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Risks to the stability of coral reefs in the South China Sea: An integrated biomarker approach to assess the physiological responses of *Trochus niloticus* to ocean acidification and warming

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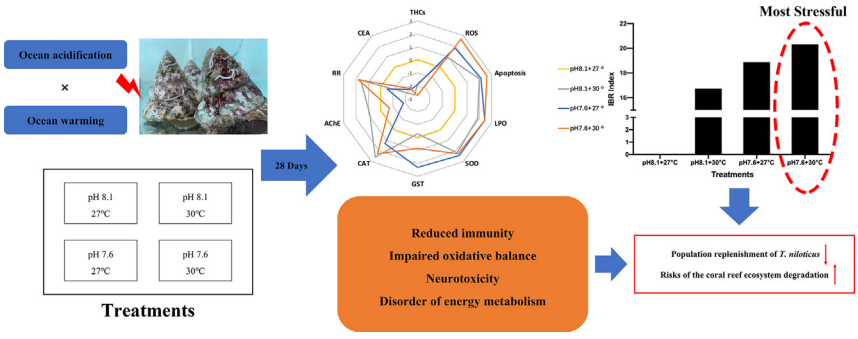
HIGHLIGHTS

- OA and OW have deleterious effects on the fitness of *T. niloticus*.
- Co-exposure of OA and OW is the most stressful condition.
- OA and OW may adversely affect population replenishment of *T. niloticus*.

GRAPHICAL ABSTRACT



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ABSTRACT

Scientific researches have clearly indicated that ocean acidification and warming poses serious threats to coral reef ecosystems. In coral reef ecosystems, herbivorous gastropods have an important function in maintaining the stability of the ecosystem due to controlling the abundance and growth of macroalgal, which compete for nutrients and space with coral. However, limited knowledge is available on the physiological responses of the specific keystone species to the increased ocean acidity and thermal stress. In this study, we evaluated the effects of ocean acidification (OA) and warming (OW) on an herbivorous gastropod *Trochus niloticus* commonly found on intertidal and shallow subtidal coral reefs in the South China Sea, on the aspect of immune responses (total hemocyte counts, reactive oxygen species level and apoptosis rate), oxidative stress (lipid peroxidation level, antioxidant enzyme activities), neurotoxicity (acetylcholinesterase activity), and energy metabolism (respiration rate and cellular energy allocation), after a 28-day exposure experiment to acidic (pH 7.6) and/or thermal (30 °C) seawater. Our results demonstrated that both OA and OW could lead to physiological disturbances of the herbivorous top-shells, including impaired immune functions and oxidative balance, neurotoxicity, and disorder of energy metabolism. Furthermore, results of integrated biomarker response (IBR) confirmed that the overall fitness of *T. niloticus* were deleteriously impacted by OA and OW, and were more stressed under the co-exposure condition. These results indicated that increased acidity and temperature in the future ocean might impair the viability of *T. niloticus* in the long-run, which will indulge the proliferation of macroalgae and lead to degradation of the coral reef ecosystem.

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1. Introduction

As a consequence of anthropogenic activities (e.g. deforestation, fossil fuel combustion), large quantities of CO_2 were discharged in atmosphere during the last two centuries (Doney et al., 2009; Vitousek et al., 1997). The rapidly increased concentrations of global atmospheric CO_2 were absorbed by ocean water, which have resulted in ocean acidification (Hasler et al., 2016; Hoegh-Guldberg et al., 2007; Sabine et al., 2004). Since preindustrial times, the average ocean surface water pH has fallen by approximately 0.1 units, from approximately 8.21 to 8.10 (Rhein et al., 2013). Meanwhile, the average global surface temperature of the ocean has increased almost 1 °C in the last 40 years due to the emissions of greenhouse gas (CO_2, N_2O and CH_4) (Intergovernmental Panel on Climate Change, 2014). Simulations of Intergovernmental Panel on Climate Change (IPCC) have indicated a 0.3–0.5 units decline in ocean pH and a 0.3–4.8 °C elevation in the average global surface temperature of the ocean by the end of the 21th century (Caldeira and Wickett, 2005; Intergovernmental Panel on Climate Change, 2014). Accumulating evidences have shown that complicated interactions between ocean acidification and warming would result in deleterious effects on the organisms in the marine ecosystems, such as increasing oxidative stress, reducing calcification rates, and disordering energy budget (Chatzinikolaou et al., 2017; Collard et al., 2016; Fang et al., 2014; Gomiero et al., 2018; Serra-Compte et al., 2018; Vehmaa et al., 2013).

Coral reef ecosystems harbor complex community structure, and they underpin a range of goods and services for human societies (Barbier et al., 2011; Moberg and Folke, 1999). In the past two decades, global climate change had led to significant decline of the coral cover across different regions and ocean basins (Déath et al., 2012; Hughes et al., 2003). Directly, global warming and ocean acidification exert negative effects on the coral health, manifesting primarily as the destruction of the symbiotic relationships necessary for coral health (also known as coral bleaching), the dissolution of coral reef $CaCO_3$ sediments, and an increase in oxidative stress in corals (Hoegh-Guldberg et al., 2007; Kornder et al., 2018; Mason, 2018; Yuan et al., 2019). Indirectly, climate change cause loss of coral cover by disrupting the healthy competitions between corals and other organisms (Wootton, 1994). For example, macroalgae proliferation occurs more frequently in warmer and acidic ocean environments due to the enhanced photosynthesis by lower pH and higher temperature, which may compete for nutrients and space with juvenile corals and coral fragments (Ferreira et al., 2019; Koch et al., 2013).

Herbivorous gastropods are widely distributed in coral reef ecosystems, and play a pivotal role in controlling macroalgal abundance, growth, and nutrient dynamics (Toh et al., 2013; Villanueva et al., 2013). Therefore, the population replenishment of herbivorous gastropods is one of key factors to predict the vulnerability and resilience of coral reef ecosystems under future climate change scenarios. Recently, several studies have tested the physiological responses of gastropods living in coral reefs to ocean acidification and warming (Lefevre et al., 2015; Milazzo et al., 2014; Grilo et al., 2018). For example, combined thermal and hypercapnia stress could have induced oxidative stresses of *Trochus histrio*, manifested by the elevated activities of antioxidant enzymes and the occurrence of cellular damage (Grilo et al., 2018).

