

Biochemical responses of the copepod *Centropages tenuiremis* to CO₂-driven acidified seawater

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ABSTRACT

An ecophysiological experiment was conducted to examine the biochemical effects of acidified seawater containing elevated concentration of CO₂ (C_{CO2} 0.08, 0.20, 0.50 and 1.00%) on the copepod *Centropages tenuiremis*. AchE, ATPase, SOD, GPx, GST, GSH level and GSH/GSSG ratio of the copepod were analyzed. The results showed that elevated C_{CO2} and the duration of culture time significantly influenced several biochemical indices in *C. tenuiremis* (ATPase, GPx, GST, GSH and SOD).

Furthermore, the principal component analysis results indicated that 72.32% of the overall variance was explained by the first three principal components (GPx, SOD and GSH). Changes in GPx and GSH levels may play a significant role in the antioxidant defense of copepods against seawater acidification. The long-term response of copepods to seawater acidification and the synergistic effects of acidification with other environmental factors, such as temperature, salinity and trace metal need further investigation.

Key words | antioxidant system, *Centropages tenuiremis*, CO₂, detoxificative defense, seawater acidification

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INTRODUCTION

The current increased atmospheric carbon dioxide (CO₂) concentration and the decreased pH of the world's oceans (i.e., ocean acidification) are important consequences of anthropogenically generated atmospheric CO₂ (Guinotte & Fabry 2009). It is estimated that atmospheric CO₂ concentration by the end of this century will reach approximately 0.075%, which exceeds the CO₂ levels over the last 650,000 years, and most likely over the past 20 million years (IPCC 2001). The accumulation of CO₂ in sea surface water due to the increase of atmospheric CO₂ from pre-industrial times has already caused a decrease of almost 0.1 pH units, and is expected to decrease by another 0.3–0.5 pH units by the end of this century (Caldeira & Wickett 2005).

Copepods, as non-calcified organisms, play an important role in food webs (Shek & Liu 2010). Studies on the impacts of seawater acidification on copepods, show that the potential deleterious effects on the reproductive performance and the developmental dynamics but not on the adult copepod survival (Kurihara *et al.* 2004a, b; Mayor *et al.* 2007; Kurihara & Ishimatsu 2008). So far, the studies have focused mainly on reproductive

performance and survival, and it is necessary to look beyond mortality as a single endpoint when considering the effects of ocean acidification, and instead look for a suite of more subtle changes, which may indirectly affect the functioning of organisms. According to our knowledge few studies have investigated the biochemical response of copepods to seawater acidification. The activities of antioxidants and other important enzymes in copepods may respond to the intracellular damage caused by seawater acidification, and so the aim of this study was to investigate how levels of acidified seawater and exposure time influence certain activities in the planktonic copepod *Centropages tenuiremis* Thompsen & Seott, which is widely distributed along the coast of China and can adapt to wide ranges of temperature and salinity.

Copepods have no special respiratory organs. Gas exchange takes place through the entire thin integument. The concentration of CO₂ between the body fluid and the extra-seawater is similar, and the buffer effect through accumulating HCO₃⁻ is not effective (Pörtner *et al.* 2004; Pörtner 2008). The antioxidant system in copepods plays

an important role in resisting the fluctuation of the environmental condition. In this paper, we hope to illuminate the change of the antioxidant system in copepods after exposure to acidified seawater. The elevated concentration of CO₂ levels (C_{CO2}) used were 0.08% (the predicted C_{CO2} by the end of this century), 0.20% (the C_{CO2} predicted for 2300), 0.50 and 1.00% (much higher C_{CO2} levels which might occur near the CO₂ sequestration sites). To examine the interaction of seawater acidification and duration of exposure, we exposed *C. tenuiremis* to elevated C_{CO2} seawater for four days, and evaluated the effects of this combination on: (1) antioxidant status, activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), reduced glutathione (GSH) and the ratio of reduced and oxidized glutathione (GSH/GSSG); (2) the activity of acetylcholinesterase (AChE), which is vital to normal behavior and muscular function and represents a prime target on which some toxicants can exert a detrimental effect; and (3) the activity of adenosinetriphosphatases (ATPase) in *C. tenuiremis*.

