The impacts of ocean warming and acidification on the behavior and muscular system of marine animals

海洋温暖化と酸性化が海産動物の行動と筋肉系に与える影響

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The impacts of ocean warming and acidification on the behavior and muscular system of marine animals

by

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CONTENTS

ABSTRACT

CHAPTER I GENERAL INTRODUCTION

There are two major sources by which ocean water is acidified; dissolution of atmospheric CO_2 and leakage from a carbon capture and storage (CCS) site. It is estimated that about 30% of anthropogenic CO_2 has dissolved into the ocean and reduced surface ocean seawater by 0.1 pH units since the beginning of the Industrial Revolution, termed ocean acidification. Intergovernmental Panel on Climate Change (IPCC) predicted that atmospheric CO_2 concentration will reach to 1000 and 1900 ppm by 2100 and 2300, respectively. The CCS was introduced for mitigating global climate change. CCS technology has been developed to reduce CO_2 emissions by separating CO_2 from flue gas from large sources and sequestering CO_2 into geological reservoirs via pipelines. However, there has been concern about accidental leakage of stored CO_2 through the seabed, which would impact benthic ecosystems around a leakage site. At the same time, emission of CO_2 and other greenhouse gases have warming the entire earth surface. IPCC predicted that sea surface could increase up to 4°C by 2100. Scientists have revealed that ocean warming and acidification have negative impacts on calcification and physiological functions of different marine organisms during the last 15 years. In comparison, our understanding is limited about how elevated temperature and CO_2 may affect the behavior and muscular system of marine animals. This study aimed to examine both the separated and combined effects of elevated CO_2 and temperature on behavior and muscle functions of Japanese anchovy, sea urchins and tiger prawn.

CHAPTER II ESCAPE RESPONSE OF THE JAPANESE ANCHOVY *Engraulis japonicus* UNDER ELEVATED TEMPERATURE AND CO_2 CONDITIONS

This study examined the escape response under elevated CO_2 concentration and temperature of the Japanese anchovy *Engraulis japonicus*. Following acclimation to four conditions (CO_2 400/1000 μatm (1 μatm = 1 ppm in a gas phase), temperature 15/19°C) for one month, the fish were tested for escape response through kinematic analysis of startle reactions to a mechanical stimulus. The response was recorded with a

high speed video camera of 500 frames per second. The result showed turning rate was significantly higher at 19°C than at 15°C. Neither CO_2 nor temperature affected the kinematic parameters analyzed (the escape trajectory, swimming velocity, acceleration, escape direction, or frequency of single and double bends), with the exception of the turning rate that was significantly higher at 19°C than at 15°C. However, we must clarify how future oceanic environmental changes affect escape responses of schooling fish and prey-predator interactions under more rigorous experimental conditions, to elaborate our prediction capacity for the trajectory of anchovy populations and thereby assess possible implications for anchovy fisheries

CHAPTER III EFFECTS OF ELEVATED CO_2 ON MUSCULAR SYSTEM AND PROTEOME COMPOSITION OF THE PINK SEA URCHINS *Pseudocentrotus depressus*

It has been reported that sea urchins are highly vulnerable to ocean acidification (early development, pH regulation etc.). However, we presently have little information on how ocean acidification affects the behavior and the muscular systems of sea urchins. This study examined the feed intake, muscle contraction and proteome composition of different muscle systems of the pink sea urchin (*Pseudocentrotus depressus*) under CO_2 concentration at 400 (control), 2000 and 10000 μatm. Feed intake significantly decreased under 10000 μatm CO_2 condition, while 2000 μatm had no effect. Histological observation showed that the cross-sectional area of the retractor muscle fibers of the masticatory apparatus was significantly smaller in 2000 and 10000 μatm than in the control. Contraction force of tube feet was significantly reduced in 2000 and 10000 μatm compared with control condition, while contraction force of the protractor muscle was not significantly affected. Two-dimensional gel electrophoresis of the proteins extracted from tube feet showed that eight spots changed in protein volume: six up-regulated, and two down-regulated. Using matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight mass spectrometry, three up-regulated spots (tubulin beta chain, tropomyosin fragment, and actin N-terminal fragment) and two down-regulated spots (actin C-terminal fragment and myosin light chain) were identified. The results suggest that elevated CO_2 could impair the tube feet muscle of sea urchins due to alteration of proteome composition, mainly

associated with post-translational processing/proteolysis of muscle-related proteins. This finding might give a clue to understand the mechanism(s) of functional impairment of sea urchin muscles by elevated CO_2.

CHAPTER IV EFFECTS OF ELEVATED TEMPERATURE AND CO_2 ON EARLY LIFE STAGE OF THE GIANT TIGER SHRIMP *Penaeus monodon* (Chapter IV)

The fertilized eggs of the giant tiger prawn, *Penaeus monodon*, were exposed to four different temperature levels (28°C (control), 30°C, 32°C and 34°C) and four different sea water pH (8.1 (control), 7.6, 7.0 and 6.0 to test effects of temperature and CO_2 on hatching, survival, and swimming performance. The results showed that the hatching rate significantly decreased below pH 7.6 and above 32°C compared with the control. The survival rate showed similar responses. The histological examination did not detect any difference in a musculature by temperature or seawater pH treatments. Swimming performance was tested only for temperature experiment, which showed that swimming speed at temperature 30°C is higher than 28°C and 34°C.

CHAPTER V GENERAL DISCUSSION AND FUTURE DIRECTION

The results of this study suggested that each species has a different sensitivity to temperature and CO_2. The mostly insiginificant impacts of elevated temperature and CO_2 on escape response of Japanese anchovy is suggestive of relative robustness of the fish's muscular and sensory system in these conditions, although we must be extremely careful in extrapolating the finding. It is known for several tropical fishes that CO_2 may disturb sensory perception in them, and that escape response of schooling fish may be different from that of solitary individual. In comparison, the muscular system in the sea urchin seems to be more vulnerable to CO_2, although muscles of different organs (tube feet vs. masticatory organ) might show different sensitivities. Changes in proteome composition by prolonged exposure to CO_2 are novel, and worth further investigation. The results on giant tiger prawn are nothing more than preliminary, but it has important implications when viewed against water conditions in aquaculture ponds. In fact, our results suggest alkalization of pond water improve embryonic hatching and larval survival.

Overall, the results of the present study demonstrated that temperature and CO_2 can affect the behavior and muscular systems of commercially important animals in different ways. Together with knowledge on the effect of ocean warming and acidification on other aspects of marine life, these should form the basis for adaptational measures to sustain fisheries and aquaculture production in the coming decades.

要約

海洋温暖化および海洋酸性化は、海洋生物の石灰化（calcification）と生理機能に悪影響を及ぼすことが知られている。しかし、海洋温暖化および海洋酸性化および相互作用が、海洋動物の行動や筋肉系にどのような影響を及ぼすかについては、ほとんど知られていない。本研究では、カタクチイワシ、アカウニ、ウシエビに対する二酸化炭素濃度および水温上昇の単独影響と相乗影響を検討することを目的とした。実験条件としては、気候変動に関する政府間パネル（Intergovernmental Panel on Climate Change, IPCC）による 2100 年および 2300 年の予測値（CO_2 濃度・温度）、および二酸化炭素貯留(Carbon Capture and Storage, CCS)施設からの遺漏時に想定される CO_2 濃度とした。

水温上昇および二酸化炭素濃度上昇に伴うカタクチイワシ *Engraulis japonicas* の逃避反応

過去数年間で、魚類に対する海洋酸性化および海洋温暖化の影響について、多くの論文が発表されている。しかし、これらの環境変化が魚類の遊泳行動にどのような影響を及ぼすのかについては、ほとんど知見が得られていない。本研究では二酸化炭素濃度および水温上昇がカタクチイワシ *Engraulis japonicas* の逃避反応にどのような影響を及ぼすかについて検討した。4 条件（二酸化炭素濃度 400μatm と 1000 μatm，水温 15°C と 19°C）への 1 ヶ月の馴致後、機械的刺激に対する逃避反応について解析した。毎秒 500 フレームのハイスピードビデオカメラで反応を記録した。回転率は CO_2 濃度に関わらず、15°C より 19°C で顕著に高かった。しかし古回転率以外については、二酸化炭素も水温も運動学的パラメータ（逃避軌道、最大遊泳速度、最大加速度、逃避方向、反応タイプの頻度）のいずれにも影響を及ぼさなかった。しかし、将来の海洋環境変化が魚群の逃避反応と、被食－捕食の相互作用にどのような影響を及ぼすのかについて、さらに検討する必要がある。