The South China Sea is one of the most important ocean areas for coral breeding in the world. As estimated, approximately 5% of the earth's coral reefs are distributed in the South China Sea, locating around several islands (e.g. Hainan, Nansha, Weizhou and Xisha Islands) (Zhang et al., 2006). Between the year 1900 and 2014, sea surface temperature of the coral regions around the Xisha Islands has raised 0.71 °C, and the seawater pH of the same regions has decreased onwards at a rate of 0.012/year from the 2005 (Yan et al., 2019). It has been increasingly known that the cumulative effect of climate changes is undermining the natural resilience of coral reefs, and is rendering them vulnerable to bleaching in the Southern Great Barrier Reef (Anthony et al., 2008). However, in the coral reef ecosystems of South China Sea, effect of ocean acidification and warming on typical marine organism has been rarely addressed. In this study, we evaluated the effects of ocean acidification (OA) and warming (OW) on *Trochus niloticus*, an herbivorous gastropod commonly found on intertidal and shallow subtidal coral reefs in the South China Sea. After exposed to ocean acidification and warming for 28 days, multiple physiological responses (immune responses, oxidative stress and neurotoxicity) and energy metabolism indicators (respiration rate and cellular energy allocation) in *T. niloticus* were evaluated. Additionally, integrated data was established by the "integrated biomarker response" approach for a better understanding of the comprehensive ecophysiological effects of ocean acidification and warming on *T. niloticus*.

2. Materials and methods

2.1. Experimental setup

Adult *T. niloticus* (shell diameter: 7.8 ± 0.84 cm) were collected nearby the Xisha islands of South China Sea (16°37′12″ N, 112°1′33″ E), and immediately transferred to the laboratory in the Hainan University. The animals were pre-acclimatized for two weeks under a medium-day photoperiod (12 h light:12 h dark). Seawater (pH at 8.13 ± 0.01, temperature at 27.0 ± 0.2 °C, salinity at 34.65 ± 0.20) used in the presented study was sand-filtered (10 μm) followed by ultraviolet (UV) irradiation at a dose of 46 mJ/cm^2. During laboratory acclimation and exposure to climate change conditions, green alga *Ulva* sp. collected from the same site of animals' collection was fed ad libitum to *T. niloticus*. During the whole experimental period, no mortality of *T. niloticus* was observed.

After the period of acclimation, *T. niloticus* were randomly divided into four groups and exposed to various combinations of two temperatures (27 °C and 30 °C) and two pH levels (8.1 and 7.6) for 28 days. These variables were created to mimic both current oceanic conditions as well as oceanic conditions for the end of the 21st century predicted by severe IPCC scenarios (Pachauri et al., 2014). Three identical aquariums containing 150 L of naturally FSW, with 10 animals per aquarium, were used for each treatment's group. Atmospheric air was bubbled into the control (pH 8.1, 27 °C) and OW treatment (pH 8.1, 30 °C), while CO_2 mixed with air was bubbled into OA treatment (pH 7.6, 27 °C) as well as co-exposure of OA and OW treatment (pH 7.6, 30 °C), controlled by an air and CO_2 gas flow adjustment system. This system could bubble dry air or a mixture of dry air and CO_2 with constant percentages into aquarium to set the pH up to the desired value of different treatments, with dissolved oxygen (DO) concentration to near saturation. Temperature of the seawater was controlled with 300 W submerged heaters (E300, EHEIM, Germany), which have been calibrated to reduce the errors by less than 0.2 °C, to meet the different experimental needs of each treatment. In addition, seawater in each aquarium was renewed daily with naturally filter seawater having same pH and temperature to its treatment.

A pH electrode (pH meter PB-10, Sartorius Instruments, Germany) calibrated with NBS standard pH solution was used to monitor the pH of every treatment three times a day. Temperature, dissolved oxygen (DO) and salinity in every aquarium were measured three times a day using an YSI meter (YSI® model 85, Yellow Springs, USA). Total alkalinity (TA) was measured weekly with the method of potentiometric titration (Gran, 1952), and certified reference material (Batch 155) from the A.G. Dickson's lab (San Diego, America) was used for calibration and accuracy assessments. To calculate other carbonate chemistry parameters, TA, temperature, salinity and pH were plotted into CO_2SYS, in which the KSO_4^- and the constants for seawater pH were gotten from previous studies (Dickson, 1990; Millero et al., 2006). The related environmental parameters of the four treatments in this study were shown in Table S1.

2.2. Hemolymph and tissue collection

After the 28-day experimental period, 3 mL of hemolymph respectively was extracted from four individuals using a sterile 5-mL syringe (Cheng and Auld, 1977). The resulting hemolymph was immediately

mixed with an equal volume of ice-cold Modified Alsever's Solution (27 mM of sodium citrate, 336 mM of sodium chloride, 115 mM of glucose and 9 mM of EDTA, pH 7.5) to prevent the hemocytes from aggregating. Then, each sample is divided into three equal parts to determine the three hemocyte-related immunological parameters. In addition, gills and foot muscles were excised from five individuals for the subsequent biological analysis and were stored at −80 °C after being frozen in liquid nitrogen until the measurement.

2.3. Hemocyte-related immunological parameters

2.3.1. Total hemocyte counts

Hemocytes collected from *T. niloticus* were stained with SYBR® Green I (Molecular Probes, Oregon, USA, 10 × final concentration diluted with DMSO commercial solution), a fluorescent dye that binds to double-stranded DNA, and then incubated in darkness for 20 min at 18 °C. The total hemocyte counts (THCs) were measured using a FACSAria flow cytometer (FCM, BD Biosciences, USA) equipped with a 488 nm argon laser, and quantified based on a defined time and flow rate. THCs were expressed as the number of cells stained by SYBR Green I per milliliter of hemolymph.

2.3.2. Intracellular reactive oxygen species (ROS) production

Intracellular ROS production was measured using 2′7′-dichlorofluorescein diacetate (DCFH-DA; Sigma) as described previously (Cao et al., 2018; Eruslanov and Kusmatsev, 2010). In brief, 500 μL of hemolymph mixed with 5 μL of fluorescent probe DCFH-DA (0.01 mM) was incubated at 18 °C in darkness for 30 min. After incubation, the hemolymph was analyzed using FACSAria FCM (BD Biosciences, USA), with excitation and emission wavelengths at 488 nm. The intracellular ROS production in the hemocytes was analyzed using FlowJo software and expressed as the geometric mean of the fluorescence (in arbitrary units, AU).

2.3.3. Detection of apoptotic hemocytes

Hemocyte apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit (Beyotime Biotechnology, Shanghai, China). Briefly, hemocytes were washed with phosphate buffered saline (PBS) buffer (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.4) and re-suspended in 195 μL binding buffer. After that, 5 μL of FITC and 10 μL of PI working solution was added into the re-suspended hemocyte solution, and incubated for 20 min at 18 °C in darkness. The hemocytes were analyzed immediately using a FACSAria FCM (BD Biosciences, USA) for their fluorescence in the FL-1 (Annexin V-FITC) and FL-2 (PI) channels. The apoptosis rate was expressed as the ratio of the number of cells that stain positive to Annexin V-FITC and negative to PI to the total number of cells.