MATERIALS AND METHODS

Sampling

C. tenuiremis was collected from Xiamen Bay, People's Republic of China (24°27.23' N, 118°04.23' E) using plankton net of mesh size 160 μm. The pH of the seawater near the collection site was 8.15 ± 0.11, the temperature was 21 ± 1 °C, and the salinity was 28–31. The plankton samples were diluted to 10 L using filtered natural seawater (S = 28–31) and taken to the laboratory within 1 h of collection. Adult females (body size 1.45–1.60 mm, with a well developed gonotome) were sorted and incubated in natural seawater before the exposure experiments.

Preparing the culture seawater

The culture seawater was aerated before the experiment and then filtered through a 0.45 μm Millipore filter into five 5 L bottles. One of these was the control without further

bubbling, the pH was measured just before the experiment. The average pH of the control seawater was 8.16 ± 0.03. The other four bottles were bubbled with air mixtures, containing 0.08, 0.20, 0.50 or 1.00% CO₂; 21.00% oxygen; and 78.92, 78.80, 78.50 or 78.00% nitrogen. The flow rate of the air mixtures was 300 mL/min, which was adjusted and measured using a flow rate meter (ZBWJ-90, ZHENGBO, China). A pH controller (HOTEC pH/ORP-101 with a Broadley James pH electrode) was used to monitor the seawater pH. After stabilization, the bubbling process was stopped. The average pH of each CO₂ level is shown in Table 1. The bubbling process was conducted twice to ensure that the seawater was acidified sufficiently for the copepod exposure experiment.

Experimental design and biochemical parameter assay

Experimental design

Glass flasks (500 mL) were filled with natural seawater or acidified seawater driven by different elevated C_{CO2}. The seawater was enriched with 4 × 10⁴ cell/mL *Isochrysis* sp. and 0.5 × 10⁴ cell/mL *Platymonas* sp., as food for *C. tenuiremis*. Fifty adult female copepods were placed into each flask, and 16 flasks were used for C_{CO2} level. Plugs were fitted in order to avoid exchange of CO₂ with the air during the exposure time. *C. tenuiremis* was exposed to CO₂-driven acidified seawater for four days at a temperature of 21 °C under natural light. Approximately 200 copepods from four flasks of every treatment were collected daily and immediately stored at –80 °C. The seawater in the other flasks was replaced with the relevant natural or acidified seawater every two days. The pH values of the seawater are shown in Table 1. The exposure experiment was conducted three times, and thus the total number of copepods sampled was 200 × 4 exposure time × 5 concentration of CO₂ levels × 3 replicates (i.e., 12,000 individuals).

Biochemical parameter assay

Before biochemical assay, the samples were processed based on the methods described by Wang & Wang (2009), modified

Table 1 | Average seawater pH of each C_{CO2} level before and after *C. tenuiremis* culture

	C _{CO2} (%) Ctrl	0.08	0.20	0.50	1.00
Before culture	8.16 ± 0.03	7.80 ± 0.01	7.34 ± 0.01	7.11 ± 0.03	6.85 ± 0.04
After culture	8.20 ± 0.05	7.82 ± 0.05	7.37 ± 0.04	7.16 ± 0.06	6.92 ± 0.05

as follows every sample (~200 copepods) was homogenized using a digital sonifier cell disrupter (model 450, Branson, USA) for 3 min with 20 mmol/L Tris-buffer (pH 7.6, containing 1 mmol/L EDTA, 0.25 mol/L sucrose, 0.15 mol/L NaCl, 1 mmol/L dithiothreitol) at 0 °C. The homogenate was centrifuged at 15,000g for 20 min at 4 °C, and the supernatant was used for biochemical parameter assay. Protein determination was performed using the method of Bradford (1976) with bovine serum albumin as a standard.