アカウニの筋肉系およびプロテオーム組成に対する二酸化炭素濃度上昇の影響

初期発生や生理機能に対する影響研究から、ウニは海洋酸性化に対して脆弱な動物であることが知られている。しかし、海洋酸性化がウニの行動および筋肉系にどのような影響を及ぼすのかについてはほとんど知見がない。本研究では、アカウニ *Pseudocentrotus depressus* を 400μatm（対照群）、2000 μatm、10000 μatm CO_2 条件に馴致し、摂餌量、管足および咀嚼器（アリストテレスの提灯）の筋収縮力、プロテオーム組成について比較した。摂餌量は、10000 μatm の CO_2 条件下では低下したが、2000 μatm では影響は見られなかった。組織学的観察では、咀嚼器の後引筋（retractor muscle）繊維の断面積が、対照群より 2000 μatm および 10000 μatm で有意に減少した。管足の筋収縮力は、対照群と比較して 2000 μatm および 10000 μatm で著しく低下したが、咀嚼器の前引筋（protractor muscle）では有意な影響はなかった。管足から抽出したタンパク質の二次元電気泳動によって、高 CO_2 条件曝露によって 8 つのスポットのタンパク質量が変化した。このうち、6 つのスポットはタンパク質量が上昇（up-regulated）、2 つのスポットは減少（down-regulated）した。レーザーイオン化四重極イオントラップ飛行時間型質量分析装置を使用して、3 つのアップレギュレートされたスポット（チューブリン β 鎖、トロポミオシン断片、アクチン N 末端断片）と 2 つのダウンレギュレートされたスポット（アクチン C 末端断片、ミオシン軽鎖）が特定された。これらの結果は、CO_2 濃度上昇が、主に筋肉関連のタンパク質の翻訳後プロセシングとタンパク質分解に関連したプロテオームの変化によりウニの管足筋に障害を与える可能性があることを示唆している。この発見は二酸化炭素濃度上昇によるウニの筋肉機能障害の仕組みを理解するための手がかりとなる可能性がある。

ウシエビの初期段階における海洋温暖化と海洋酸性化の影響

海洋温暖化と海洋酸性化の甲殻類に対する影響はあまり知られていない。特に、商業的に重要なエビ類に対する海洋温暖化と海洋酸性化の影響について知見は乏しい。従って、本研究では 4 つの異なる水温（28°C (対照群)、30°C、32°C、34°C）と 4 つの異なる海水 pH（8.1(対照群)、7.6、7.0、6.8）のもとで、ウシエビの初期成長段階に対する影響を孵化率、生残率、遊泳速度、筋肉組織について検討した。孵化率は、pH7.6 以下および水温 32°C で有意に低下した。生残率についても同様の影響が見られた。筋肉の組織学的検索によっては、海水 pH や水温の影響を認めることができなかった。遊泳速度は、水温 30°C で極大となり、28°C と 32°C で差は見られなかった。

Chapter I

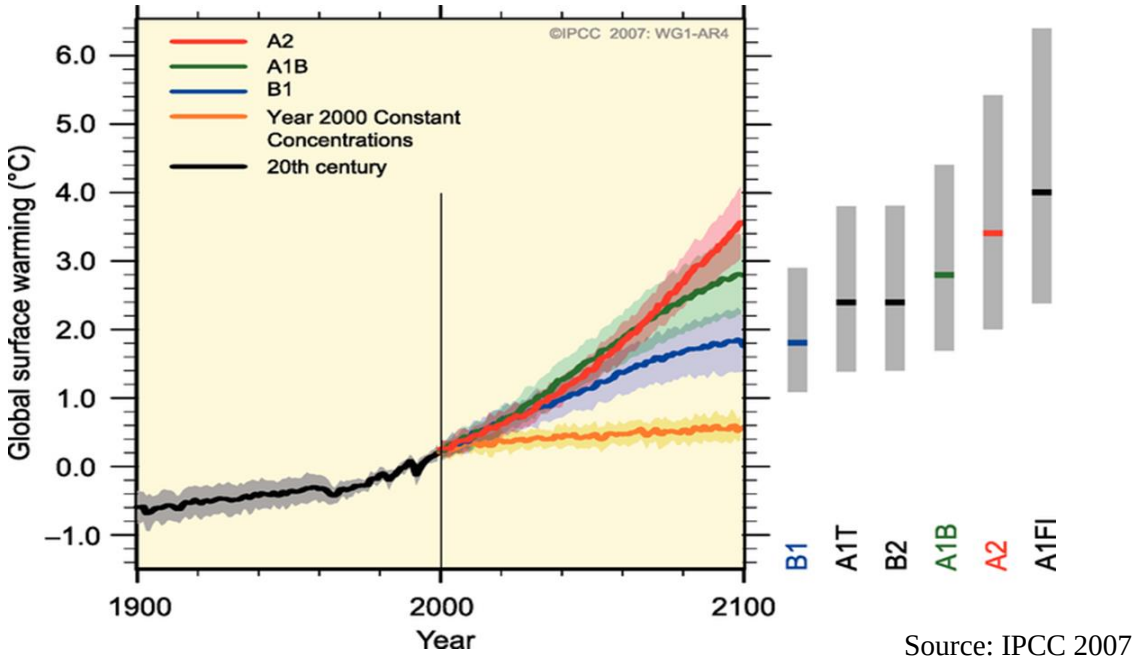
GENERAL INTRODUCTION

Greenhouse gas emission caused by the burning of fossil fuels has been identified as the major reason for the change of temperature in the atmosphere (global warming). This leads to global climate change which manifested in a number of effects including sea level rise, seasonal monsoon/rainfall variations, increased and stronger incidence of storms and typhoons and sea-surface temperature increases (IPCC, 2013). The effects are significant to humans, including the threat to food supplies due to decreasing crop yields and the loss of habitat from sea level rise (Kang et al., 2009). Intergovernmental Panel on Climate Change (IPCC) has reported that mean global temperature rise has been approximately 0.85°C during the last century. The recent climate models forecast a further increase of the mean global temperature of 1.5 to 4°C by the year 2100 relative to 1850 to 1900 with all Representative Concentration Pathways (RCPs) scenarios (IPCC, 2007) (Fig I-1). Seawater surface temperature is related to air temperature, so warming also results in seawater surface temperature rise possibly to a depth of 700 m, with the most significant changes in the upper 75 m from the surface. IPCC reported that the Arctic and eastern Pacific are the largest regions affected by rising seawater temperature (IPCC, 2013).

Temperature controls the rates of fundamental biochemical processes in organisms, and consequently, changes in the environmental temperature can influence population, species and community-level processes. At high temperatures, aquatic life has excessive oxygen demands that cause insufficient oxygen in the body fluids of the animals. Hypoxia conditions cause a reduction in both ventilation and circulation. This could affect all higher functions of the animals such as muscular activity, behavior, growth, and reproduction (Pötner, 2016). Each organism has a thermal death point as well as a different critical thermal range that produces an optimum abundance of each species depending on their habitat. Cold water animals have a narrower critical thermal range than warm water animals (Pötner, 2016). For example, the range of temperatures of survival of brown trout is between 4 – 12°C (Réalis-Doyelle et al, 2016), the

optimum temperatures for salmonid egg survival ranges from 6 - 10°C (Carter, 2005) and the spawning temperature of the Japanese anchovy (*Engraulis japonicus*) ranges from 15 - 28°C, while the Japanese sardine (*Sardinops melanostictus*) ranges from 13 - 20°C (Takasuka et al., 2008). Temperature is associated with habitat selection, for example, cod fish select vegetated habitat when the water temperature is lower than 16°C, but they live in rock and sand beds in deeper water when the water temperature is higher than 16°C (Freitas et al., 2016). At the cellular level, heat shock protein is synthesized to protect cellular molecules when the animal was exposed to extreme thermal events (Tomanek and Somero, 2000).

Gattuso et al. (2015) stated that average surface temperature rise of only 2°C could affect marine organisms from mid-latitude to high latitude (mid-latitude seagrass, high-latitude pteropods and krill, mid-latitude bivalves, and finfish). The marine animals in tropical and polar regions are at the greatest risk due to the change in thermal conditions.



Source: IPCC 2007

Fig I-1 Mean global temperature of RCP scenarios for the year 2100

Burning of fossil fuels is a major cause of the reduction of seawater pH. CO_2 represents about 77% of the total of emitted greenhouse gases. The anthropogenic CO_2 emission is approximately 8 Gt per year, of which 30-50 % dissolve into the oceans (IPCC, 2007; Sabine et al., 2004). The oceans play a crucial role in the global carbon cycle, forming an important sink for anthropogenic CO_2. CO_2 reacts with seawater to form carbonic acid (H_2CO_3), and then dissociates to bicarbonate and hydrogen ions. This leads to a lowering of seawater pH and other chemical changes collectively termed as ocean acidification (Calderia and Wickett. 2003). The increase in hydrogen ion concentration causes carbonate ions to react with hydrogen to become bicarbonate. Therefore, the dissolution of CO_2 in seawater increases the concentrations of hydrogen ions, carbonic acid and bicarbonate whilst decreasing the concentration of carbonate (Fig. I-2).

