforts have been made to illustrate the impacts of ocean acidification on bivalves or other marine invertebrates with integrated behavioural, physiological, and molecular data.

The razor clam *Sinonovacula constricta* (Lamarck, 1818) is an important aquaculture species that is widely distributed in the estuarine and intertidal zones of the Indo-Pacific (Feng et al. 2010, Li et al. 2011, Niu et al. 2013). As an important member of the food chain, razor clams also play an ecological role in the stability of the coastal ecosystem (Dumbauld et al. 2009). Encompassing a slender body shape, a muscular foot, and a thin shell, the razor clam is well adapted to a bottom-dwelling lifestyle. The burrowing behaviour is crucial for *S. constricta* to avoid predation and harsh environments and results from the muscular movement of the foot, which is closely related to the biological energy availability of the foot muscle (Gosling 2008, Peng et al. 2016). This foot energy is mainly provided through metabolic pathways such as glycolysis and the tricarboxylic acid (TCA) cycle (Peng et al. 2016). Current knowledge of *S. constricta* focuses on its ecological traits (Chung et al. 2008), nutritional contents (Tamari & Kandatsu 1986), immune characteristics (Feng et al. 2010, Li et al. 2011, Niu et al. 2013), and population genetics (Niu et al. 2012). To the best of our knowledge, it remains unknown whether ocean acidification will affect the burrowing behaviour of the razor clam through inducing physiological and altering molecular signatures.

Therefore, to reveal the impacts of ocean acidification on marine organisms comprehensively, a multiple approach including behavioural, physiological, and molecular analysis was conducted with the razor clam in the present study. The main objectives were to explore whether ocean acidification would affect the burrowing behaviour of the razor clam and, if so, whether the observed impacts could be partially explained by alterations in animal metabolism, energy supply, and the expression of energy-production-related genes.

MATERIALS AND METHODS

Animal collection and maintenance

Adult razor clams were collected from Yueqing Bay (28.28° N, 121.11° E), Wenzhou, China, before the