The measurement of AchE activity was based on the colorimetric method of Ellman *et al.* (1961) with acetylthiocholine iodide as substrate and dithiobisnitrobenzoate as reagent at 37 °C. One unit of AchE activity (U/mg prot) was defined as the number of μmol of substrate broken down per mg of protein tissue at 37 °C in a hydrolyzed reaction system.

The ATPase activity was determined using the method used by Pullman *et al.* (1960). One unit of ATPase activity ($\mu\text{molPi}/\text{mg prot}/\text{hour}$) was defined as the number of μmol of inorganic phosphorus used per hour per mg of protein tissue during conjugation with ATPase to catalyze ATP.

Total SOD activity was determined as previously described (Spitz & Oberley 1989). One unit of SOD activity (U/mg prot) was defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium reduction per mg of protein.

The GPx activity was measured according to the modified method of Xia & Zhu (1987). One unit of GPx activity (U/mg prot) was defined as the amount of enzyme decreasing 1 $\mu\text{mol}/\text{L}$ of GSH in a catalyzed reaction system per mg of protein tissue conjugated per min.

The GST activity was determined using 1-chloro-2, 4-dinitrobenzene as the substrate, as previously described by Habig *et al.* (1974). One unit of GST activity (U/mg prot) was defined as the amount of enzyme decreasing 1 $\mu\text{mol}/\text{L}$ of GSH in a catalyzed reaction system per mg of protein tissue conjugated per min at 37 °C.

The GSH and GSSG contents were determined using a spectrofluorimetric assay, as previously described by Wang & Wang (2009). They were expressed as micrograms per milligram of protein.

Statistical analysis

All measurements were replicated at least three times and the data were expressed as mean values \pm standard deviation (SD). Prior to any statistical analysis, data were log-transformed to meet ANOVA assumptions of normality. After being transformed and the normality test, only data

meeting the normality requirements were used in the two-way ANOVA and LSD test. The non-normality data were analyzed using a nonparametric ANOVA in order to check whether the differently acidified groups had significant impacts on the biochemical parameters. Significant differences were indicated at $p < 0.05$. The Pearson's test was used to compute any correlation between these variables. All the data except the GSH/GSSG ratio, were subjected to a principal component analysis (PCA, significant at a factorial weight > 0.7). Statistical analysis was performed using SPSS v. 16.0.

RESULTS

Effects of elevated p_{CO_2} and culture time on various biochemical parameters in the copepod *C. tenuiremis*

Figure 1(a) shows the response of GPx activity in *C. tenuiremis* to different elevated C_{CO_2} levels. Meanwhile, the results of two-way ANOVA indicated that the elevated C_{CO_2} levels had significant impacts on the GPx activity (Table 2, $p < 0.05$). However, the culture time had a negligible effect on the GPx activity in all groups (Table 2, $p > 0.05$). The GPx activity in the C_{CO_2} 0.08% group was significantly higher than that of the control group on days 1, 2 and 3, even though it was lower than the control groups on the last day of exposure, when it was significantly impacted by the near-future C_{CO_2} level (Table 3, $p < 0.05$). In the C_{CO_2} 0.20 and 0.50% groups, the GPx activity was significantly stimulated (Table 3, $p < 0.05$) during the four day exposure. Its active peak was in the C_{CO_2} 1.00% group on day 3, and it was significantly stimulated ($p < 0.05$).

In response to elevated C_{CO_2} levels, GST activity in the copepods was depressed during the whole exposure period (Figure 1(b)), but there was no significant difference between the control and the treatments (Tables 2 and 3, $p > 0.05$). However, the culture time had a significant effect on the activity of GST (Table 2, $p < 0.001$).