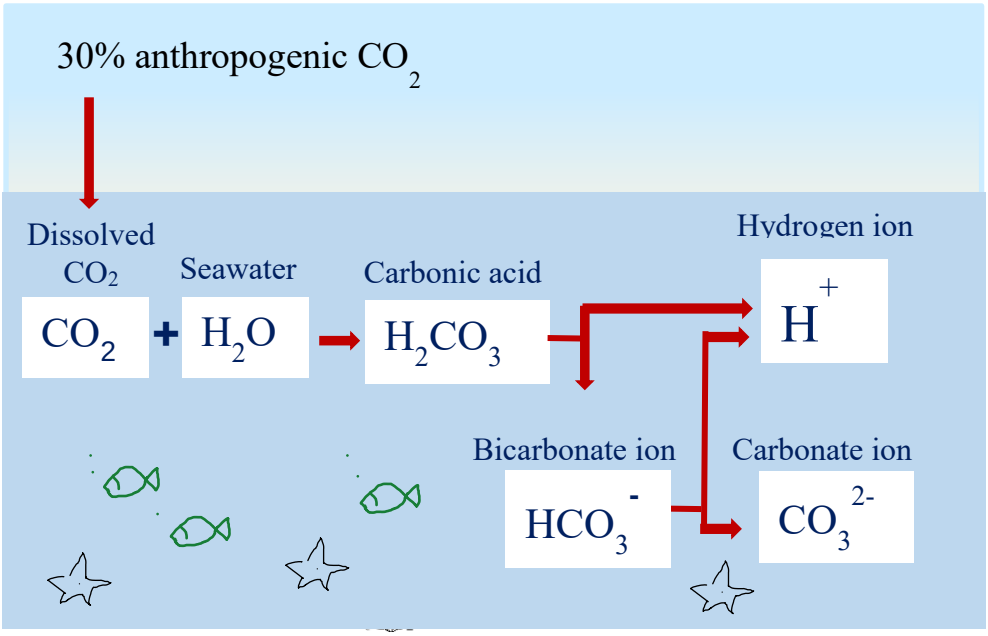


Fig. I-2 Mechanism of anthropogenic CO_2 reduction seawater pH

The anthropogenic CO_2 is projected to increase by 0.5 % per year throughout the 21st century, which is 100 times faster than what has occurred in the past 650,000 years (Meehl et al., 2007). The current concentration of atmospheric CO_2 is about 400 ppm (NASA, accessed 26 October 2016), having increased from pre-industrial levels of 280 ppm, and an emissions scenario predict a continued increase to 936 (RPC

8.5) and 1900 ppm (IS92a) by 2100 and 2300 respectively (IPCC, 2013; Caldeira and Wickett, 2003). The pH of ocean surface water has decreased 0.1 unit since the beginning of the industrial era and predicted that an average surface ocean pH will further decrease by 0.30 and 0.8 units by the year 2100 and 2300 respectively (IPCC, 2013; Caldeira and Wickett, 2003).

The accidental leakage of CO_2 from carbon capture and storage (CCS) sites is another source of CO_2 which reduces sea water pH. The CCS was introduced in many regions around the world, aiming to mitigate global climate change. The CCS can reduce CO_2 emissions by isolating CO_2 from flue gases at large emission sites and injecting CO_2 into geological reservoirs via pipelines (Schrag, 2009). Globally, there are 16 large-scale projects in operation, with a further six projects under construction (Global CCS institute, 2015). CCS can lead to CO_2 leakage from the pipeline either during transferal or at the seabed when the pressure and temperature of the reservoir increase and the cap-rock seal is fractured (Taylor et al., 2014). Accidental leakage of stored CO_2 through seabed, which would impact benthic ecosystems near a leakage site, has been one of the major concerns about the CCS technology (Taylor et al., 2015). When CO_2 leakage occurs, CO_2 concentrations in the surrounding seawater can be much higher than those predicted under ocean acidification. Therefore, the CO_2 levels used in the recent risk assessment studies of leakage from CCS sites are as high as 18325 µatm (Murry et al., 2013), 20000 µatm (Rastelli et al., 2015), 29000 µatm (Ishida et al., 2013), or even up to 313862 µatm (De Orte et al., 2014), an order of one or two higher than in ocean acidification studies (typically up to 1000 or 2000 µatm). The rapidly changing oceanic environmental conditions will likely alter the structure and function of marine ecosystems (Fabry et al., 2008; Hoegh-Guldberg and Bruno, 2010), which will undermine fisheries productivity and other ecosystem services of the ocean (IPCC, 2013).

Effects of CO_2 on marine organisms have become a focus of marine biological research during the past 20 years, mainly because of the increasing concern for the impacts of ocean acidification (Gattuso and Hansson, 2011) and CO_2 leakage from a CCS site (Noble et al., 2012). As a result of much research effort, it is now generally accepted that calcification (i.e., the formation of $CaCO_3$ structures from Ca^{2+} absorbed from seawater and HCO_3^- mainly generated by metabolism but also originated from seawater carbon pool,

(Furla et al., 2000) is one of the most sensitive biological processes negatively affected by seawater acidification (Kroeker et al., 2013), although different organisms may show different sensitivities and response patterns (Ries et al., 2009). Similarly, relatively rich information is now available on the effect of ocean acidification on biological processes such as early development, growth, metabolism, photosynthesis, and survival (Kroeker et al., 2013). By comparison, much less is known about how CO_2 affects the integrity and functionality of muscular systems. There are only several papers that studied histology and physiological responses of muscular systems to high CO_2 conditions. Wood et al. (2008) reported that the amount of muscles in the arms of the brittlestar, *Amphiura filiformis*, decreased in lowered pH (7.7, 7.3 and 6.8) but without structural changes in muscles. In contrast, the brittlestar *Ophiura ophiura* showed no difference in muscle density in lowered pH (7.7 and 7.3; PCO_2 1300 - 1400 and 2300 - 2500 μatm, Wood et al., 2010). Schalkhausser et al. (2013) found significant declines of force generated by the adductor muscle in the king scallop, *Pecten maximanus*, collected in Norway and reared under PCO_2 of 1120 μatm at 10°C. But the effect was not detected for the same species from France under the same experimental conditions (Schalkhausser et al., 2014). Chambers et al. (2014) and Frommel et al. (2016) showed subtle histology alterations in fish larvae (*Paralichthys dentatus* and *Thunnus albacares*) under elevated CO_2 conditions (1800 and 4700 μatm, and 2000-9600 μatm, respectively), but the functional significance of these changes was not studied. We have recently found that CO_2 significantly reduced the locomotion speed of the sea urchin, *Hemicentrotus pulcherrimus*, reared under 1000 μatm for seven months (Yin, Lee, Kurihara and Ishimatsu, in preparation).

The combined effect of ocean warming and acidification has become an important issue to understand how multiple-stressor interactions affect marine organisms and ecosystems. Increasing temperature has a stimulatory effect on development, whereas hypercapnia can depress developmental processes (Byrne and Przeslawski, 2013). Recent papers found that combination between temperature and CO_2 reduced carbonate mineral saturation and their interactive effects will have significant impacts on calcifying marine invertebrates (Byrne, 2011; Byrne and Przeslawski, 2013). Simultaneous exposure to increased temperature and CO_2 significantly reduced larval metabolism and triggered a widespread downregulation of histone

encoding genes in sea urchin larvae (Padilla-Gamiño et al., 2013). Respiration rates and ammonium excretion rates of mussels (*Mytilus coruscus*) were significantly lower in pH 7.7 and 7.3 than 8.1 (control) for both temperatures of 25°C and 30°C and significantly lower at 30°C than 25°C (control) after exposure for 14 days (Wang et al., 2015). This also found the interaction of both factors have increased predation rate of coral reef fish when acclimated at PCO_2 995 µatm and increased temperature of 3°C (Ferrari et al., 2015).

Three species were selected for this study. The Japanese anchovy *Engraulis japonicus* was selected in this study for the following two reasons. Firstly, it is very important commercially, being ranked ninth in the list of major marine fish species in production, amounting to an annual global catch of 1.3 million tonnes in 2012 (FAO., 2014b). In fact, both the top and 21st species in the list are also from the genus *Engraulis* (Peruvian anchovy *E. ringens* and European anchovy *E. encrasicolus*, respectively), underpinning the importance of the genus in fisheries production. Secondly, the Japanese anchovy plays an important role in the marine ecosystem as prey of higher trophic predators such as skipjack tuna (Takasuka et al., 2004) and, as such, experiences a high mortality rate due to predation. Sea urchin was selected for the second study because they have calcifying exoskeletons, play a critical role in controlling the balance between kelp ecosystems (Pearse, 2006) and are a species with a high demand of the Japanese consumer market (FAO, 2015). Marine invertebrates have been extensively studied as bioindicators of environment stress (Jha, 2004). Sea urchins have been frequently used in ocean acidification studies. The present knowledge on the effect of ocean acidification is somewhat biased to early developmental stages, while less is known for juvenile and adult stages (Dunpont and Thorndyke, 2013). Giant tiger shrimp (*Penaeus monodon*) was selected for the third study. Shrimp is an important species of the world market. Globally about 7.4 million tonnes was consumed in 2014 and global demand is increasing year by year. In 2014, southeast Asia produced about 32% of world shrimp production. Major countries of production are Indonesia, Vietnam and Thailand (FAO, 2014a). Giant tiger shrimp was selected for this study because it is one major species for farming and a wild brood stock is commonly used for production. If the predicted temperature and CO_2 conditions affect the giant tiger shrimp, then this could increase mortality in this species, affecting the fishery market and availability for human consumption.

The aim of this study was to examine both separate and combined effects of elevated CO_2 and temperature on the behavior and muscular system of the three species. Locomotion is one important behavior that helps animals move between locations. Vertebrates have two major systems related to movement and locomotion, the skeleton system and the muscular system. In general, invertebrates only have a muscular system for movement, with the exception being crustaceans that have a exoskeleton and muscular system. Animals move for a variety of reasons, such as to find food sources, to mate and to escape from predators. Seawater pH and temperature were set based on the prediction scenarios for the years 2100, 2300 and the accidental leakage from the CCS sites. For the Japanese anchovy, the aim of this study was to investigate ocean warming (OW) and OA on fast start escape response. The anchovies were exposed to four conditions, control (CO_2 at ambient, temp. 15°C), high CO_2 (CO_2 at 1000 μatm, temp. 15°C), high temperature (CO_2 at ambient, temp. 19°C) and combined (CO_2 at 1000 μatm, temp. 19°C). The study on sea urchins investigated the effect of elevated CO_2 on muscular system and proteome composition. The sea urchins were exposed to three different CO_2 levels, 400 μatm (control), 2000 μatm (2300 prediction) and 10000 (CCS leakage). For giant tiger shrimp, I examined the effect of OW and OA on the early life stage. The fertilized eggs were exposed to four different sea water pH (8.2, 7.3, 7.0 and 6.8) and four different temperature levels (28°C, 30°C, 32°C and 36°C).