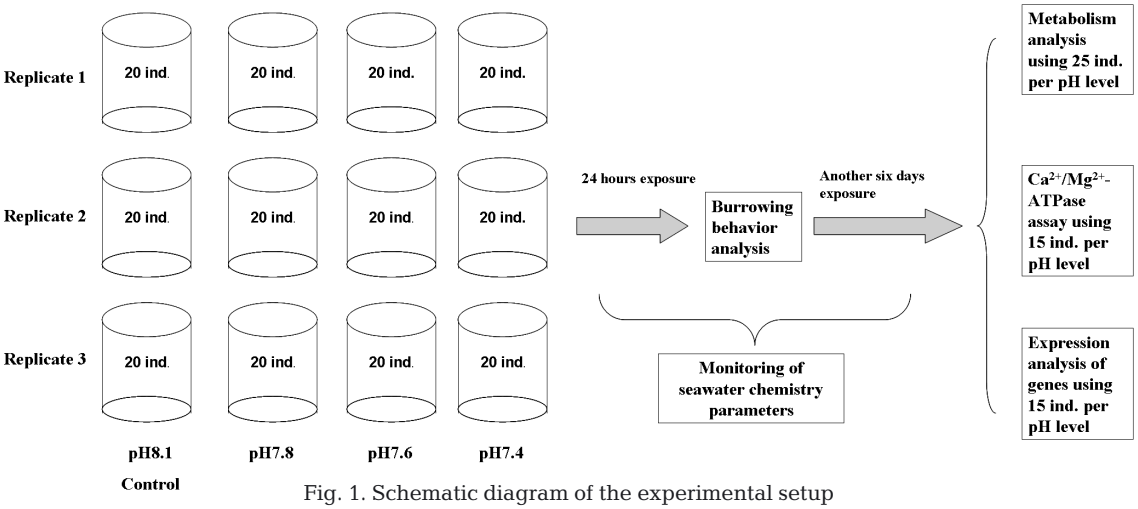

Fig. 1. Schematic diagram of the experimental setup

spawning season from late May to early July 2014. Once transported to the laboratory, clams were acclimated in a 2000 l tank with 500 l of flowing sand-filtered aerated seawater (temperature 23.9 ± 1.0°C, pH 8.10 ± 0.03, salinity 20.0 ± 0.5 PSU, dissolved oxygen 8.01 ± 0.3 mg l^{-1}, and total alkalinity 1.91 ± 0.40 mol l^{-1}) for 1 wk before commencement of the experiment. The clams were fed with microalgae *Platymonas subcordiformis* at the satiation feed rate twice a day at 08:00 and 20:00 h (Shi et al. 2017, Zhao et al. 2017a). Healthy individuals without shell damage and of similar sizes (shell length 5.3 ± 1.1 cm) were used for the experiments.

Burrowing behaviour trials

The burrowing behaviour experiments were carried out in 160 l plastic buckets containing 20 cm of mud (with particle diameters of 7.34 ± 0.76 µm) covered by 30 cm of sand-filtered seawater (Fig. 1). In total, 240 razor clam individuals equally assigned to 12 experimental buckets (3 replicates for pH 8.1, 7.8, 7.6, and 7.4) were used for the burrowing behaviour analysis. Following the method described by Peng et al. (2016), a fishing line (0.18 mm in diameter and 1.5 m in length) was attached to the shell of each razor clam in a longitudinal direction for the estimation of digging depth. As indicated by the preliminary experiment, in which all digging behaviour of the razor clams was completed within 24 h once they were seeded to the bucket and remained barely changed afterwards, an experimental period of 24 h was applied for the burrowing behaviour analysis. The seawater of each trial was aerated with a corresponding air–CO_2 mixture throughout the entire experiment to obtain the respective desired acidification conditions. The simulation of ocean acidification scenarios and the monitoring of seawater chemistry parameters were conducted as described in the following subsection.

Ocean acidification simulation

Seawater aerated with ambient dry air was used as a control (pH at 8.1). Three desired experimental pHs at 7.8, 7.6, and 7.4, levels which are predicted to occur in the near future (IPCC 2014), were achieved by continuous aeration with air–CO_2 mixtures obtained by mixing dry CO_2-free air and pure CO_2 gas at a known flow rate using flow controllers (Shi et al. 2016, Zhao et al. 2017b). To ensure there was no substantial change in the seawater chemistry parameters in each tank during the entire experiment, pH on the National Bureau of Standards scale (pH_{NBS}), salinity, temperature, and total alkalinity (TA) were measured daily. The pH_{NBS} of each level was checked by a pH meter (PB-10, Sartorius) calibrated with NBS standard buffers. Salinity was determined using a conductivity meter (Multi 3410, WTW). Temperature was measured with a mercury thermometer. TA was obtained by potentiometric titration (Anderson & Robinson 1946). Carbonate system parameters were calculated from the measured pH_{NBS}, salinity, temperature, and TA values using the open-source program CO2SYS (Pierrot et al. 2006), with the constants supplied by Mehrbach et al. (1973) and refitted by Dickson & Millero (1987) and the KSO_4 dissociation constant from Dickson (1990). Both measured and calculated seawater parameters of the experimental trials are summarized in Table 1.

Table 1. Seawater parameters during the 1 wk incubation of *Sinonovacula constricta* (mean ± SE). Partial pressure of CO_2, dissolved inorganic carbon, and saturation state of aragonite and calcite were calculated from measured pH_{NBS}, salinity, temperature, and TA values using the open-source program CO2SYS. T: temperature; Sal: salinity; pH_{NBS}: pH on the National Bureau of Standards scale; TA: total alkalinity; pCO_2: CO_2 partial pressure; DIC: dissolved inorganic carbon; Ωara: aragonite saturation state; Ωcal: calcite saturation state