2.4. Antioxidant responses and acetylcholinesterase activities

The gills of 5 *T. niloticus* in each treatment were dissected sterilely and homogenized in 1:9 (w:v) PBS (50 mM KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA, pH 7.4) with IKA homogenizer (Ultra Turrax IKA T10 basic, Staufen, Germany). The homogenates were centrifuged at 10,000*g*, at 4 °C for 20 min, and the resultant supernatants were used for the assay of lipid peroxidation (LPO) levels and antioxidant enzymes activities.

LPO levels were measured using the thiobarbituric acid (TBA) reaction method (Ohkawa et al., 1979). Quantification of TBA reactive substances was determined by measuring malondialdehyde (MDA) concentrations spectrophotometrically at OD 532 nm, and the results were expressed as nmol MDA (mg protein)$^{-1}$. Measurements of superoxide dismutase (SOD) and glutathione S-transferase (GST) and catalase (CAT) activities were performed using corresponding enzymatic colorimetric assay kits following the manufacturer's instructions (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). The units of antioxidant enzyme activities were per mg of protein (U/mg), where one unit denoted the change in absorbance spectra per milligram of protein in 1 min.

To determine the activity of acetylcholinesterase (AChE), gill samples were homogenized in a 1:2 (w:v) Tris-HCl buffer solution (0.1 M Tris-HCl containing 0.1% Triton X-100, pH 7.0), and the homogenates were centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was extracted to measure the AChE activity with corresponding enzymatic colorimetric assay kits following the manufacturer's instructions (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). Results were expressed in nmol (min mg protein)$^{-1}$ using a molar extinction coefficient ($\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$).

To obtain normalized results of the enzymatic activities mentioned above, the total protein content of each gills sample was determined following the Bradford method (Bradford, 1976), using bovine γ-globuline as a standard.

2.5. Energy metabolism parameters

2.5.1. Respiration rate

Respiration rate of *T. niloticus* was measured according to the method described by Sui et al. (2016). Briefly, five *T. niloticus* were randomly selected from each treatment, and their respiration rates were measured with an instrument purchased from Loligo Systems Inc., respectively. There were three phases in the measurement process: a water circulation phase (200 s), a wait phase to stabilize the instrument (100 s) and a measurement phase (600 s). Concentrations of dissolved oxygen were recorded by the AutoResp software (version 2.0.1; Loligo Systems Inc.) and the r^2 of all slopes were >0.95. After measurement, the soft tissues were dissected from the shell and dried at 60 °C for 48 h to a constant weight. The respiration rate of *T. niloticus* was expressed in mg O_2 consumed per hour per g of dry weight and corrected to O_2 variation in blanks (chambers with no organisms).

2.5.2. Cellular energy allocation

Cellular energy allocation (CEA) was measured following the method described in Verslycke and Janssen (2002) by calculating the ratio of available energy reserve (Ea) and energy consumption (Ec) in foot muscle samples (n = 5 per treatment). Ea has three components, including whole body protein, lipid and glycogen contents (which are the major energy reserves in the foot muscle). Protein content in the foot muscles of *T. niloticus* was determined by the Biuret method (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was chosen as the standard. The total lipids in the foot muscles of *T. niloticus* were extracted following the method described by Bligh and Dyer (1959), and measured at 400 nm using tripalmitine as standard. The total glycogen content in the muscles of *T. niloticus* was determined using the method colorimetry of anthrone-sulfuric acid and glucose was used as the standard material (Frings and Dunn, 1970). The units of three components were unified to $mJ/mg_{w.\ w.}$ assuming 39,500 J/g for lipid, 17,500 J/g for glycogen, and 24,000 J/g for protein. In addition, Ec was determined by the electron transport system (ETS) activity, which was measured using the method developed by Kenner and Ahmed (1975) and Owens and King (1975). In brief, 100 μL of 0.1 M BSS (Trizma HCl/base buffer pH 7.5, 0.2% Triton X) and 50 μL NAD(P)H solution (1.17 mM NADH, 0.25 mM NADPH dissolved in distilled water) were added into 50 μL of homogenized muscle sample. The reaction was initiated by adding 100 μL 0.8 mM iodonitrotetrazolium chloride (INT), and the absorbance at 490 nm was measured immediately at 20 °C every 30 s for 10 min. The slope of absorbance was calculated representing O_2 consumption rate by electron transport system, and the outcomes were transformed into energetic equivalents by using the parameter of 484 kJ/mol O_2, where the unit was $mJ/mg_{w.\ w.}$ h.

Table 1
Results of two-way ANOVA: Effects of ocean acidification and warming on the biomarkers in *T. niloticus*. Significant effects ($p < 0.05$) are highlighted in bold.

		Factors/interaction		
		pH	Tempe0072ature	pH × Temperature
Hemocyte-related immunological parameters	THCs	F(1,12) = 0.038 $p = 0.849$	F(1,12) = 0.018 $p = 0.896$	F(1,12) = 1.526 $p = 0.240$
	ROS	**F(1,12) = 26.191** **$p < 0.001$**	**F(1,12) = 3.945** **$p = 0.032$**	F(1,12) = 0.002 $p = 0.573$
	Apoptosis	**F(1,12) = 5.229** **$p = 0.041$**	F(1,12) = 3.948 $p = 0.070$	F(1,12) = 0.516 $p = 0.486$
Antioxidant enzyme activity	LPO	**F(1,16) = 42.68** **$p < 0.001$**	**F(1,16) = 12.76** **$p = 0.003$**	**F(1,16) = 13.93** **$p = 0.002$**
	SOD	F(1,16) = 1.673 $p = 0.214$	F(1,16) = 0.990 $p = 0.335$	F(1,16) = 1.325 $p = 0.267$
	GST	**F(1,16) = 65.84** **$p < 0.001$**	**F(1,16) = 21.75** **$p < 0.001$**	**F(1,16) = 13.67** **$p = 0.002$**
	CAT	F(1,16) = 0.824 $p = 0.378$	**F(1,16) = 28.683** **$p < 0.001$**	**F(1,16) = 4.934** **$p = 0.041$**
Neurotoxicity	AChE	**F(1,16) = 13.553** **$p = 0.002$**	**F(1,16) = 4.637** **$p = 0.047$**	F(1,16) = 0.001 $p = 0.972$
Energy Metabolism parameters	RR	F(1,16) = 0.07 $p = 0.794$	**F(1,16) = 24.033** **$p < 0.001$**	F(1,16) = 0.585 $p = 0.455$
	CEA	F(1,16) = 1.919 $p = 0.185$	**F(1,16) = 4.652** **$p = 0.047$**	**F(1,16) = 5.433** **$p = 0.033$**

2.6. Integrated biological response index

The integrated biomarker response version2 (IBRv2) was calculated for each treatment with physiological parameters in the present study following the method described in previous studies (Beliaeff and Burgeot, 2002; Sanchez et al., 2013). For each treatment, the ratio of the individual biomarker data (Xi) and the reference data ($X0$) was logged to reduce variance, so $Yi = \log(Xi / X0)$. Yi was then standardized with general mean (μ) and standard deviation (s): $Zi = (Yi - \mu) / s$, and the mean of the standardized biomarker response (Zi) minus the mean of reference biomarker data ($Z0$) was used to obtain the biomarker deviation index (A): $Ai = Zi - Z0$. Finally, the absolute values of all A parameters were added to get the IBRv2 index: $\text{IBRv2} = \Sigma|A|$. Star plots were depicted using Microsoft Excel software to evaluate the physiological state from a whole organism perspective in *T. niloticus*.