Chapter II

ESCAPE RESPONSE OF JAPANESE ANCHOVY *Engraulis japonicus* UNDER ELEVATED TEMPERATURE AND CO_2 CONDITIONS

(Nasuchon et al. 2016)

1. Introduction

Scientist recognized that ocean warming and acidification have negative impacts to marine organism. Reflecting mounting concern about the fate of marine ecosystems in the coming decades, there has been a rapid growth of scientific literature attempting to predict how marine species and ecosystems will respond to these environment changes (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012; Branch et al., 2013; Harvey et al., 2013). Although earlier studies of ocean acidification focused mainly on invertebrates, more attention has been paid to fishes during the last few years. Recent papers on the ocean acidification effect on fish have demonstrated that ocean acidification disrupts olfactory discrimination of chemical cues (Munday et al., 2009), visual perception of a predator fish (Ferrari et al., 2012a) which may be related to impaired retinal function (Chung et al., 2014), and auditory ability to discriminate sounds appropriate for settling (Simpson et al., 2011). In addition to these findings on sensory functions, several studies have also shown that CO_2 could have detrimental impacts on the brain function of fish, which may have direct implications for fish survival. For example, Ferrari et al. (2012b) demonstrated clear evidence that acclimation to CO_2 (850 μatm) would suppress the learning ability of juvenile fish. Elevated CO_2 also disrupts the behavioral lateralization of fish, which may influence efficacy of an escape response (Domenici et al., 2012). More directly, coral reef fish larvae pretreated with CO_2 (700 and 850 μatm) and released to a natural reef showed significantly higher mortality, though the cause-and-effect relationship of this finding is somewhat obscure (Munday et al., 2010). In contrast, the data by Allan et al. (2013) demonstrated that when predators acclimated to control CO_2 conditions are interacted with prey fish acclimated to 880 μatm CO_2, capture success rate decreased with no change in predation rate (capture success/number of attack). There is some evidence for the involvement of a neurotransmitter, gamma-aminobutyric acid (GABA), in alterations of sensory and behavioral functions under elevated CO_2 (Nilsson et al., 2012; Chivers et al.,

2014; Hamilton et al., 2004), but the mechanism(s) for the cognitive and behavioral disturbance by CO_2 needs more scrutiny. In comparison, much less is known about swimming responses to elevated ambient CO_2 (Melzner et al., 2009; Bignami et al., 2013; Maneja et al., 2013; Bignami et al., 2014).

One crucial behavior that determines survivorship of fish is the escape response (Domenici, 2010a). In response to a predation risk, fish generally show a behavior termed C-start, which determines whether or not fish can avoid a predator. The escape response (C-start) is the highest swimming speed attainable by a fish and occurs during the 'flight or fight' response to predators. C-start begins with unilateral contraction of trunk musculature, bending the fish's body into a C shape (stage 1) and may be followed by contraction of the contralateral trunk musculature (stage 2) (Domenici and Blake, 1997). An escape response consists of non-locomotion variables, which relate to sensory perception of a stimulus and processing of the sensory input to trigger a response (responsiveness, i.e., whether a fish respond to a stimulus or not, escape latency and directionality) and locomotion variables, which directly govern kinematics of the escape response (turning rate, distance, swimming speed and acceleration) (Domenici, 2010b). Because escape responses are usually triggered by a specific type of cells in the central nervous system (the Mauthner cells) (Eaton et al., 2001), one might expect that CO_2 would somehow modify or disrupt escape responses of fish.

Since ocean acidification will proceed concurrently with temperature rise (IPCC, 2013; Meinshausen et al., 2011), it is crucial to understand how these two environmental changes will affect marine species and ecosystems in concert. Under the RCP 8.5 pathway, surface ocean temperature is projected to rise 2°C by the year 2100 (Meinshausen et al., 2011). Numerous papers have already been published on the effect of temperature on various aspects of fish biology [see Wood and McDonald, 1997; Currie and Schulte, 2014 for review] because temperature is one of the most powerful environmental factors to modulate biological activities (Willmer et al., 2005). Similar to all other animal groups, temperature almost invariably exerts strong influences on development (Boucher et al., 2014; Ma, 2014; Politis et al., 2014), behavior (Malavasi et al., 2013; Bartolini et al., 2014; Johansen et al., 2014), metabolism (Clarke and Johnston, 1999; Tirsgaard et al., 2015), growth (Khan et al., 2004; Neuheimer et al., 2011; Sun and Chen, 2014), and swimming (Pang et al., 2013; Almeida et al., 2014; Cai et al., 2014) of fishes. It has also been reported that temperature

affected tail-beat frequency, muscle twitch contraction and aversive behavior of fish (Batty et al., 1993; Johnson and Bennett, 1995; Manciocco et al., 2015), and that increasing CO_2 levels could reduce the swimming ability of both predators and prey (Allan et al., 2013; Allan et al., 2014). Domenici et al. (2014) showed that elevated temperature attenuated the magnitude of lateralization in a marine damselfish without affecting the directionality of turning behavior, whereas elevated CO_2 significantly reversed the turning bias. To our knowledge, no study has been conducted on the interactions of temperature and CO_2 on the escape response of fish.

The aim of this study was to examine both separate and combined effects of temperature and CO_2 on the escape responses of fish. The Japanese anchovy *Engraulis japonicus* was selected in my study for the following two reasons. First, it is commercially very important, as being ranked ninth in the list of major marine fish species in production, amounting to the annual global catch of 1.3 million tonnes in 2012 (FAO., 2014a). In fact, both the top and 21st species in the list are from the genus *Engraulis* (Peruvian anchovy *E. ringens* and European anchovy *E. encrasicolus*, respectively), underpinning the importance of the genus in fisheries production. Second, the Japanese anchovy plays an important role in the marine ecosystem as prey of higher tropic predators such as skipjack tuna (Takasuka et al., 2004) and, as such, experiences a high mortality rate due to predation. The Japanese anchovy is distributed in the western north and central Pacific including the Yellow Sea, East China Sea and Sea of Japan, showing changes in distribution pattern with resource abundance (Whitehead et al., 1988; Funamoto and Aoki, 2002). The main distribution areas of the Japanese anchovy, the East China Sea and the Sea of Japan, are among the most rapidly warming large marine ecosystems around the world (Belkin, 2009). Thus, studying the escape response of Japanese anchovy to elevated CO_2 and temperature should provide useful information to foresee the trajectory of this important fishery species into the future ocean.

2. Material and methods

2.1 Experimental animals

In January 2013, approximately 200 adult Japanese anchovies (total length = 99.3 ± 9.9 mm (mean ± SD, N = 25) were purchased from a fisherman in Saikai-shi, Nagasaki, Japan (33°05' N, 129°41' E), who maintained the anchovies in fish cages as bait for skipjack tuna fishing. The fish were transferred to the Institute for East China Sea Research of Nagasaki University, Japan and stocked in two 500 L tanks supplied with a continuous flow of fresh seawater at a flow rate of 4 L min^{-1}. Fish were fed artificial pellet (diameter 2.3 mm, Otohime EP2, Marubeni Nisshin Co. Ltd., Tokyo) by using a custom-made automatic feeding machines every 30 min from 9 am to 5 pm with a daily ration of 5% body weight. The Japanese anchovy turned out to be highly sensitive to handling stress, and a high mortality occurred within three days of the transportation. The fish were maintained for two weeks before being acclimated to the experimental conditions described below.

2.2 Acclimation

After two weeks of transportation, eighty fish were randomly chosen, divided into four groups of 20 each and acclimated to four conditions; control condition (seawater equilibrated with ambient air containing 400 μatm CO_2 and temperature at 15°C), high CO_2 condition (seawater equilibrated with air containing CO_2 at a concentration of 1000 μatm and temperature at 15°C), high temperature condition (CO_2 same as control and temperature at 19°C) and combined condition (CO_2 1000 μatm and temperature at 19°C. There were two acclimation tanks for each treatment (a total of eight tanks) and 10 fish were stocked in each tank (20 fish per treatment). The acclimation tanks were 100 L in capacity and supplied with filtered (1 μm) seawater at a flow rate 0.4 L min^{-1}. The overflow from the tanks was drained without recirculation. Seawater in the acclimation tanks was bubbled with atmospheric air for control or CO_2-enriched air for high CO_2 conditions at a flow rate of 20 L min^{-1}. Water temperatures of 15ºC and 19ºC were controlled by a submersible heater and a thermostat. The fish were fed with artificial pellets at a daily ration of 5% of body

weight in three portions. Excess food was removed daily by siphoning. Water quality in each tank was monitored at 10:00 am daily for dissolved oxygen (DO) concentration, pH (NBS scale) and salinity. DO concentration was measured with a digital DO meter (YSI proODO dissolved oxygen meter, USA) and never fell below 85% saturation. Salinity was measured with a digital salinometer (Mettler-Toledo GmbH SG3, Switzerland). Water pH was measured with a digital pH meter (Mettler-Toledo GmbH SG8, Switzerland), calibrated with standard buffer solutions of pH 4.01, 6.86 and 9.18 (Nacalai Tesque Inc. Kyoto Japan). Alkalinity was measured with a total alkalinity titrator (Kimoto, ATT-05, Japan) at weekly intervals. Partial pressure of CO_2 (PCO_2) was calculated from measured seawater pH, temperature and total alkalinity by using the program CO2SYS (Pierrot et al., 2006). The concentration of ammonia in seawater was measured twice weekly with an ammonia electrode (Orion 9512, Thermo Scientific, USA), calibrated with a standard solution of 1000 μatm NH_3. The fish were acclimated to the four experimental conditions for one month before testing between 4 February and 7 March 2013. The data of seawater temperature, oxygen saturation and carbonate chemistry during acclimation are shown in Table II-1.