Target pH	T (°C)	Sal (PSU)	pH_{NBS}	TA (μmol kg^{-1})	pCO_2 (μatm)	DIC (μmol kg^{-1})	Ωara	Ωcal
8.1	23.9 ± 0.2	20.83 ± 0.25	8.10 ± 0.03	2074.77 ± 11.55	549.63 ± 2.86	1933.21 ± 18.45	1.97 ± 0.01	3.17 ± 0.02
7.8	24.0 ± 0.1	20.66 ± 0.21	7.78 ± 0.02	2092.65 ± 7.33	1187.72 ± 6.01	2044.56 ± 13.33	1.06 ± 0.01	1.71 ± 0.01
7.6	24.0 ± 0.4	20.76 ± 0.45	7.61 ± 0.01	2097.38 ± 11.54	1934.88 ± 4.67	2101.96 ± 18.73	0.69 ± 0.01	1.11 ± 0.01
7.4	24.0 ± 0.3	20.87 ± 0.21	7.41 ± 0.03	2062.17 ± 17.32	3064.16 ± 27.21	2122.46 ± 31.18	0.44 ± 0.01	0.70 ± 0.01

Oxygen consumption rate and ammonia excretion rate

After 1 wk of exposure to seawater at experimental and control pHs, 25 individuals from each pH exposure trial were divided equally into 5 closed-glass respirometers (2 l) filled with oxygen-saturated filtered seawater pre-equilibrated to the corresponding pH values following the method described by Zhao et al. (2017b). In total, there were 5 replicates for each pH tested containing 5 individuals in each replicate, and a blank trial was conducted with no individual assigned. After an experimental duration of 1 h, the oxygen consumption rate and the ammonia excretion rate were analyzed following the methods described by Peng et al. (2016) and Zhao et al. (2017b). To obtain the oxygen consumption rate, the dissolved oxygen concentrations before and after the experiment were determined by an oxygen meter (Multi 3410 SET4, WTW). To obtain the ammonia excretion rate, the seawater ammonia concentrations before and after the experiment were measured using the standard indophenol blue photometric method (Ivančič & Degobbis 1984). The differences between the final oxygen (or ammonium) concentration value of the experimental trial and the mean concentration of the blank controls (without razor clams) were used for estimation of the oxygen consumption (or ammonium excretion) rate. After the measurement, the soft tissue of each individual was excised carefully with a scalpel and then dehydrated in an 80°C oven for 2 wk. Dry weights of the soft tissues were determined using a Sartorius electronic balance (BSA2245). The oxygen consumption rate and ammonia excretion rate were calculated using the following equation (Peng et al. 2016, Zhao et al. 2017b).

$$R(E) = \frac{(C_{t0} - C_{t1}) \cdot V}{w \cdot t} \quad (1)$$

where $R(E)$ is the oxygen consumption (or ammonium excretion) rate, C_{t0} and C_{t1} represent the concentrations of oxygen (or ammonium) before and after the experiment, t is the total respiration (or excretion) time, w is the dry weight of the soft tissues, and V is the volume of the respiratory (or excretion) chamber.

Ca^{2+}/Mg^{2+}-ATPase activity assays

After a 1 wk exposure to the experimental and control seawater, 5 individuals from each experimental bucket were used as 1 replicate for the Ca^{2+}/Mg^{2+}-ATPase activity assay following the method described by Peng et al. (2016). The foot of each razor clam was carefully excised on ice and then used for enzyme activity analysis following the protocol of the Minim ATP enzyme test kit (Ca^{2+}/Mg^{2+}-ATPase) from Nanjing Jiancheng Bioengineering Institute®. Ca^{2+}/Mg^{2+}-ATPase hydrolyzes ATP into ADP and inorganic phosphate. The concentrations of the inorganic phosphate produced from ATP hydrolysis were determined using a spectrophotometer (UV-2100, Shanghai Jinghua Instruments) at 636 nm, and subsequently the Ca^{2+}/Mg^{2+}-ATPase activities were estimated according to Eq. (2). One Ca^{2+}/Mg^{2+}-ATPase activity unit was defined as the amount of enzyme decomposing 1 μmol ATP per milligram tissue protein per hour. Total protein concentrations were determined following the method described by Bradford (1976) and Peng et al. (2015).