2.7. Statistical analysis

For all tested parameters, data is represented as the means ± standard deviation (SD). Shapiro-Wilk test and Bartlett's test were used respectively to assess the normality and homogeneity of the data. For eligible data, two-way analysis of variance (two-way ANOVA) was used to determine the main effect and interaction effect of OA and OW. Post-hoc Tukey HSD tests were subsequently carried out to identify significant differences between each treatment when the interaction was not significant. Statistical significance difference was accepted when $p < 0.05$. All statistical analyses were performed using RStudio (version 1.1.463).

3. Result

3.1. Biochemical biomarker in T. niloticus

3.1.1. Hemocyte-mediated parameters

The result of the two-way ANOVA indicated that no significant interactive effects of OA and OW were detected on the THCs, intracellular ROS production and apoptosis rate of hemocytes in *T. niloticus* (Table 1). Either exposure to OA or to OW has a significant main effect on the intracellular ROS production in hemocytes of *T. niloticus*. And exposure to OA also has a significant main effect on the apoptosis rate of hemocytes in *T. niloticus* (Table 1). In addition, no statistically significant difference was observed in the result of THCs after the 28 days of OA and/or OW exposure (Fig. 1A). Compared to the control, the intracellular ROS production and apoptosis rate of hemocytes increased significantly in *T. niloticus* under the co-exposure of OA and OW treatment (Fig. 1B, C).

3.1.2. Indicators of oxidative stress

Obviously, OA and OW led to negative effects on the status of oxidative stress of *T. niloticus*. Significantly interactive effects of OA and OW were detected on the CAT and GST activity, and LPO level (Table 1). Compared with the control, significant increases of LPO level were observed in all stressor-exposed treatments in comparison to the control (Fig. 2A). A slight but not significant upward of the SOD activity were observed after the 28 days' exposure to OA and OW (Fig. 2B). In addition, GST activity was enhanced significantly at OA treatment (pH 7.6, 27 °C), which was inhibited at the co-exposure treatment (pH 7.6, 30 °C) due to the antagonistic effects (Fig. 2C). Similarly, the CAT activity was also significantly enhanced at the OW treatment (pH 8.1, 30 °C), nevertheless it was inhibited at the co-exposure treatment (pH 7.6, 30 °C) conversely (Fig. 2D).

3.1.3. Indicators of neurotoxicity

According to the two-way ANOVA analysis, no significant interaction was detected on the AChE activity between OA and OW (Table 1). However, both OA and OW have significant main effect on the activity of AChE. Exposure to OA inhibited the AChE activity in gills of *T. niloticus*, while exposure to OW induced enhancement of the AChE activity in this study (Fig. 3).

3.1.4. Respiration rate and cellular energy allocation

No significant interaction of OA and OW was observed on the respiration rates of *T. niloticus*. However, OW does show significant main effect on the respiration rate of *T. niloticus* (Table 1). Significant stimulatory effects of increased temperature on the respiration rates was observed in *T. niloticus* (Fig. 4).

The results of cellular energy allocation (CEA) of *T. niloticus* to OA and OW was shown in Table 2. In general, the CEA values were decreased under all the stressor-exposed treatments compared to the control (Table 2). In terms of the available energy reserves in foot muscle of *T. niloticus*, increased glycogen consumption and lipid accumulation were showed under the OW treatments. In addition, the energy consumption in foot muscle of *T. niloticus* was stimulated under all the stressor-exposed treatments.

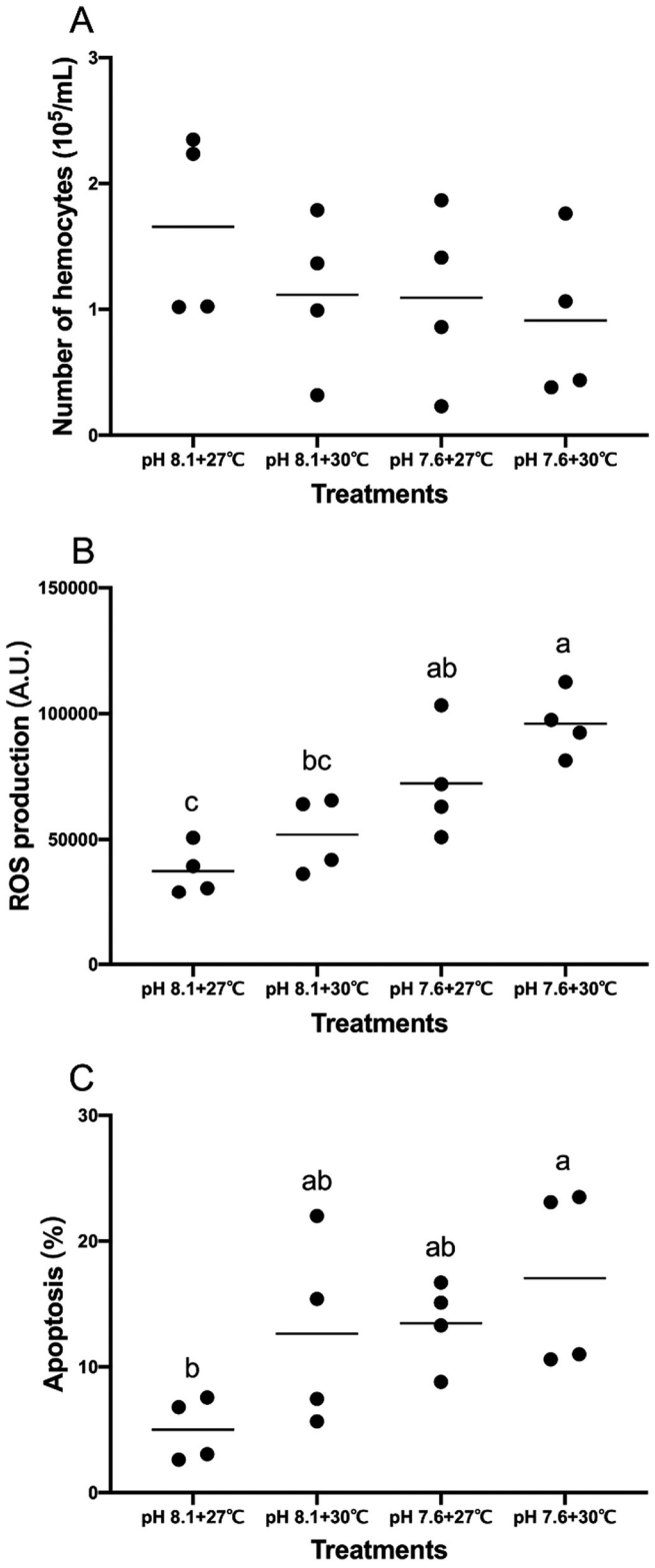

Fig. 1. The immune-related parameters in hemocytes of *T. niloticus* after exposure to ocean acidification and/or warming. A—THCs; B—ROS; C—Apoptosis; Point represents one of the repeats in the treatment. Horizontal line represents mean of all the repeats in the treatment. Different letters indicate significant differences among treatments ($p < 0.05$).