In the case of the GSH level, the elevated p_{CO_2} displayed a significant effect (Figure 1(c), Table 2, $p < 0.05$). Amongst the elevated p_{CO_2} groups, the content of GSH decreased on days 1 and 2, whereas it increased in the C_{CO_2} 0.08, 0.20 and 0.50% groups on days 3 and 4. During the four day exposure, there was significant difference between the C_{CO_2} 0.20 and 0.50% groups and the control (Table 3, $p < 0.05$). The culture time also had a significant impact on the GSH content among the treated versus the control groups (Table 2, $p < 0.05$).

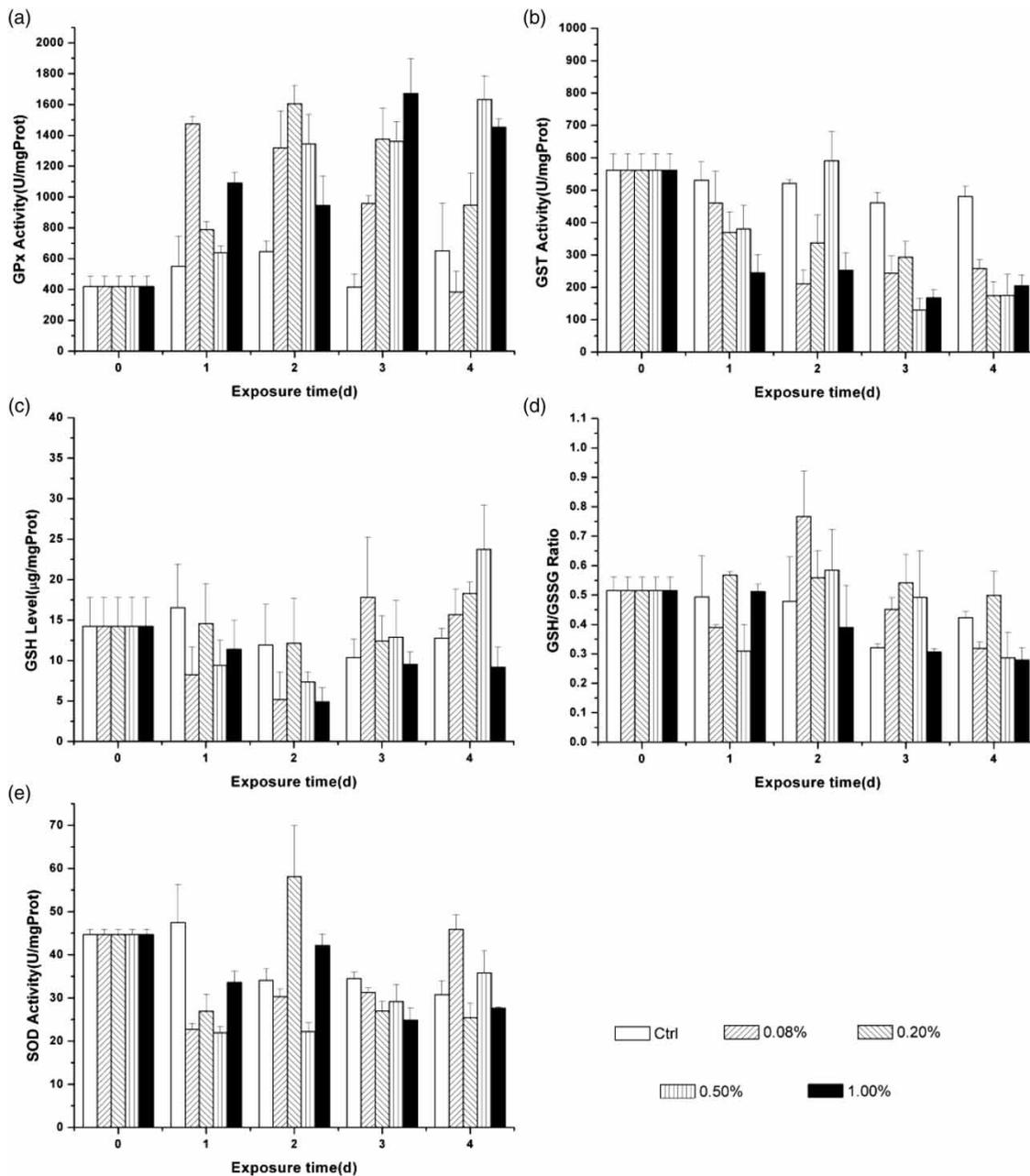


Figure 1 | Influence of elevated C_{CO_2} levels on enzyme activities in *C. tenuiremis*. (a) GPx, (b) GST, (c) GSH, (d) GSH/GSSG, (e) SOD. Data are described as means \pm SD ($n=3$).

The response of the elevated p_{CO_2} levels on the GSH/GSSG ratio in *C. tenuiremis* is shown in Figure 1(d). As shown, the ratio was independent of the different elevated C_{CO_2} levels. Furthermore, there was no significant difference between the elevated C_{CO_2} groups and the control group during the whole exposure time (Tables 2 and 3, $p > 0.05$), and nor did the culture time have any significant impact on the GSH and GSSG ratio (Table 2, $p > 0.05$).

The elevated C_{CO_2} initiated an inhibitive effect on the SOD activity of *C. tenuiremis* on day 1 (Figure 1(e)). Its

activity was stimulated by C_{CO_2} 0.20% on day 2, but decreased and remained at a lower level during the remaining exposure time. Table 2 shows that elevated C_{CO_2} had significant impact on the SOD activity during the 4 day exposure ($p < 0.05$).