$$\text{Activity} = \frac{(OD_{\text{measured}} - OD_{\text{control}}) / (OD_{\text{standard}} - OD_{\text{blank}}) \cdot C}{C_{\text{protein}} \cdot t} \quad (2)$$

where OD_{measured}, OD_{control}, OD_{standard}, and OD_{blank} indicate the optical density values obtained for the tested sample, control, standard sample, and blank, respectively; C and C_{protein} indicate the concentra-

Table 2. Primer sequences for the 6 transcripts investigated and the internal reference 18S rRNA

Primers for the genes analyzed	Sequence (5′ to 3′)	Accession No.
Citrate synthase-F	CAGTTCAGTGCTGCCATA	JZ897771
Citrate synthase-R	CAAGTTACGGTAGATGATAGAC	
Isocitrate dehydrogenase (NAD^+)-F	GCAGGCAAGATGGTATGA	JZ897772
Isocitrate dehydrogenase (NAD^+)-R	GATGCTGAGATGTCTATGGA	
Isocitrate dehydrogenase ($NADP^+$)-F	ATGTTGCTAAGGATGTTACC	JZ897773
Isocitrate dehydrogenase ($NADP^+$)-R	TTAGGAGATGGACTGTTCTT	
Oxoglutarate dehydrogenase-F	GGCATTACAACAGAAGAGAAG	JZ897774
Oxoglutarate dehydrogenase-R	GTAGACACGCTGGAAGATG	
Dihydrolipoamide succinyltransferase-F	GCATAGGTCTGGATAGCA	JZ897775
Dihydrolipoamide succinyltransferase-R	CTTGTTGTTCTACCATGTTG	
Dihydrolipoamide dehydrogenase-F	ACAGGCTCTGAAGTCACA	JZ897776
Dihydrolipoamide dehydrogenase-R	GCACCAATCACAATCATCTT	
18S ribosomal RNA-F	TCGGTTCTATTGCGTTGGTTTT	AY695800.2
18S ribosomal RNA-R	CAGTTGGCATCGTTTATGGTCA	

tions of protein in the standard and sample, respectively; and t is the reaction time.

Quantitative real-time PCR of energy-producing-related genes

After exposure to pH 7.4, 7.6, 7.8, and 8.1 for 1 wk, 5 razor clams from each experimental bucket were dissected on ice and used as 1 replicate for the corresponding pH tested. The foot of each razor clam was carefully excised with a scalpel and immediately frozen in liquid nitrogen for real-time PCR analysis following the method described by Peng et al. (2016). Total RNA from each foot tissue was extracted using TriPure reagent (Aidlab Biotechnologies) according to the manufacturer's instructions. RNA quality was checked by gel electrophoresis, and RNA quantity was determined on a NanoDrop 1000 spectrophotometer (Thermo Scientific®). High-quality total RNA was then reverse transcribed (M-MLV, Invitrogen). The transcripts citrate synthase, isocitrate dehydrogenase, oxoglutarate dehydrogenase, dihydrolipoamide succinyltransferase, and dihydrolipoamide dehydrogenase were investigated in the present study. The 18S ribosomal RNA gene was used as an internal reference, and all corresponding primer sequences (Table 2) were obtained from published literature (Peng et al. 2016). Quantitative real-time PCR was performed with a CFX 96™ real-time system. PCR amplifications were carried out in triplicate in a total volume of 10 µl containing 5 µl SsoFast EvaGreen Supermix (Bio-Rad®), 3 µl PCR-grade water, 1 µl cDNA, and 1 µl of each primer (100 µM). The cycling included an initial denaturation at 95°C for 5 min followed by 39 cycles of 95°C for 20 s, 61°C for 20 s, and elongation at 72°C for 20 s. Melting-curve analysis was performed with a Bio-Rad CFX Manager to confirm that a single PCR product was produced. The $2^{-\Delta\Delta CT}$ method was then applied to analyze the relative gene expression of the tested genes.