3.2. Principal component analysis

Ten common physiological biomarkers were analyzed by Principal Component Analysis (PCA), which explained 87.4% of the total variance (Fig. 5). Among these, PC1 explained 59.5% of the total variance, highlighting distinct relationship between OA treatments and the ambient pH treatments. PC2 accounted for 27.9% of the total variance, separating the OW treatments from the other two treatments (Fig. 5). The PCA results clearly demonstrated a positive correlation between co-exposure treatments and most of the biomarkers (Apoptosis rate, intracellular ROS production in hemocytes, SOD activity, CAT activity and LPO level) and a negative correlation between co-exposure treatment and the CEA value.

3.3. Integration of biomarker responses

The star plots constructed based on 10 common physiological biomarkers (Fig. 6A) indicated that most parameters were induced by OA and/or OW. The integrated biomarker response (IBR) indexes of the three stress treatments (OA, OW and co-exposure) were much higher than those of the control (Fig. 6B), among which the index of the co-exposure was the highest.

4. Discussion

Coral reefs worldwide have experienced unprecedented decline over the last several decades, primarily due to their disproportionate susceptibility to the emerging effects of global climate change (Hoegh-Guldberg et al., 2007), as well as the anthropogenic exploitation (Wilkinson, 1999). In addition to the direct inducement of coral mortality, global climate change has also increased the susceptibility of reef communities, leading to degradation and losses caused by disrupted interspecies relationships between corals and other organisms (Wootton, 1994). Typically, climate changes are considered to favour the proliferation of opportunistic algae (e.g. algal turfs) living in coral reefs, which allows them to turn from subordinates to dominants (Bender et al., 2014). As the major grazers of algae in coral reef ecosystems of the South China Sea, *T. niloticus* was regard as the keystone species to maintain the stability of the ecosystem under future climate change scenarios due to their ability to enhance the survivorship of coral through algae grazing (Omori, 2005; Villanueva et al., 2013). In this work, we provide with the first study to evaluate the effects of OA and OW on *T. niloticus* in South China Sea, and the result will enrich our understanding to the physiological response of this key species in the ecosystem to climate changes.

4.1. Effects of OA and/or OW on immune responses of T. niloticus

Hemocytes constitute the first line of the immune system in gastropods, and variation of its concentration in vivo was commonly regarded as a sequel to alteration in inflammatory mobilization or hemocyte proliferation to peripheral organizations induced by stressful environment (Yu et al., 2010; Cheng and Auld, 1977). Generally, OA and OW exposure would decrease the THCs in marine invertebrates, which has been reported in the blood clams *Tegillarca granosa* (Liu et al., 2016) and the blue mussel *Mytilus edulis* L. (Mackenzie et al., 2014). This could be a consequence of hemocytes apoptosis or a reduced migration of hemocytes from tissues to hemolymph in response to stressful conditions. However, no significant difference in THCs of *T. niloticus* after exposure to OA and/or OW was observed in the present study. This discrepancy may be caused by the different adaptive capacities of species to environmental stress. In alignment with our finding, THCs of the spiny lobster *Jasus lalandii* did not affected by chronic exposure to OA and/or OW (Knapp et al., 2019). But co-exposure to OA and OW weakened the reactivity of *J. lalandii* to the challenge of the pathogenic bacteria *Vibrio anguillarum*, which is reflected on the significant decrease of THCs under this stressful condition (Knapp et al., 2019). Therefore, although we did not find significant decrease of THCs in the present study, the capability to cope with pathogen of *T. niloticus* could also have been impaired by the exposure to OA and OW.

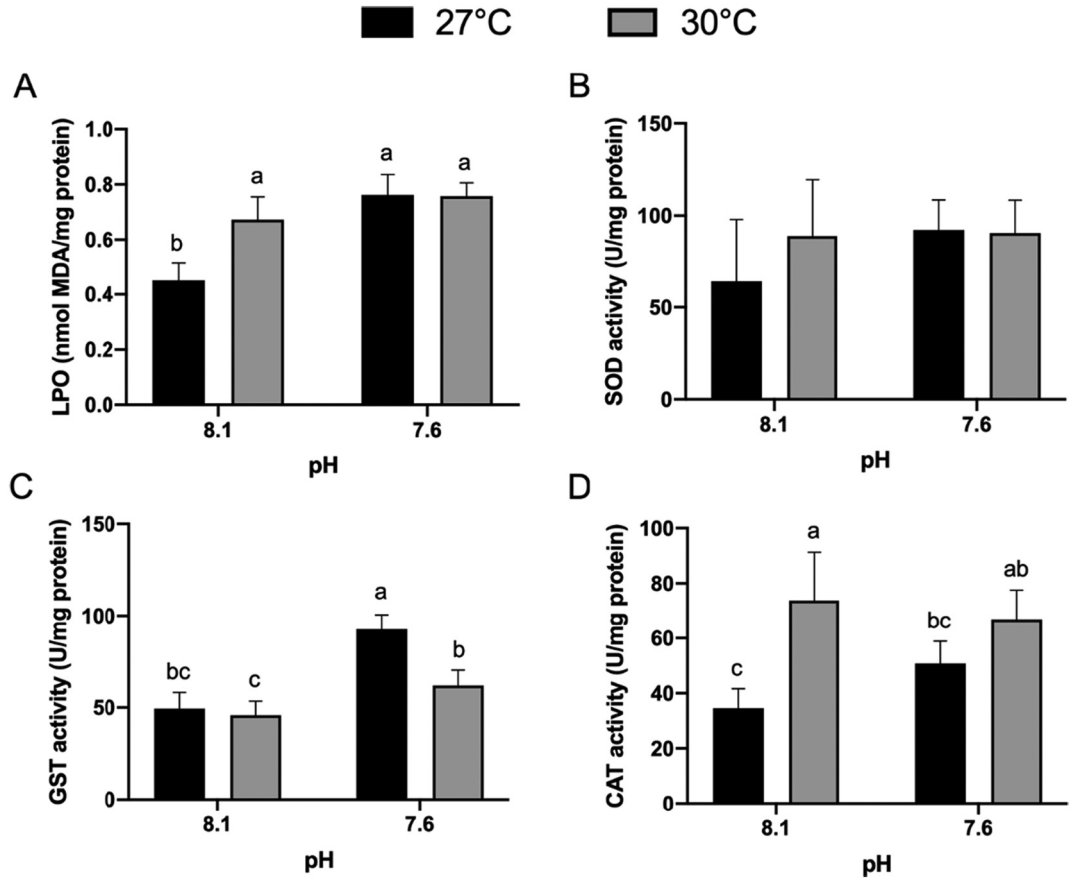

Fig. 2. LPO level and antioxidant enzyme activities in gills of *T. niloticus* in response to ocean acidification and/or warming. A—LPO; B—SOD; C—GST; D—CAT. Different letters mean significant differences among treatments ($p < 0.05$).