The AchE activity of *C. tenuiremis* showed no difference between the elevated C_{CO_2} groups and the control groups or between the different C_{CO_2} treatments (Figure 2(a), Table 2, $p > 0.05$, Table 3, $p > 0.05$). The culture time also played an insignificant role (Table 2, $p > 0.05$).

Table 2 | Synthesis of the two-way ANOVA displaying the effect of acidification and exposure time on biochemical parameters in *C. tenuiremis*

Biological parameter	Effect of acidified treatment			Effect of exposure time			Treatment × time	
	F	df	p	F	df	p	F	p
AChE	2.71	4	0.774	0.53	3	0.135	2.15	0.063
ATPase	85.61	4	0.185	188.68	3	0.021	226.88	0.000
GPx	21.34	4	0.009	8.28	3	0.215	12.36	0.000
GST	1.92	4	0.686	25.81	3	0.000	11.23	0.000
GSH	8.35	4	0.003	6.51	3	0.021	4.69	0.000
GSH/GSSG	5.19	4	0.141	4.37	3	0.217	5.77	0.000
SOD	–	–	0.049	–	–	–	–	–

Table 3 | Influence of different C_{CO2} levels on each *C. tenuiremis* parameter

C _{CO2} (%)	Parameters						
	AChE	ATPase	GPx	GST	GSH	GSH/GSSG	SOD
Ctrl	1.000	1.000	1.000	1.000	1.000	1.000	–
0.08	0.126	0.051	0.030 ^a	0.617	0.737	0.370	–
0.20	0.429	0.241	0.003 ^a	0.415	0.004 ^a	0.520	–
0.50	0.464	0.925	0.012 ^a	0.628	0.034 ^a	0.769	–
1.00	0.680	0.464	0.001 ^a	0.524	0.405	0.195	–

Note: ^aIndicates a significant difference between treatments (LSD test, $p < 0.05$).

Elevated C_{CO2} had an insignificant effect on ATPase activity during the four day exposure (Table 2, $p > 0.05$, Table 3, $p > 0.05$). Its peak on day 2 in the C_{CO2} 0.20% group (18.32 μmol Pi (mg prot)⁻¹ h⁻¹) was double that of other treatments (Figure 2(b)). However, the culture time had significant impact on its activity in all the elevated C_{CO2} and the control groups (Table 2, $p < 0.05$).