Statistics

To investigate the effect of different pHs (pCO_2) on the burrowing behaviour of *Sinonovacula constricta*, the digging depths of razor clams exposed to various pHs were analyzed using a 1-way ANOVA followed by Tukey's post hoc tests. The same statistical tests were conducted to investigate the effects of ocean acidification on the oxygen consumption rates, ammonia excretion rates, Ca^{2+}/Mg^{2+}-ATPase activities, and expression of energy-producing-related genes. For all analyses, the assumptions of normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's tests, respectively. In cases where the above assumptions were not satisfied by raw data, the data were log transformed prior to analysis (Zhao et al. 2017b). All statistics were conducted with the statistical package R (www.r-project.org), and all data were presented as mean ± SE. A p-value < 0.05 was accepted as statistically significant.

RESULTS

As shown in Table 3 and Fig. 2, although no significant difference between pH 7.8 and the control

Table 3. One-way ANOVA for the digging depths of razor clams after 24 h exposure to various pHs (pCO_2)

Factor	df	SS	MS	*F*-value	p-value
Model	3	2.30	0.77	$F_{3,12} = 7.46$	0.01
Error	8	0.82	0.10		
Total	11	3.12			

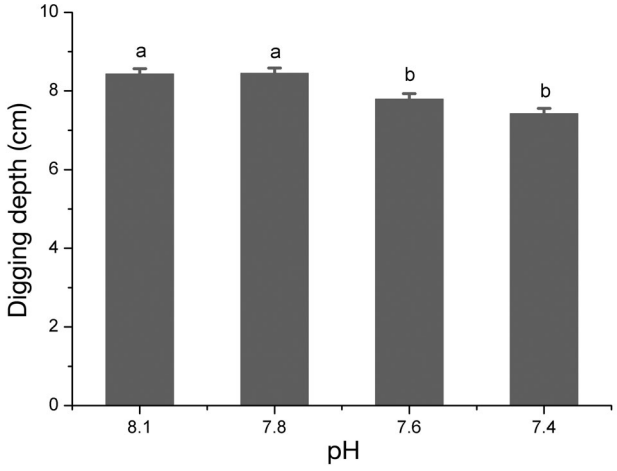

Fig. 2. Digging depths (mean ± SE) of razor clams exposed to various pHs (CO_2 partial pressure) for an experimental period of 24 h. Different letters indicate significant differences between trials by Tukey's test

(pH 8.1) groups was detected, the digging depths of the razor clams exposed to pH 7.6 and pH 7.4 were significantly less than those exposed to pH 8.1 and 7.8 ($p < 0.05$), and the reduction in depth corresponded about 11.78 and 18.87% of the body length of the razor clams.

The oxygen consumption and ammonium excretion rates of razor clams exposed to pH 8.1, 7.8, 7.6, and 7.4 for 1 wk are listed in Table 4. Results of 1-way ANOVA analysis showed that the oxygen consumption rates and the ammonium excretion rates were significantly affected by pH. When exposed to pCO_2-

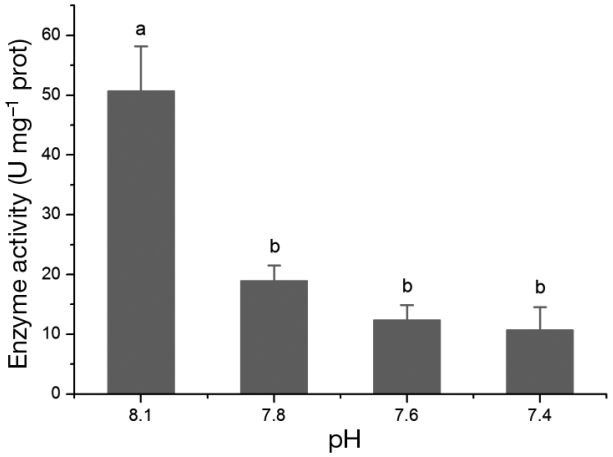

Fig. 3. Enzyme activities of the Ca^{2+}/Mg^{2+}-ATPase (mean ± SE) of razor clams after 1 wk of exposure to simulated ocean acidification (pH at 7.8, 7.6, 7.4) and the ambient control (pH at 8.1). Different letters indicate significant differences between trials by Tukey's test

acidified seawater at pH 7.8, 7.6, and 7.4, the oxygen consumption rates of razor clams were about 85.12, 83.47, and 76.86%, respectively, of that of the control. Similarly, compared to that of control, exposure to pCO_2-acidified seawater at pH 7.8, 7.6, and 7.4 brought about 19.45, 33.68, and 33.55% reductions, respectively, in the ammonium excretion rates.