Hemocytes are involved in a number of immune defense responses in marine invertebrate, such as phagocytosis, apoptosis, intracellular killing of bacteria, and wound healing (Terahara et al., 2008). In our study, increases in intracellular ROS production were observed in hemocytes of *T. niloticus* exposed to OA or OW. However, the mechanisms that the increased ROS production level induced by OA and OW were different. OA exposure might lead to intracellular acidosis which triggered dysfunction of mitochondrial electron transport chain and non-enzymatic generation of free radicals (Murphy, 2009), while OW exposure might depend on the decrease of mitochondrial coupling decreased and the increase of oxygen consumption in organisms (Abele et al., 2002). Increase of intracellular ROS production in hemocytes could lead to different consequences. Firstly, it could react with biomacromolecules, including DNA and proteins, leading to the oxidative damage of these molecules (Kimura et al., 2005). Meanwhile, the extra ROS produced in hemocytes could be released into hemolymph to inactivate invasive microorganisms including pathogenic bacteria and viruses (Babior et al., 1973), or be a signal molecule induced other immune system function like apoptosis (Desagher and Martinou, 2000). Therefore, we inferred that the increases of apoptosis rate induced by exposure of OA or OW in the present study both depend on the mitochondrial apoptosis pathway activated by extra ROS (Desagher and Martinou, 2000). Specifically, excessive ROS could stimulate mitochondria to release Cytochrome c (an essential component of

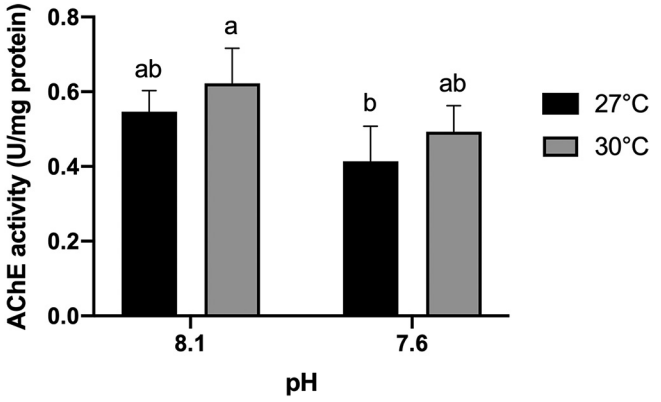

Fig. 3. AChE activity in gills of *T. niloticus* in response to ocean acidification and/or warming. Different letters mean significant differences among treatments ($p < 0.05$).

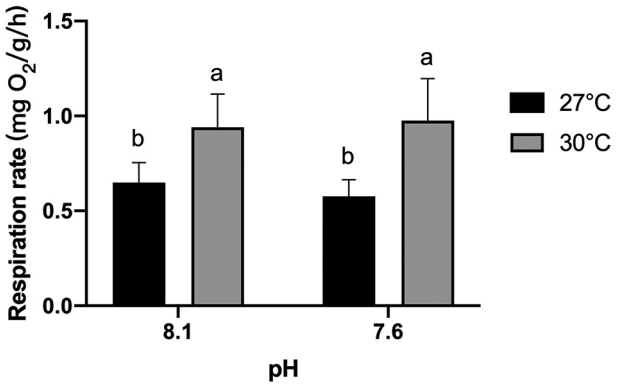

Fig. 4. Respiration rate of *T. niloticus* in response to ocean acidification and/or warming. Different letters mean significant differences among treatments ($p < 0.05$).

Table 2
The results of cellular energy allocation (CEA). Available energy reserve (Ea) in the muscle of *T. niloticus* calculated from the different fractions: contents of lipid, protein and glycogen. Energy consumption determined by the electron transport system activity. The Ea divided by the energy consumption (Ec) was used to calculate the CEA index (mean ± SD, n = 5). Different letters after the mean of CEA indicate significant differences among treatments ($p < 0.05$).

Treatments	Glycogen ($mJ/mg_{w.w.}$)		Protein ($mJ/mg_{w.w.}$)		Lipid ($mJ/mg_{w.w.}$)		Ea ($mJ/mg_{w.w.}$)		Ec ($mJ/mg_{w.w.}/h$)		CEA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
pH 8.1 + 27 °C	33.39	11.96	1608.62	84.98	272.58	61.94	1914.59	131.09	5.89	1.17	332.79^{a}	49.65
pH 8.1 + 30 °C	23.35	2.10	1477.26	158.69	600.41	110.28	2101.02	192.95	9.03	1.95	240.46^{b}	51.92
pH 7.6 + 27 °C	27.82	4.33	1467.05	66.16	275.08	47.70	1769.95	93.60	7.01	0.92	256.32^{ab}	39.07
pH 7.6 + 30 °C	23.80	4.24	1486.12	139.60	388.03	88.99	1897.95	132.01	7.53	1.81	259.95^{ab}	42.08

the electron transport chain) into cytoplasm, which could bind the adaptor molecule apoptotic peptidase activating factor 1 to form a complex called the apoptosome (Polster and Fiskum, 2004; Bock and Tait, 2020). The apoptosome, in turn, binds to and activates the initiator caspase 9, which subsequently cleaves and activates the executioner caspases to induce apoptosis (Bock and Tait, 2020). This is consistent to previous studies in the Pacific oyster *Crassostrea gigas*, showing that excessive production of ROS induced by environmental stresses caused oxidative damage and ultimately cell death (Wang et al., 2016; Lushchak, 2011). In addition, both the intracellular ROS production and apoptosis rate in *T. niloticus* were significantly increased under the co-exposure of OA and OW, which could be explained by the additive effect between the two factors. Similarly, significant increases of intracellular ROS production and apoptosis rate were also observed in the hemocytes of the mussel *Mytilus coruscus* after co-exposure of OA and OW (Wu et al., 2016).