I used every precaution to avoid any stressful treatment to the anchovies during acclimation, but daily cleaning of the tanks was apparently stressful to them. Many fish jumped out from the tanks or into the net covering the tanks. Thus, the number of fish decreased from 80 to 48 (Control 15, high CO_2 12, high temperature 10, and combined 10) during the 1-month acclimation period.

Table II-1 Seawater temperature, pH_{NBS}, salinity, oxygen saturation (SO_2), ammonia (NH_3) concentration, total alkalinity (A_T) and calculated CO_2 partial pressure (PCO_2) values (mean ± SD) during acclimation

Treatments	Measured						Calculated
	Temperature (Range, °C)	pH_{NBS}	Salinity PSU	SO_2 %	$[NH_3]$ µatm	A_T µmol/l	PCO_2* µatm
Control	14.8 - 16.3	8.12 ± 0.03	34.61 ± 0.49	89 ± 2	0.0017 ± 0.0004	2246 ± 60	424 ± 26
High CO_2	14.7 - 16.2	7.83 ± 0.02	34.61 ± 0.49	90 ± 2	0.0036 ± 0.0009	2233 ± 29	939 ± 31
High temp.	18.3 - 20.1	8.11 ± 0.03	34.61 ± 0.49	90 ± 3	0.0017 ± 0.0005	2227 ± 35	443 ± 15
Combined	18.2 - 20.2	7.84 ± 0.02	34.61 ± 0.49	87 ± 3	0.0044 ± 0.0007	2237 ± 28	946 ± 23

*Calculated from measured temperature, pH_{NBS}, salinity and A_T. In a gas phase, 1 µatm = 1 µatm at the barometric pressure of 1 atm. N = 56

2.3 Procedure

Seven dead fish (total length = 98.4 ± 4.5 mm and wet body mass = 8.80 ± 0.71 g; mean ± SD) were used to estimate the position of the center of mass (CM). The CM was estimated by hanging dorsally each dead fish with a needle. The balancing point of the body indicated the position of the CM at 43.64 ± 1.70 mm, i.e., 44.3 ± 0.2% of the total length, from the snout. A black polyethylene tank (capacity 500 l, diameter 150 cm, height 80 cm) with a light yellow sheeting on the bottom was used for recording escape responses. The tank was filled with seawater to a depth of 20 cm. Escape responses were studied under the same CO_2 and temperature conditions as during acclimation. The stimulus (a rubber parcel) was released through a 75 cm long PVC tube with a diameter of 6 cm by triggering an electromagnetic device. To avoid visual stimulation, the PVC tube was positioned at the center of the experimental tank, with the lower end 5 mm above water surface. A high-speed video camera (HAS-L1, Ditect Co., Japan) was installed two meters above the experimental tank. A 180 W spotlight was mounted on the upper edge of the experimental tank and two 32 W fluorescent lamps were installed above the tank. The light intensity at the water surface of the testing tank was about 1800 lx. Each fish was tested individually and only once. Before triggering the

electromagnetic stimulus, the anchovies were allowed to habituate themselves in the test tank for about 10 minutes until they started to swim in a normal manner. When the fish swam to the center of the tank, the stimulus was activated. A high-speed video recorded the escape response at 500 frames/second. The video recording was triggered two seconds before the stimulus was dropped and lasted for four seconds.

2.4 Measured variables

The fish movement was sequentially tracked frame by frame by using ImageJ 1.46r (National Institute of Health, USA). The x-y coordinates of the CM and the tip of the head were digitized in each frame. The following variables were calculated according to Lefrançois et al. (2005): (1) response latency, i.e. the time interval between the stimulus hitting the surface and the first detectable movement of the escape behavior; (2) response type, termed single bend (SB), when the tail did not recoil completely after the formation of the C or double bend (DB), when a full return flip of the tail occurred after initial contraction; (3) directionality, the 'away' and 'toward' responses which were defined on the basis of the first detectable movement of fish being either oriented away or toward the stimulus; (4) stage 1 duration, the time between the first detectable fish movement and the onset of the return tail flip; (5) stage 2 duration, the time between the end of stage 1 and the end of the return tail flip; (6) total duration, the sum of the stage 1 and stage 2 durations; (7) initial orientation (A0), the angle between the line passing through the stimulus and the CM, and the line passing through the CM and the tip of the head at the onset of stage 1 (as shown in Fig. II-1); (8) stage 1 angle (A1), the angle between the lines passing through the CM and the tip of the head at the onset and the end of stage 1; (9) stage 2 angle (A2), the angle between the lines passing through the CM and the tip of the head at the end of stage 1 and at the end of stage 2; (10) escape angle (A3), the sum of A1 and A2; (11) escape trajectory angle (A4), the sum of A0 and A3; (12) turning rate, calculated by dividing A1 by stage 1 duration; and (13) the cumulative distance (D), maximum speed (V_{max}), and maximum acceleration (A_{max}) were determined within a fixed 48 ms duration which was the time needed to complete stages 1 and 2 (see Results). Speed and acceleration were calculated by differentiation and double differentiation, respectively, by the cumulative distance for the time-series. A five points smoothing with

polynomial regression was applied to calculate the speed and acceleration using QuickSAND software (Walker, 1998).

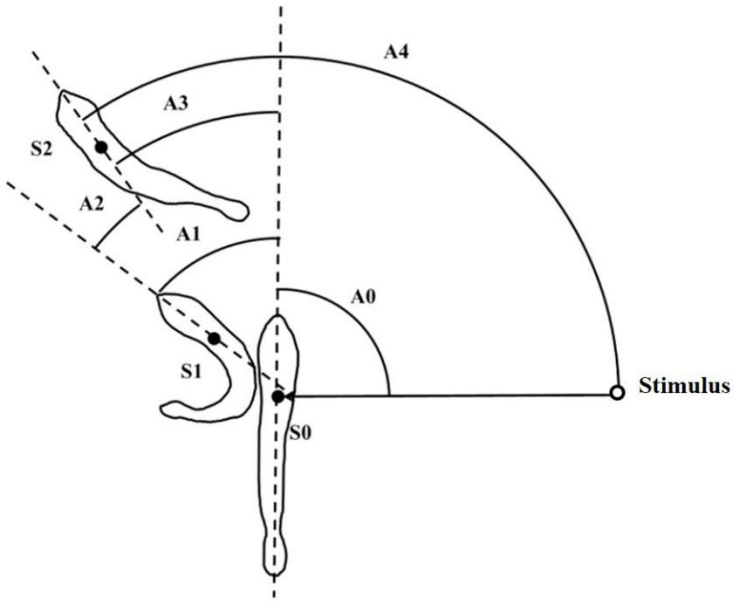


Fig. II-1 Schematic drawing of angular variable. The solid circles indicate the positions of the center of mass. The open circle indicates the position of stimulus hitting the water surface. S0, position of fish at the onset of stage 1; S1, position at the end of stage 1; S2, position at the end of stage 2; A0, initial orientation; A1, stage 1 angle; A2, stage 2 angle; A3, escape angle; A4, escape trajectory angle.

2.5 Statistical analysis

Due to the unexpected mortality during acclimation period, a total of 48 fish were used for testing C-start response. One fish in the high temperature group failed to respond to the stimulus. Therefore, data from 47 individuals were used for analysis (15 in the control treatment, 12 in the high CO_2 treatment, 10 each in high temperature and combined treatments). The SPSS 16 was used to analyze all the measurement parameters. Two-way ANOVA with temperature and CO_2 as fixed factors was used to compare response latency. A binary logistic regression was applied to analyze response type and directionality (away and toward the stimulus), in which the response type or directionality was regarded as the objective variable,

and temperature, CO_2 and their interaction were regarded as explanatory variables. The significances of these explanatory variables were assessed by using the Wald *t*-test. The initial orientation was compared by a Kruskal-Wallis test, followed by Mann-Whitney *U* test for pairwise comparison. Mardia-Watson-Wheeler test was applied to detect differences in angular variance of escape direction between treatments. Because of multiple pairwise testing, Bonferroni correction was applied to adjust the alpha for each comparison. There were six hypotheses being tested at *P* value 0.05, and therefore the new critical *P* value equals 0.05/6 or 0.008. Two-way ANCOVA was applied to assess the effect of temperature and CO_2 on the s1 and s2 durations, turning rate, cumulative distance, maximum velocity and maximum acceleration. Webb (1976) observed that fast-start increased with size while Domenici and Blake (1993) interpreted that the initial orientation of the fish had affected the directionality of escape. Therefore, the effect of both fish size and initial orientation were also included as explanatory variables to estimate all the parameters.