As shown in Fig. 3, the activities of Ca^{2+}/Mg^{2+}-ATPase were significantly affected by acidification ($p_{3,12} = 1.32 \times 10^{-4}$, ANOVA). The Ca^{2+}/Mg^{2+}-ATPase activities of the foot tissue of razor clams exposed for 1 wk to pCO_2-acidified seawater were significantly lower (about one-fifth to two-fifths) than that of the control.

All of the 6 investigated genes of the TCA cycle (Fig. 4) showed a similar relative expression pattern, with the highest gene expression (about 9.75 to 22.30 times those of control) detected in razor clams exposed to pCO_2-acidified seawater at pH 7.8. When exposed to more strongly acidified experimental seawater, the gene expression of all genes examined

Table 4. Oxygen consumption rates and ammonium excretion rates (mean ± SE) of *Sinonovacula constricta* after 1 wk of exposure to simulated acidification (pH at 7.8, 7.6, 7.4) and the ambient control (pH at 8.1). Different superscript letters indicate significant differences between trials by Tukey's test

	pH				*F*-value	p-value
	8.1	7.8	7.6	7.4		
Oxygen consumption rate (μmol g^{-1} h^{-1})	37.81 ± 0.94^{a}	32.19 ± 0.31^{a}	31.56 ± 1.88^{b}	29.06 ± 1.56^{b}	$F_{3,12} = 7.28$	1.13×10^{-2}
Ammonium excretion rate (μmol g^{-1} h^{-1})	7.66 ± 0.52^{a}	6.17 ± 0.38^{a}	5.08 ± 0.65^{b}	5.09 ± 0.80^{b}	$F_{3,12} = 7.66$	9.74×10^{-3}