Multivariate analysis of the biochemical responses in the copepod *C. tenuiremis*

The PCA was run from matrix data including AchE, ATPase, SOD, GPx, GST and GSH (Table 4). The results showed that 72.32% of the overall variance was explained by the first three principal components. The first component (PC1, 29.39% of overall variance) was GPx; the second (PC2, 25.21% of overall variance) was formed by SOD; and the third (PC3, 17.73% of overall variance) was GSH level. The results of Pearson's test indicated that GST activity had a negative relationship (correlation coefficient, -0.444) with GPx activity ($p < 0.05$), whereas GPx activity showed a significantly positive correlation (correlation coefficient, 0.425) with GSH/GSSG ratio ($p < 0.05$).

DISCUSSION

In our study, PCA indicated that the biochemical variables were divided into three principal components (Table 4). PC1, 2, 3 were formed by the GPx, SOD and GSH levels, which indicated that these of *C. tenuiremis* enzymes played important roles during acidified culture. GPx, which reduces both H₂O₂ and lipid hydroperoxides, are some of the most common detoxification enzymes in organisms. In present study, its activity was significantly stimulated in *C. tenuiremis* by the elevated p_{CO2} levels but not impacted by the duration of the culture time (Figure 1(a), Table 2). This might be the significant defense of copepods to seawater acidification. Many organisms could improve the GPx activity to protect the damage from the environmental stress. For example, reduced blood pH typically decreases the affinity of respiratory proteins for oxygen (Seibel & Walsh 2001), and *Hyphessobrycon callistus* can increase the GPx activity in response to hypoxia (Pan *et al.* 2010). Furthermore, the expression of the gene of GPx in the liver of Atlantic cod is known to be upregulated under hypoxic conditions. The GPx might be released and becomes involved in the antioxidant defense processes during the acidified exposure.

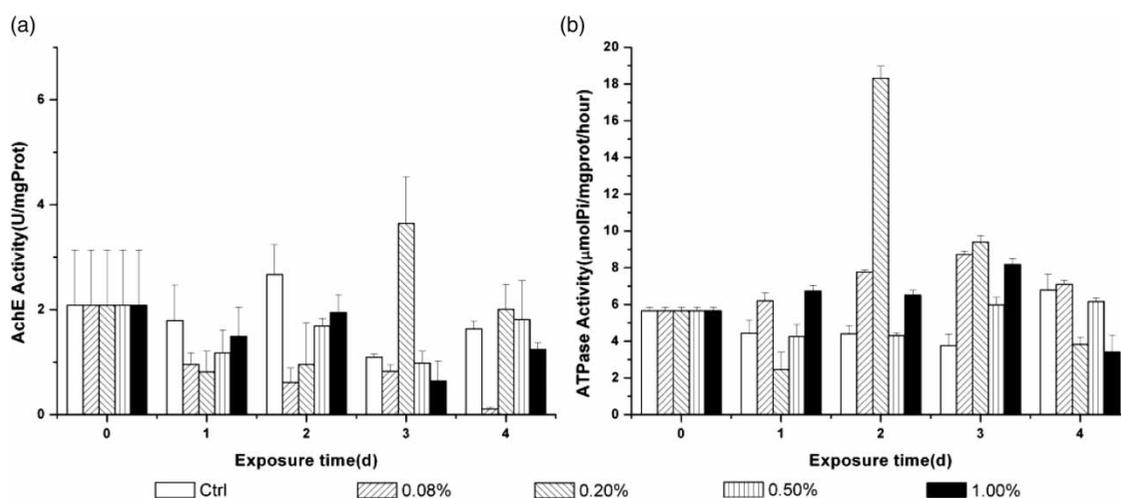


Figure 2 | Influence of elevated C_{CO_2} levels on AchE (a) and ATPase (b) activities in *C. tenuiremis*. Data are described as means \pm SD ($n = 3$).