4.2. Effects of OA and/or OW on antioxidative system of T. niloticus

LPO, caused by excessive ROS production, has been widely used as an indicator of oxidative damage in marine organisms (Ferreira et al., 2015; Madeira et al., 2018b; Munari et al., 2018; Tomanek et al., 2011). In this study, the higher LPO levels in all stressor-exposed treatments indicate that excessive ROS was not counterbalanced in time by the antioxidant defense mechanisms and that oxidative substance accumulated in cells, resulting in progressive oxidative damage (Pannunzio and Storey, 1998). Similar results have also been found in the octocoral *Veretillum cynomorium*, experienced severe oxidative stress (high LPO levels) under exposure to OA and/or OW (Lopes et al., 2018).

To combat oxidative stress, hosts have developed various antioxidant enzymes to neutralize free radicals and repair the ROS-derived damage. For example, SOD converts superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), then CAT converts H_2O_2 into oxygen (O_2) and water (H_2O) (Lesser, 2006). GST serves as a major second phase detoxification enzyme, catalyzing the conjugation of various electrophiles with glutathione and detoxifying both exogenously and endogenously derived toxic compounds (Sheehan et al., 2001). Significantly up-regulated activities of the antioxidant enzyme GST were observed in *T. niloticus* after exposures to OA, accompanied by a slight increase in SOD activity. This result is concordant to the previous findings in the Pacific oyster *C. gigas* and the polychaete *Hediste diversicolor*, which showed similar anti-oxidative responses under seawater acidification (Cao et al., 2019; Freitas et al., 2016). As proposed by Tomanek et al., these anti-oxidative responses could be induced by oxidative stress in several ways: (1) increasing the release of transition metals, Fe^{2+}, especially as it activates the Fenton reaction and the production of hydroxyl radicals, (2) stimulating electron transport chain to produce ROS, and (3) CO_2 reacting with ROS to produce other additional radicals (Tomanek et al., 2011). In addition, CAT activity was significantly elevated in *T. niloticus* exposed to OW, indicating that the oxidative stress could have been induced by H_2O_2 under high temperature conditions. Similarly, CAT activity enhancement was found in the

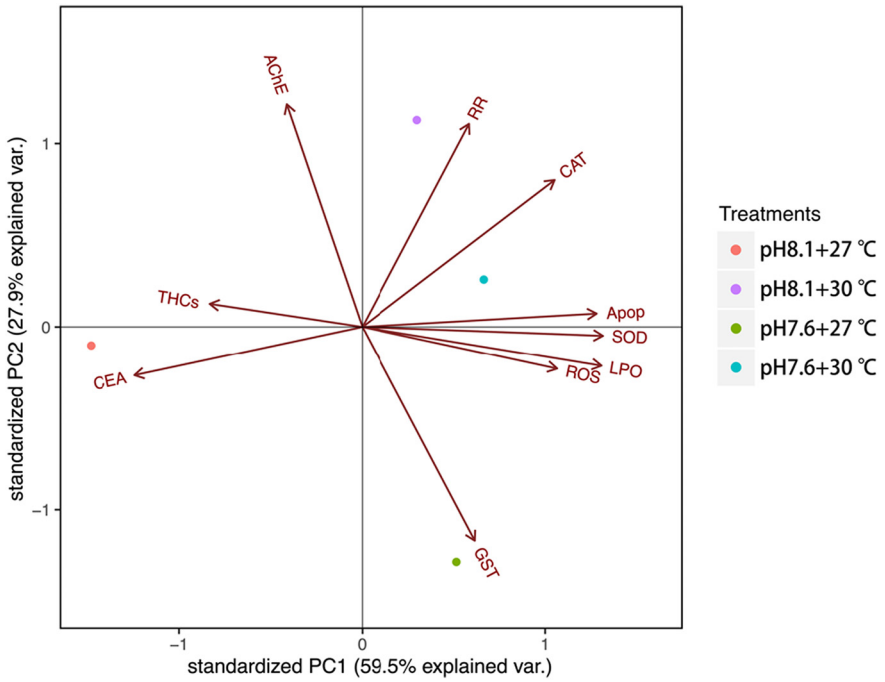

Fig. 5. Principle component analysis (PCA) ordination biplot of ocean acidification and/or warming treatments for *T. niloticus*.

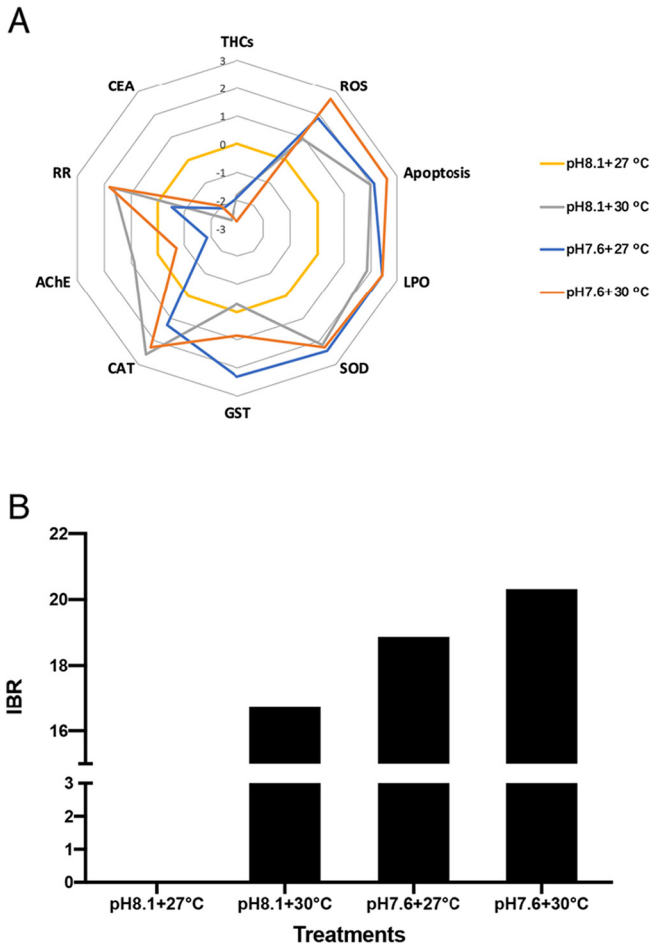

Fig. 6. The integrated biomarker response of *T. niloticus* exposed to ocean acidification and/or warming. A—Biomarker star plots; B—IBR values for each treatment.

Rock goby *Gobius paganellus* under the increased temperature exposure condition (Vinagre et al., 2014), while abundant one DyP-type peroxidase isoform (which catalyzes the degradation of H_2O_2 to H_2O) were detected in the mussels *Mytilus trossulus* under a chronic heat exposure (Fields et al., 2012). Interestingly, antagonistic effects were found between OA and OW on GST and CAT activity in *T. niloticus*. Compared to single exposures, GST and CAT activity were inhibited under co-exposure condition, indicating that the antioxidant system of the organism could have been damaged. This is unsurprised because when animals are close to their thermal limits, even a small disturbance (low seawater pH) may reduce their ability to cope with oxidative stress (Range et al., 2014; Talmage and Gobler, 2011).