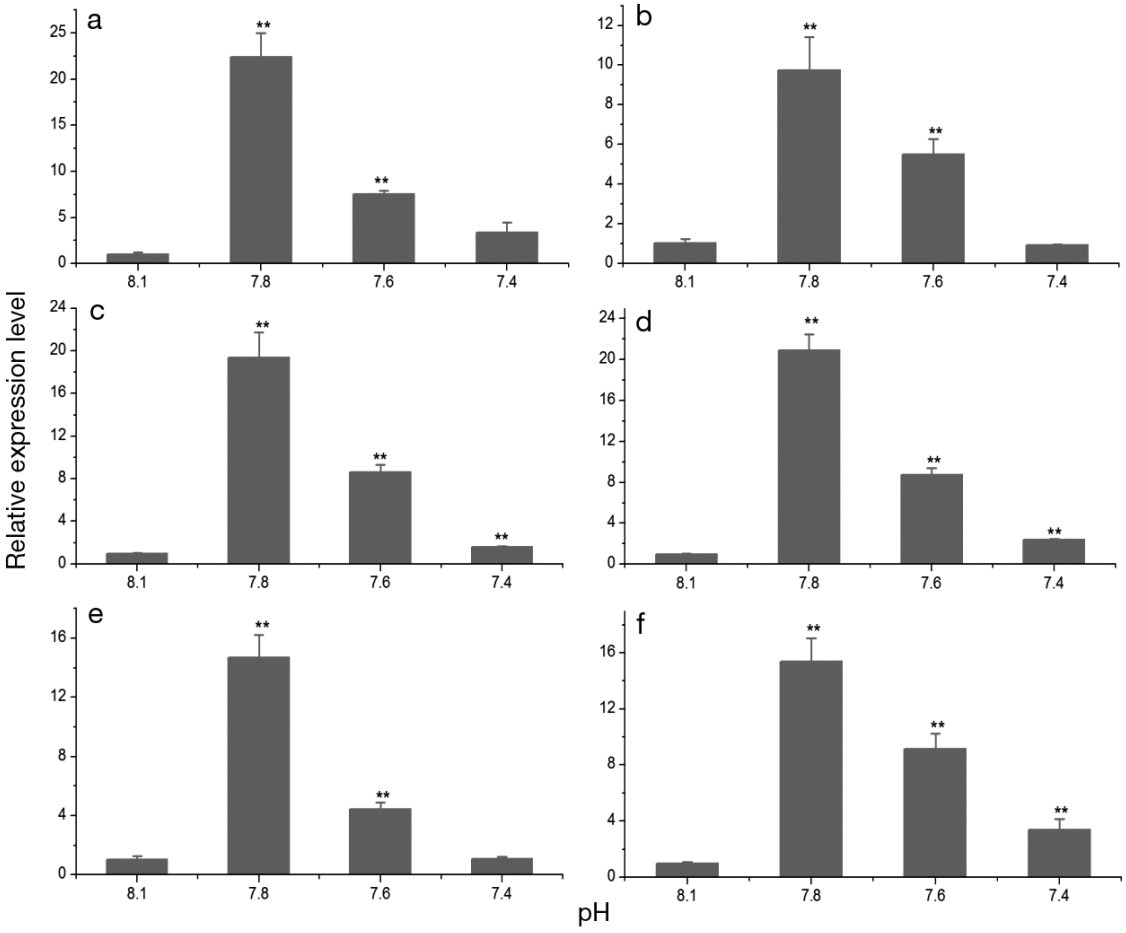

Fig. 4. Relative gene expression levels (mean ± SE) of genes of the tricarboxylic acid pathway. (a) Citrate synthase, (b) NAD^+-dependent isocitrate dehydrogenase, (c) $NADP^+$-dependent isocitrate dehydrogenase, (d) oxoglutarate dehydrogenase, (e) dihydrolipoamide succinyltransferase, and (f) dihydrolipoamide dehydrogenase of razor clams exposed to 1 wk of manipulated acidification (pH at 7.8, 7.6, 7.4) and the ambient control (pH 8.1). ** Significant differences ($p < 0.01$) relative to the control by 1-way ANOVA

decreased with declining experimental pHs (an increase in pCO_2) and were about 4.43 to 9.20 and 0.97 to 3.40 times those of the control for pH 7.6 and pH 7.4, respectively. The relative expression levels of isocitrate dehydrogenase, oxoglutarate dehydrogenase, and dihydrolipoamide dehydrogenase exposed to the simulated ocean acidification at pH 7.4 were significantly higher than those of the control.

DISCUSSION

Since the introduction of the concept of ocean acidification (Caldeira & Wickett 2003, Raven et al. 2005), there is a growing international concern about the effects of ocean acidification on marine organisms. Although various biological impacts of ocean acidification on different marine species have been increasingly well documented (Riebesell et al. 2000, Gazeau et al. 2007, Bibby et al. 2008, Lannig et al. 2010, Waldbusser et al. 2011, Liu et al. 2016, Zhao et al. 2017a,b), comprehensive investigation integrating behavioural, physiological, and molecular data is still scarce.

Different behavioural alterations due to the effects of ocean acidification on various marine mollusks including *Mercenaria mercenaria*, *Mercenaria arenaria*, *Ruditapes decussatus*, *Mytilus galloprovincialis*, *Mytilus chilensis*, and *Concholepas concholepas* have been revealed in recent years (Fernández-Reiriz et al. 2011, 2012, Green et al. 2013, Navarro et al. 2013, Vargas et al. 2015, Clements et al. 2016, 2017); however, the susceptibility of the burrowing behaviour of the razor clam remains unknown. The burrowing and/or emerging behaviours of bottom-burrower bivalves are crucial for individuals to avoid

predation and harsh environments and are directly controlled by foot muscular movement through an energy-consuming process (Gosling 2008, Peng et al. 2016). Alteration in the burrowing behaviour of razor clams detected in the present study may be due to a series of interdependent and coordinated physiological changes induced by elevated pCO_2.