Table 4 | Component matrix of all parameter variables in *C. tenuiremis* via a PCA

	Correlation coefficients		
	Component 1	Component 2	Component 3
SOD	0.083	0.796	0.333
ATPase	0.492	0.580	-0.441
AchE	0.270	-0.691	-0.122
GST	-0.656	-0.104	0.113
GPx	0.900	-0.200	-0.093
GSH	0.448	-0.118	0.849
% of variance	29.39	25.21	17.73

Note: The bold type represents parameters having significant factorial weights >0.7 .

If the C_{CO_2} level increases in seawater, most organisms are likely to have difficulty in reducing their internal CO_2 concentrations, resulting in the accumulation of CO_2 and H^+ in the body tissues and fluids, viz. hypercapnia, which can inhibit the transportation of oxygen (Pörtner *et al.* 2004). The level of reactive oxygen species (ROS), especially the superoxide anion (O_2^-), generated as obligatory by products of oxidative metabolism and depends on the O_2 uptake of the organism, ca. 2% of the whole O_2 is accounted for by ROS generated as obligatory byproducts of oxidative metabolism (Turrens 2003). Under the low pO_2 level caused by seawater acidification, the O_2^- quantity may decrease, and the SOD activity change correspondingly. In our study, the SOD activity of *C. tenuiremis* was inhibited for the most of the exposure time. This might be because: (1) the deteriorated intracellular environment inhibited its activity; and (2) the amount of O_2^- decreased. The activity of SOD was inhibited on the first day of exposure, after

that, SOD activity in the C_{CO_2} 0.08% group increased and resumed its initial levels on the last exposure day in order to perform the antioxidative function. The cause might be that *C. tenuiremis*, which is a coastal copepod, can tolerate a certain level of environmental perturbation and SOD activity is more easily regulated. However, the activity of SOD in the other higher C_{CO_2} (0.20, 0.50 and 1.00%) group was inhibited, which indicated that the elevated C_{CO_2} may cause oxidantive damage to *C. tenuiremis*. Although the SOD activity in the near-future C_{CO_2} group recovered after four day exposure, more attention should be paid to potential synergistic effects of low pH with other fluctuating environmental factors. For instance, Sampaio *et al.* (2010) showed that SOD activity in pacu, *Piaractus mesopotamicus*, exposed to copper-enriched and acidified water, was significantly inhibited.

GSH can act as an antioxidant on its own or as a co-substrate with GPx and GST in the detoxification process, and this might explain our result whereby GSH level was inhibited and GSH/GSSG ratio decreased in all the elevated C_{CO_2} levels at the beginning of exposure. The GSH level of copepod in C_{CO_2} 0.08% groups increased with exposure time, however, the GPx activity was inhibited at the same time. This may be because C_{CO_2} 0.08% hardly affects the coastal *C. tenuiremis*, which had high tolerance to environmental fluctuation. Under homeostasis, more than 90% of GSH is present in reduced form. Under 1% C_{CO_2} condition, GSH kept a lower level during the exposure time, but the GPx activity was significantly stimulated. From this we may deduce that the higher C_{CO_2} level could cause oxidative damage on copepods. However, the GST, which is the most important phase II enzyme in metabolic detoxification

processes (Wang & Wang 2009) was inhibited during the whole exposure experiment. Todgham & Hofmann (2009) show that elevated C_{CO_2} (pH 7.96 and 7.88) significantly decreases GST mRNA expression in the sea urchin *Strongylocentrotus purpuratus* larvae. Thus, GST might be downregulated by the elevated C_{CO_2} , but the exact mechanism of the impact on this enzyme is still unknown and needs more study. Therefore, the copepods might be forced to undergo oxidative stress due to the increase in C_{CO_2} level, even though the antioxidant (e.g., GPx and GSH) enzymes worked together during the four-day exposure. This effective cooperation might be suggested by the strongly positive correlation among several antioxidant enzymes.