4.3. Effects of OA and/or OW on AChE activity of T. niloticus

AChE is one of the most common biomarkers of neurotoxicity used to study aquatic organisms (Sogorb and Vilanova, 2002). In recent years, a lot of studies have found that climate stressors could also have neurotoxic effects on marine organisms (Bebianno et al., 2004; Madeira et al., 2018a; Sampaio et al., 2016). In this study, OA and OW independently impacted on AChE activity of *T. niloticus*, which did not interact with each other. Firstly, we found that OA inhibited AChE activity in *T. niloticus*. Previous studies have suggested that CO_2 could disrupt basal inhibitory potential on GABA-A receptors in clownfish *Amphiprion percula*, leading to shifts in ionic concentrations of Cl^- and HCO_3^- (Nilsson et al., 2012). This shift stimulates the release of GABA neurotransmitters, which might cause the inhibition of cholinergic activity in organisms (Giorgetti et al., 2000). Therefore, the inhibition of AChE activity observed in the OA treatments were perhaps related to increased concentrations of GABA neurotransmitters. Similarly, a study conducted by Dionísio et al. (2020) showed that AChE activity of the marine gastropod *Gibbula umbilicalis* could be inhibited after exposure to acidified seawater. By contrast, OW led to increased AChE activity in *T. niloticus* held at both pH 8.1 and pH 7.6. This is in line with studies to the tropical gastropod *Stramonita haemastoma* (Madeira et al., 2018a), where increased temperature enhanced AChE activity. The stimulated AChE activities in marine organisms was likely due to increased metabolic rates caused by high temperatures (Sampaio et al., 2016). However, exceeding the thermal threshold of organisms is expected to cause adverse effects.

4.4. Effects of OA and/or OW on energy metabolism of T. niloticus

Generally, organisms distribute most of their available energy to three areas (basal metabolism, growth, and reproduction) and strive to maintain a balance between each area. However, the energy homeostasis of organisms can be easily disrupted by external factors, leading to a substantial decline in fitness and survival. As such, respiration rates are often used as an indicator of energy metabolism at the whole organism level (Brooks et al., 2015; Kim et al., 2014; Leung et al., 2018). In this study, OA had little effect on the RR of *T. niloticus*, which is consistent with the results of the pheasant snail *P. australis* (Leung et al., 2018), the Manila clam *Ruditapes philippinarum* (De Marchi et al., 2019) and the Pacific oyster *C. gigas* (Cao et al., 2019). However, OW could significantly increase the respiration rate of *T. niloticus*. This metabolic promotion of temperature is considered a common physiological response in marine poikilotherm and was also found in the gastropod *Crepidula fornicata* and the edible cockle *Cerastoderma edule* (Noisette et al., 2016; Ong et al., 2017).

The cellular energy allocation (CEA) measures a net cellular energy budget of the organisms at a cellular level of biological organization, by quantifying the available energy reserves and energy consumption in tissues (Erk et al., 2011). Presently, the CEA has been widely used as a biomarker reflected the metabolic processes of an organism under stressful conditions (Verheyen and Stoks, 2020; Gandar et al., 2017; Kühnhold et al., 2017). In terms of the available energy reserves, only exposure to OW led to an increase in total lipid content and a decrease in glycogen content in the muscles of *T. niloticus*. This indicates that *T. niloticus* adopted an active glycolytic metabolism to cope with the OW exposure, by reducing utilization of lipid reserves. It has been reported that glycogen is primarily used for maintaining fitness of animals under stressful conditions (Bayne et al., 1976; Patrick et al., 2006). Similar results were also observed in the Pacific oysters *C. gigas* exposed to variable pH and temperature conditions, preserved lipids and consumed glycogen has been reported to sustain metabolic functions given the stressful conditions (Clark et al., 2013). In addition, we found energy consumption of *T. niloticus* was significantly increased after a 28-day OA and/or OW exposure, reflected on the increases of electron transfer system activity. This means more energy accumulated in the proton gradient is used by complex V (ATP synthase) to produce ATP (Zhao et al., 2019). To cope with the stressful condition, it is common to find that elevated energy demands were mobilized by organisms (Kang et al., 2019). In line with our results, several environmental stressors have been found to induce the ETS activities of the mussels *M. trossulus* and the crustacean *Daphnia magna* (Kim et al., 2014; Turja et al., 2014). Corresponding to the changes in energy reserves and energy consumption mentioned above, decreased CEA values were observed in all stressful treatments of the present study. It can be explained by that the metabolism rate of *T. niloticus* under increased OA and/or OW were regulated to allocate more energy on the physiological activities, such as acid base regulation, antioxidant reaction and repair processes.

4.5. Comprehensive analysis of multiple biomarkers

An IBR index was adopted to assess the integrated impact of environmental stressors between treatments (Maulvault et al., 2019; Xu et al., 2016). Generally, higher IBR values indicated that the experimental condition was more stressful to the organisms (Leinio and Lehtonen, 2005). In this study, *T. niloticus* exposed to OA and/or OW displayed a notably higher IBR value than the control group, in which the co-exposure was most stressful. This is consistent with the result of PCA analysis, which has shown that most physiological alterations are related to the co-exposure treatment, such as impaired immune functions and oxidative balance accompanying to increased metabolic rates. According to the dynamic energy budget theory, organisms assimilate and utilize energy and elemental matters from food for its maintenance, growth, reproduction and development (Kooijman, 1986). Under the future climate change scenario, *T. niloticus* would perhaps allocate less energy to the reproduction and development due to the increased energy consumption by other physiological activities, implying deleterious effects on the population replenishment. It was suggested that loss of this key herbivorous gastropods would favour proliferation of macroalgae in coral reefs, which would be a threat to stability of coral reef ecosystem in the future.

5. Conclusion

In conclusion, the *T. niloticus* studied herein are susceptible to OA and OW. The stressors have led to impaired immune functions, oxidative stress, neurotoxicity and disturbance in energy metabolism of the organism. The IBR results showed that co-exposure of OA and OW posed the most severe stress to *T. niloticus*. According to the dynamic energy budget theory, these results suggest that OA and OW would perhaps affect the population replenishment of *T. niloticus* in the long run, and the loss of this keystone species would cause further degradation of coral reefs in South China Sea.

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CRediT authorship contribution statement

Tianyu Zhang: Investigation, Formal analysis, Data curation, Writing – original draft. **Yi Qu:** Data curation, Validation. **Qianqian Zhang:** Funding acquisition, Writing – review & editing. **Jia Tang:** Visualization. **Ruiwen Cao:** Methodology. **Zhijun Dong:** Supervision. **Qing Wang:** Supervision. **Jianmin Zhao:** Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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