pCO_2-acidified seawater may affect the transmission of neural signals that control the activation of muscle cells. It has been suggested that as a consequence of maintaining *in vivo* acid–base balance, ocean acidification may lead to alterations in the concentration of *in vivo* ions such as H^+, HCO_3^-, Ca^{2+}, Mg^{2+}, and Cl^- of marine invertebrates (Pörtner et al. 1998, Miles et al. 2007, Stumpp et al. 2012, Clements et al. 2017, Zhao et al. 2017b). Hence, the alterations in specific ions may affect the transmission of neural signals and subsequently change animal behaviour such as the burrowing of razor clams, as investigated in the present study. Notably, since neural signal transmission through GABA–GABA type A receptors is sensitive to *in vivo* HCO_3^- and Cl^- concentration changes and is directly related to muscle movement (Nilsson et al. 2012, Watson et al. 2014, Clements et al. 2017), ocean acidification-induced corresponding ion concentration changes could also disrupt neural signal transmissions and subsequently affect movement of the foot muscle.

Ocean acidification renders individuals in a stressed condition and therefore may put a constraint in energy supply for foot muscular movement. It is possible that ocean acidification depresses individual metabolism and therefore reduces the overall energy budget. Bivalve species are poor iono- and osmoregulators and possess limited physiological capacities to compensate for acid–base disturbances (Whiteley 2011). Therefore, the pCO_2 increase in coelomic fluid caused by ocean acidification may hamper blood oxygen transportation and subsequently respiration of marine invertebrates (Spicer et al. 2011, Dupont & Thorndyke 2012, Zhao et al. 2017b). For instance, in the present study, a significant decline in both the oxygen consumption and ammonia excretion of razor clams exposed to elevated pCO_2 was detected, indicating that the metabolism of the razor clams was inhibited (Carter et al. 2013, Hennige et al. 2014). On the other hand, to compensate for the negative influences brought by elevated pCO_2, marine invertebrates may reallocate their finite energy resources for more fundamental life processes (Beesley et al. 2008, Evans & Watson-Wynn 2014, Shi et al. 2016), hence putting a restriction on the energy available to burrowing behaviour. In the present study, it has been shown that ocean acidification significantly depressed the activity of Ca^{2+}/Mg^{2+}-ATPase, a high-affinity Ca^{2+}-stimulated, Mg^{2+}-dependent ATP-hydrolyzing enzyme directly related to muscular contraction (Migdalis et al. 2000, Ogi et al. 2000, Yokomori et al. 2001, Ji et al. 2009, Hammer & Sellers 2012). Since the Ca^{2+}/Mg^{2+}-ATPase catalyzes the decomposition of ATP, providing energy for muscular movement (Peng et al. 2016), data obtained in the present study indicated a reduced energy availability for foot muscular contraction.

Ocean acidification-induced gene expression change has been well documented in various marine organisms and has been suggested as a mechanism by which ocean acidification exerts corresponding impacts at the molecular level (O'Donnell et al. 2010, Liu et al. 2016, Shi et al. 2016, Zhao et al. 2017a). However, results obtained in the present study indicated that the expression of metabolism-related genes investigated failed to explain the measured metabolism results. Although the metabolism of razor clams was depressed in pCO_2-acidified seawater, expression of the 6 energy-producing-related genes of the TCA cycle was induced. It has been shown that different adaptive responses can be induced by different levels of pCO_2 (Zhao et al. 2017a). In the present study, razor clam individuals may attempt to boost the TCA cycle via upregulating the expression of energy-producing-related genes to compensate for the hampered metabolism challenged by moderate elevated pCO_2. However, when significant metabolism reduction is induced by severe pCO_2 induction, this basal response may become insufficient and be taken over by other reactions in the body.

The present study showed that ocean acidification exerts significant impacts on the razor clam *Sinonovacula constricta*, not only at the behavioural level but also at the physiological and molecular levels. The burrowing behaviour alteration detected may be partially explained by the reduction in energy availability for foot muscular movement due to significant hampered metabolism rates and Ca^{2+}/Mg^{2+}-ATPase activity.

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