AchE is the enzyme that degrades the neurotransmitter acetylcholine in the cholinergic synapses of the nervous system, and it is vital to normal behavior and muscular function and represents a prime target on which some toxicants can exert a detrimental effect (Forget *et al.* 2003). The AchE activity was insignificantly impacted by the elevated C_{CO_2} or culture time in this study, although its activity was induced or reduced during the whole experiment. The acidified seawater had no neurotoxicity effects on *C. tenuiremis* in present study. However, several studies show that AchE activity is prominently stimulated or inhibited by contaminants including metals and pesticides (Galgani & Bocquene 1990; Forget *et al.* 2003). Therefore, these studies highlight the fact that the responses of AchE activity to environmental stress are different, depending on the species and type of toxicant, and so a further challenge would be to investigate the exact mechanism with regard to elevated C_{CO_2} effects on AchE activity in this copepod.

When C_{CO_2} levels increase in seawater, dissolved CO_2 more readily diffuses across animal surfaces and equilibrates in both intra- and extracellular spaces. CO_2 reacts with internal body fluids causing H^+ to increase, and so pH to decrease. The mechanisms available to counteract this intracellular acidification include: (1) passive buffering of intra- and extracellular fluids; (2) transport and exchange of relevant ions; (3) transport of CO_2 in the blood in those species that have respiratory pigments; and (4) metabolic suppression to wait out periods of elevated CO_2 (Fabry *et al.* 2008). The ATPase plays an important role during the transportation and exchanging of relevant ions and maintaining normal life activities. There are several types of ATPase in crustaceans, such as Na^+ , K^+ -ATPase; Mg^{2+} -ATPase; Ca^{2+} -ATPase; Ca^{2+} , Mg^{2+} -ATPase; H^+ -ATPase and so on. However, we only measured the total ATPase in our study. Although the ATPase activity appeared to be stimulated by some of the elevated C_{CO_2} levels on certain

days, it was negligibly impacted by the elevated C_{CO_2} (Figure 2(b), Table 2, $p > 0.05$). It was difficult to explain the functioning of different types of ATPase during the anti-acidification process. Data are rare concerning the effects of seawater acidification on ATPase activity in copepods, whereas an increasing amount of literature is available on related organisms. Christen *et al.* (1983) show that ATPase activity in the mitochondria of the sperm of the sea urchin *S. purpuratus* is inhibited by the decreased internal pH due to its dilution at low external pH (pH 5.5). When the internal pH is alkalinized, the ATPase activity is increased. However, the fertilization and motility of the sea urchin *Heliocidaris erythrogramma* is not negatively impacted by the decreased pH (7.6, 7.8) (Byrne *et al.* 2010). Furthermore, Hayashi *et al.* (2004) suggest that the Na^+/K^+ ATPase activity in the gills of the Japanese flounder, when exposed to 1 and 5% CO_2 acidified seawater, increases significantly. As the various types of ATPase have different functions, they may respond differently to the elevated C_{CO_2} , and therefore this question requires more investigation.

CONCLUSION

During our short-term exposure experiment, none of the parameters of *C. tenuiremis* were significantly impacted by the near-future C_{CO_2} level (0.08%). However, several enzymes (GPx, SOD activity and GSH level) showed a significant response to C_{CO_2} 0.20% and other elevated C_{CO_2} levels of *C. tenuiremis*. Moreover, the duration of the culture time in the acidified seawater is also an important factor to the biochemical processes of copepods. Ocean acidification is lasting and the interacting of ocean acidification with time is important when we estimate its impacts on these organisms. Furthermore, these organisms live in a complicated environment, not only suffering from ocean acidification, but also from other environmental fluctuations, such as temperature and salinity, as well as other contamination. Hence, the synergistic effect of seawater acidification and other environmental factors on marine organisms deserves further study.

ACKNOWLEDGEMENTS

We thank colleagues at our laboratory for assistance in collecting and sorting the samples. Professor John Hodgkiss is thanked for his help with English.

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