3. Results

3.1 Initial orientation and timing variable

The median of initial orientation angle was found to be 150.8, 98.8, 146.7 and 113.8 degrees in each of the control, high CO_2, high temperature and combined treatments, respectively. The initial orientation angle was significantly smaller in high CO_2 treatment, compared to the control and high temperature treatments (Table II-2). The data on response latency, s1 and s2 durations are shown in Table II-3. Two-way ANOVA detected no significant effect of temperature, CO_2 or their interaction on the response latency (Table II-4). Similarly, two-way ANCOVA failed to detect significant effects of temperature, CO_2, or their interaction on the s1 duration or s2 duration (Table II-4).

3.2 Directionality and escape trajectory

The proportions of fish whose first detectable movement was away from the stimulus in control, high CO_2, high temperature and combined treatments were 40, 50, 60 and 60%, respectively. Binary logistic

regression indicated that temperature or CO_2 did not have statistically significant effect on directionality (Table II-4). Multiple Mardia-Watson-Wheeler test with adjusted *P* value showed no significant difference in escape trajectory angle between treatments (Table II-2, Fig. II-2).

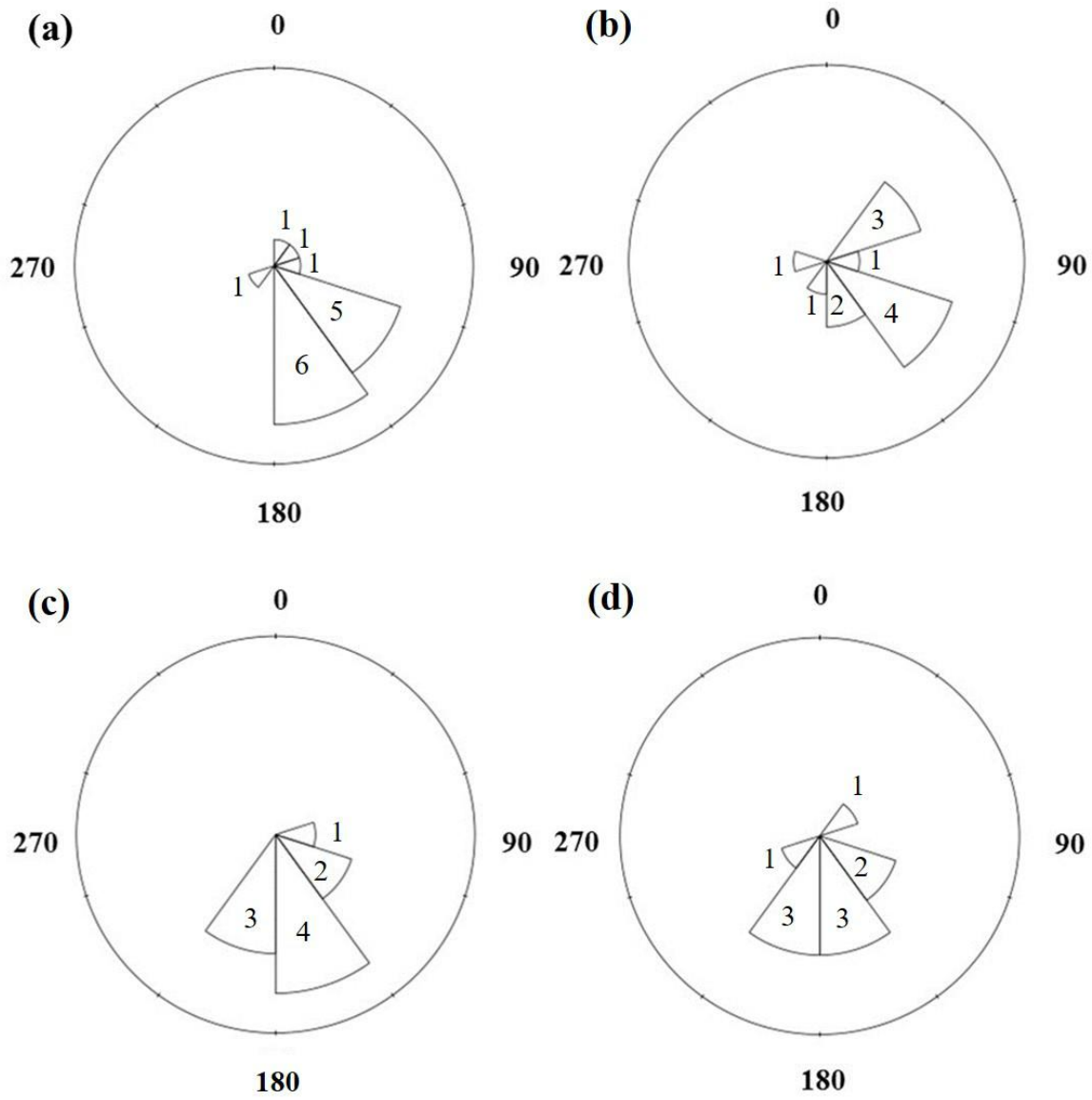


Fig II-2 Circular frequency distribution of escape trajectory angle of the Japanese anchovy defined as swimming direction at the end of stage 2 with respect to the stimulus orientation at 0°. The number beside a sector represents the number of fish that had an escape trajectory angle within each central angle division of 36°. (a) control, (b) high CO_2, (c) high temperature and (d) combined.

Table II-2 Statistical analysis of the effects of temperature, CO_2 and their interaction on the escape response of the Japanese anchovy *Engraulis japonicas*

Treatments	Initial orientation (A0)		Escape trajectory angle (A4)	
	U	*P*	*W*	*P*
Control-High CO_2	33.0	0.005*	3.1898	0.3183
Control-High temp.	73.0	0.921	1.4708	0.4936
Control-Combined	51.0	0.183	1.7159	0.4388
High CO_2-High temp.	18.0	0.006*	4.0339	0.200
High CO_2-Combined	51.0	0.553	4.3998	0.1225
High temp.-Combined	28.0	0.096	0.4584	0.8044

*Significantly different. Mann-Whitney *U* test and Mardia-Watson-Wheeler test were used for the pairwise comparisons of initial orientation and escape trajectory angle between treatments, respectively. N = 47

Table II-3 Response latency and durations of stage 1 (s1) and stage 2 (s2) of the Japanese anchovy *Engraulis japonicas* in control, high CO_2, high temperature and combined treatments (mean ± SD)

Treatments	Response latency (ms)	s1 duration (ms)	s2 duration (ms)
Control	6.3 ± 4.0	22.1 ± 5.4	21.7 ± 3.4
High CO_2	7.3 ± 2.6	24.8 ± 10.6	21.4 ± 4.1
High temp.	4.8 ± 1.7	21.8 ± 9.4	22.3 ± 3.5
Combined	4.8 ± 3.0	27.1 ± 10.9	21.3 ± 3.0

3.3 Turning rate and response type

The turning rate was calculated for both single bend (SB) and double bend (DB) responses. The turning rate at 19°C was significantly higher than those at 15°C (Table II-4, Fig. II-3a). Binary logistic regression showed neither temperature nor CO_2 affected response type (Table 4; Fig. II-3b).

3.4 Kinematics

The mean (± SD) values of cumulative distance (*D*), maximum velocity (V_{max}) and maximum acceleration (A_{max}) within the average s2 duration (48 ms) of control, high CO_2, high temperature and combined treatments are shown in Fig. II-4. ANCOVA showed that there were no significant effects of temperature, CO_2 or their interaction on *D*, V_{max} or A_{max} (Table II-4).

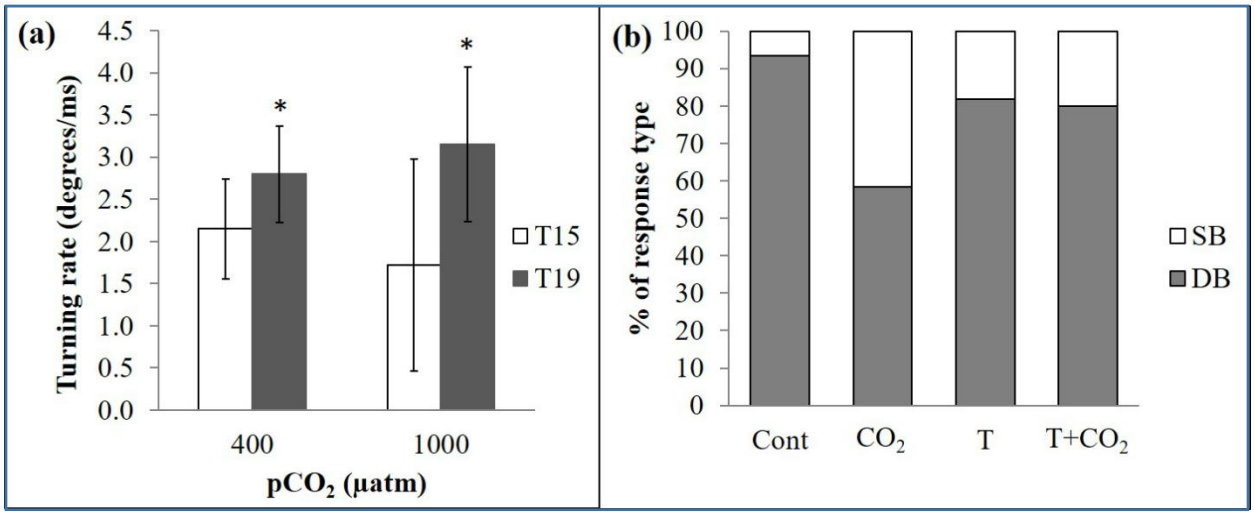


Fig. II-3 (a) Comparison of turning rate between the four treatments (mean ± SD). (b) Percent frequency of single (SB) and double bend (DB) response in each treatment. *Significant difference between 15°C and 19°C data ($P < 0.05$, Two-way ANOVA). Cont: control, CO_2: high CO_2, T: high temperature, T+CO_2: combined.

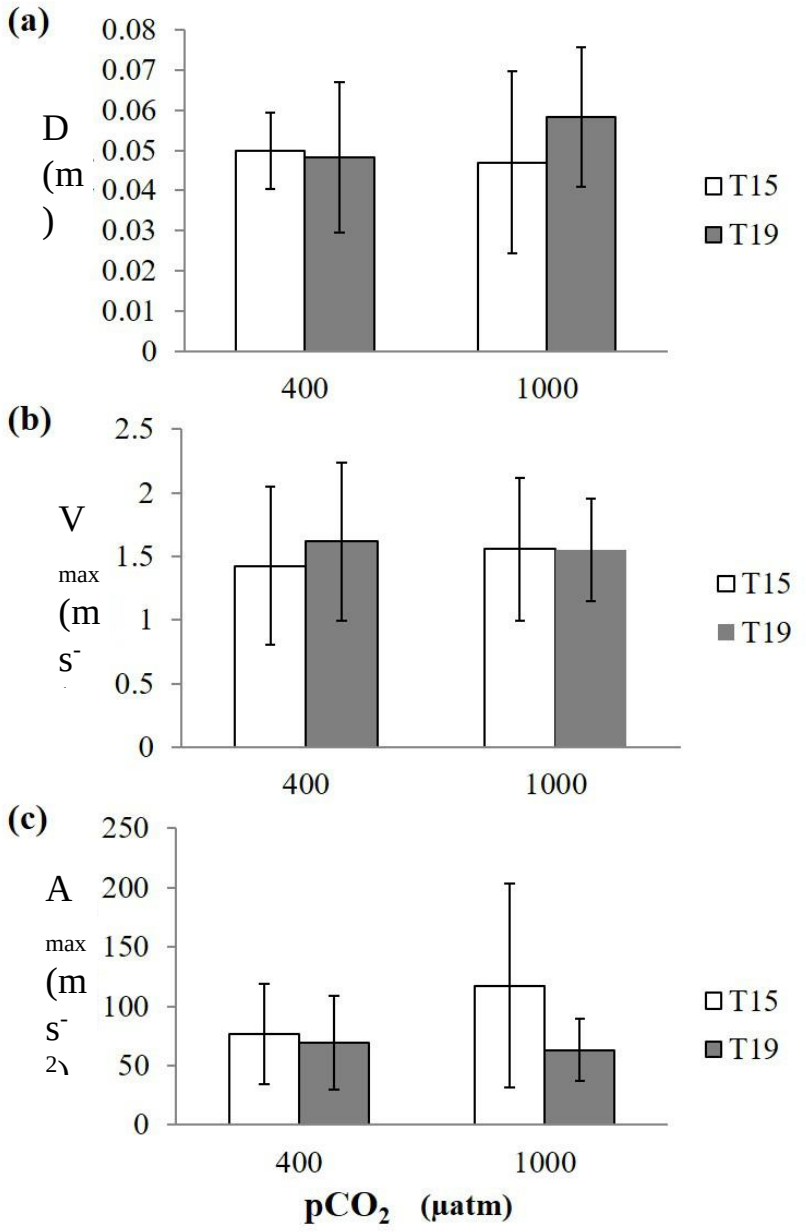


Fig. II-4 Comparison of swimming kinematics of the Japanese anchovy in four treatments (mean ± SD). (a) cumulative distance, (b) maximum velocity, and (c) maximum acceleration.

Table II-4 Statistical analysis of the effects of temperature, CO_2 and their interaction on the escape response of the Japanese anchovy *Engraulis japonicas*.

Factors	L_t		T_r		s1 duration		s2 duration		D		V_{max}		A_{max}		D_t		R_t	
	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	Wald t	P	Wald t	P
Temp.	3.417	0.071	16.18	0.001*	0.185	0.670	0.055	0.816	0.690	0.415	0.076	0.785	1.043	0.313	2.275	0.131	0.057	0.812
CO_2	0.329	0.057	0.180	0.893	0.780	0.781	1.127	0.266	0.044	0.835	0.519	0.476	0.002	0.961	0.872	0.351	0.893	0.345
Temp.×CO_2	0.329	0.057	2.291	0.137	0.300	0.864	1.193	0.281	3.090	0.860	1.259	0.268	2.873	0.096				

*Significantly different. Two-way ANOVA was used for the analysis of response latency (L_t), two-way ANCOVA for turning rate (T_r), stage 1 (s1) and stage 2 (s2) durations, cumulative distance (D), maximum velocity (V_{max}) and maximum acceleration (A_{max}), and binary logistic regression for directionality (D_t) and response type (R_t)

4. **Discussion**

This is the first study that has examined separate and combined effects of elevated temperature and CO_2 on the escape response of fish. The results demonstrated that neither elevated temperature nor CO_2 concentration or their combination affected any of the measured variables of solitary anchovies except turning rate, which was significantly higher at 19°C than at 15°C irrespectively of CO_2 levels.

The maximum velocity (1.4 to 1.6 m s^{-1}) found in this study is in good agreement with the relationship between body length and V_{max} obtained from the data from five species of fish (1.3 m s^{-1} for body length 0.1 m) (Dominici, 2001). The observed ranges of A_{max} (63 to 117 m s^{-2}) and turning rate (1.7 to 3.2 degrees ms^{-1}) are both well within the range reported for other fishes. Both the sum of stage 1 and stage 2 durations (44 to 48 ms) and response latency (5 to 7 ms) are relatively short but are still within the range reported for other fishes from other studies (Dominici and Blake, 1997; Dominici, 2010b). The relatively low proportion of away response (40 to 60%) may have resulted from the initial orientation angles being larger than 90°. It has been shown that the proportion of away response can be as high as 90% when a fish is stimulated sideways but it decreases when the initial orientation angles deviate from 90° (Dominici, 2010b). My video recording showed that 50 to 75% of fish was stimulated sideways.

Response latency is a behavioral measure directly correlated to the activity in the brainstem escape network that includes the two Mauthner cells (Eaton et al., 2001) and thus the measurement of response latency yields important information with respect to the temperature dependence of the system. It has been reported that response latency decreases with temperature (Preuss and Faber, 2003; Webb, 1978). The response latency in my study tended to be shorter at higher temperature (Table II-3), but the difference was not statistically significant. This may have been due to the relatively small temperature difference used (4°C) as compared with the much wider habitat temperature range of the Japanese anchovy (15 to 29ºC) (Hayasi, 1967).

The escape responses observed for the Japanese anchovy are somewhat different from those reported by Allan et al. (2014), who studied the effects of ocean acidification on escape response in juvenile coral reef fish, *Amphiprion melanopus*. These authors tested escape responses in control (400 µatm) and high (1087 µatm) CO_2 conditions, using juveniles produced by the parents that had been kept in the respective conditions for 11 days. The comparison between control and CO_2-treated juveniles from control

parents detected significant reductions in: (1) response distance (the total distance covered by the fish during the entire escape response until the fish stopped movement); (2) mean swimming speed; (3) V_{max}; (4) the proportion of fish that showed away response in directionality; and (5) response duration (the duration of entire escape response). In addition, a significant increase was detected for the proportion of non-reactive fish, while no difference was found in response latency. CO_2-treated juveniles from the parents acclimatized to high CO_2 conditions largely showed intermediate responses. Data from this study showed no change in V_{max} or the proportion of non-reacting fish (with only one fish in high temperature group failing to respond). It is not clear whether these differences in escape response between the species are due to the differences in life stages of test fish (i.e. juveniles vs. adults), test temperature (15 and 19°C vs. 28.5°C) or acclimation period (11 days vs. 1 month) or the neuromechanical nature of the kinematic behavior inherent to each species.

The turning rate was the only parameter which showed a significant difference between treatment groups in this study (Fig. II-3a). Turning rate determines how fast a fish turns its body into C shape following a stimulus, and therefore a higher turning rate would potentially lead to a higher rate of escape from predators. Walker et al. (2005) demonstrated that the following abilities of the prey affected evasion outcome; the ability of the prey to generate rapid tangential acceleration (V_{max} and A_{max}), and the ability of the prey to rapidly rotate during the initial stage of the fast start (i.e., a higher turning rate), together with the evasion path of the prey relative to the strike path. An elevation of 4 °C increased turning rate from 0.65 degrees ms^{-1} (400 μatm CO_2) to 1.44 degrees ms^{-1} (1000 μatm CO_2). Temperature is also known to increase reaction distance, latency, responsiveness, distance, speed and acceleration in fish (Dominici, 2010b). These effects may be substantial when a test fish is subjected to acute temperature change, but will usually subside after temperature acclimation (Batty et al., 1993). Considering the range of temperature rise projected (4 to 5°C), which will gradually occur over the next decades, temperature will probably exert a minor, if any, direct influence on fish escape behavior. It is likely that increasing daily temperature fluctuations during extreme weather events would have much stronger effects on the behavior.

Although the data of my study demonstrated the relative robustness of escape response by the Japanese anchovy under simulated future conditions, this does not imply that the fish will be subjected to a lower risk when attacked by a predator in the high CO_2, warmer oceans. For instance, there is now ample

evidence that the predicted CO_2 level in 2100 will affect the sensory perception of external stimuli in fish, which is a crucial step in initiating an escape response (see Introduction). Thus, there is a possibility that prey fish would incur a higher risk of predation through impaired perception of approaching predators. On the other hand, how future oceanic environment may affect attack behavior of predators needs to be examined for the mechanistic understanding of prey-predator interaction in changing marine conditions. Another important consideration is each species' tendency to form schools or aggregations. The Japanese anchovy, like many other small pelagic fishes, forms a large school in nature (Whitehead et al., 1988). Although schooling individuals can become solitary when they are sequentially attacked by predators (Major, 1978), Domenici and Batty (1997) reported that escape responses shown by fish in school may differ quantitatively in kinematics from those of solitary individuals, and speculated that a solitary herring (a schooling species) may employ more toward responses, as a result of an alternative strategy aiming at increasing the unpredictability of the response. Namely, schooling fish can take advantage of the confusion effect while solitary fish cannot. However, these authors also pointed out that individual herrings in a school can be in effect solitary for a brief period. Therefore, to understand how these small pelagic fish will be affected by the future oceanic environmental changes and how the effect will propagate to higher trophic levels, it is crucial to analyze escape responses of the fish in both school and solitary conditions, and to investigate how prey-predator interaction of solitary and schooling fish will be influenced under simulated future oceanic conditions.

Recently, Cornwall and Hurd (2015) reported that 94% of papers published since 1993 in the field of ocean acidification research had employed experimental designs that fall short of the rigorous statistical requirements; treatments within manipulation experiments must all contain adequate numbers of randomly interspersed and independent treatment replicates (Cornwall and Hurd, 2015). To satisfy the level of independence of each experimental unit these authors recommend, each anchovy must have been acclimated to an experimental condition in a separate tank, or multiple anchovies must have been acclimated to multiple tanks with 3 or more header tanks per treatment. Though ideal, these designs would have been logistically highly demanding, and practically quite difficult to achieve because the Japanese anchovy is the species that would not easily acclimate to laboratory conditions and need extreme care to handle, which was evident from the unfortunate high mortality we experienced in this study. Repeated investigations from independent

research institutions on different species of commercially important, small pelagic fishes would improve our prediction capacity for the trajectory of these fishes and fishery resources in the future oceanic environment, as long as they meet statistical requirements to the maximum feasible extent to prevent pseudoreplications in the experimental design. With these limitations in mind, I still hope that the present results would form a basis for further research to predict how commercially important, small pelagic fish will be affected by warmer and more acidic future oceans.

Chapter III

EFFECTS OF ELEVATED CO_2 ON MUSCULAR SYSTEM AND PROTEOME COMPOSITION OF SEA URCHINS

III-A

Effects of elevated CO_2 on masticatory muscles of sea urchin *Pseudocentrotus depressus*

1. Introduction

In the past 20 years, it has been reported that ocean acidification affected physiological process in marine animals, particularly the calcify marine animals. Kroeker et al. (2013) reviewed that CO_2 decreased survival, calcification, growth, development and abundance in marine animals. Low ability in acid-base regulation animals should be a high vulnerable species to ocean acidification. Sea urchins lack of the system using for regulating acid - base in the coelomic fluid, for example, absence of respiratory pigment (or any substantial protein in the coelomic fluid) and dependency on a magnesium calcite test (Stumpp et al., 2012, Miles et al., 2007). Previous studies indicated that CO_2 caused change in the concentration of Mg^{2+} and Ca^{2+} in the coelomic fluid of sea urchin (Miles et al., 2007, Spicer et al., 2011; Kurihara et al., 2013; Wang et al., 2013). It is possible that sea urchins might be a high vulnerable species to CO_2. It has been reported that CO_2 decreased feed intake of *Strongylocentrotus. droebachiensis, S. droebachiensis* and *Anthocidaris crassispina* (Siikavuopio et al. 2007; Stumpp et al., 2012; Wang et al., 2013), affected larvae development stage of *Hemicentrotus pulcherrimus* (Kurihara et al., 2004), delayed gonad maturation of *H. pulcherrimus* (Kurihara et. al., 2013), reduced tube feet contraction force of *Pseudocentrotus depressus* (Nasuchon et al., 2017).

The entire chewing organ of sea urchins is known as Aristotle's lantern. The mouth of sea urchins is made up of 5 calcium carbonate teeth. There are two major muscles involved in mastication system, the

protractor muscle, whose role is to push the teeth down. and retractor muscle which pulls the teeth upwards. Fitness of the masticatory muscles would affect sea urchins feeding and then result in growth. Wood et al. (2008) reported that elevated CO_2 reduced muscle mass in a starfish (Wood et al., 2008) and Nasuchon et al. (2017) also found that elevated CO_2 reduced contraction force of sea urchin tube feet. A review paper of I hypothesized that elevated CO_2 could damage mastication muscles of sea urchin.

Sea urchins is a commercially important species. Japan is being the world's largest market. Japan consumes about 80% of the world total catch, mostly produced for sushi. It has been reported that the total catch of sea urchins in Japan has been declining since 1983. The declining catch in Japan has forced Japan to import sea urchins from different countries around the world, including USA, Canada and China. The increasing market demand has led to the overfishing of sea urchins in 1992 (FAO, 2015). It is possible that over fishing associated with ocean acidification reduce sea urchin population. The pink sea urchins *P. depressus* were selected for this study because of the location of its habitat and it being the most important fishery species in the south of Japan (Agatsuma, 2013). The aim of this study was to observe feed intake, the histology of retractor muscles and contraction force of the protractor muscles under three different CO_2 concentrations based on present concentration (400 μatm), OA prediction by 2300 (2000 μatm) and CCS leakage (10000 μatm).

2. Materials and methods

2.1 Animal collection and maintenance

This study was performed over two years (July 2014 and June 2015). The wild sea urchins *P. depressus* were collected by fisherman in Saga prefecture at latitude 33°32' 55.82" N and longitude 129°50'56.74" E. Their test diameter and body wet weight were 54.26 ± 2.26 mm and 56.12 ± 6.24 g for 2014 and 50.50 ± 2.36 mm and 51.32 ± 6.71 mm (Mean ± SD) for 2015 respectively. The sea urchins were transferred to the Institute for East China Sea Research of Nagasaki University, Japan and stocked in a 100 L tank supplied with a continuous flow of filtered seawater at 2 L min^{-1} at ambient pH (8.17 pH unit) and

temperature 22 – 24°C. Sea urchins were fed with around 10 g artificial food pellets described by Hiratsuka and Uehara (2007) every second day. The sea urchins were maintained for 10 days before acclimation to the experimental conditions following Stumpp et al. (2012).

2.1 Environmental treatments

The experiment was performed in 3 different CO_2 concentrations, 400 µatm (pH 8.2), 2000 µatm (pH 7.6) and 10000 µatm (pH 7.0). The sea urchins were reared individually using 14 containers (14 x 22 x 14 cm) of each treatment. The containers were placed in a tray (120 x 75 x 20 cm) in water of 12 cm depth (Fig III-A-1). In the control conditions, sea water was bubbled continuously with an outside air at flow rate 10 L min^{-1}, while CO_2 concentrations at 2000 and 10000 µatm were prepared with a gas blender (Kofloc, GB-2C, Japan) by mixing dried air and pure CO_2 at flow rate 10 L min^{-1}. The sea water temperature of the rearing containers was kept stable at the level of the first acclimation day (25°C). Storage seawater was first filtered with a net (mesh size 150 µm) and then, secondly, with string wound filter cartridges before being supplied to the header tanks. The filtered seawater was gravity-fed from the header tank to each of the 14 rearing containers at a flow rate of 50 mL min^{-1}. The rearing containers were bubbled gently with air, for the control treatment, and with mixed gas, for elevated CO_2 treatments. Seawater pH (NBS) and dissolved oxygen were checked daily with a digital multi-parameter (WTW multi 3420, Germany), and calibrated with a standard buffer solutions (pH 4.01, 6.86 and 9.18). Salinity and temperature were accurately monitored throughout the experiment using a salinity refractometer (S/Mill-E, Japan). Alkalinity was measured weekly with a total alkalinity titrator (Kimoto, ATT-05, Japan). Partial pressure of CO_2 (PCO_2) was calculated from the data of seawater pH, temperature, salinity and alkalinity, using the CO2SYS program (Lewis and Wallace, 2006) (see Table III-A-1). The sea urchins were acclimated for 48 days before testing (13 July to 27 August 2014 and 27 June to 10 August 2015). Feed intake and retractor muscle areas were measured in 2014, while protractor muscles contraction force was measured in 2015.

2.3 Measured parameters

2.3.1 Feed intake

Feed intake was measured from 14 July to 12 August 2014. Artificial food pellets (about 10 g) were prepared every week based on the descriptions of Hiratsuka and Uehara (2007). The pellets were refrigerated until used. Preliminary study demonstrated that the percentage weight loss of the pellets after 24 h of immersion in running water (50 mL min^{-1}) was 4.42 ± 1.99 % (n = 10), and this was taken into account in the calculation of the feed intake. The feed intake was measured every second day from fourteen sea urchins of each treatment throughout the experiment**.** Each sea urchin received one pre-weighed pellet in the afternoon. After 24 h, the residual pellet was removed and put on a tissue tower to remove any excess water and then weighed. The feed intake was calculated as dry weight per individual by converting from wet to dry weight, which was determined by drying pre-weighed 10 moist food pellets at 50°C for 48 h to a constant weight.

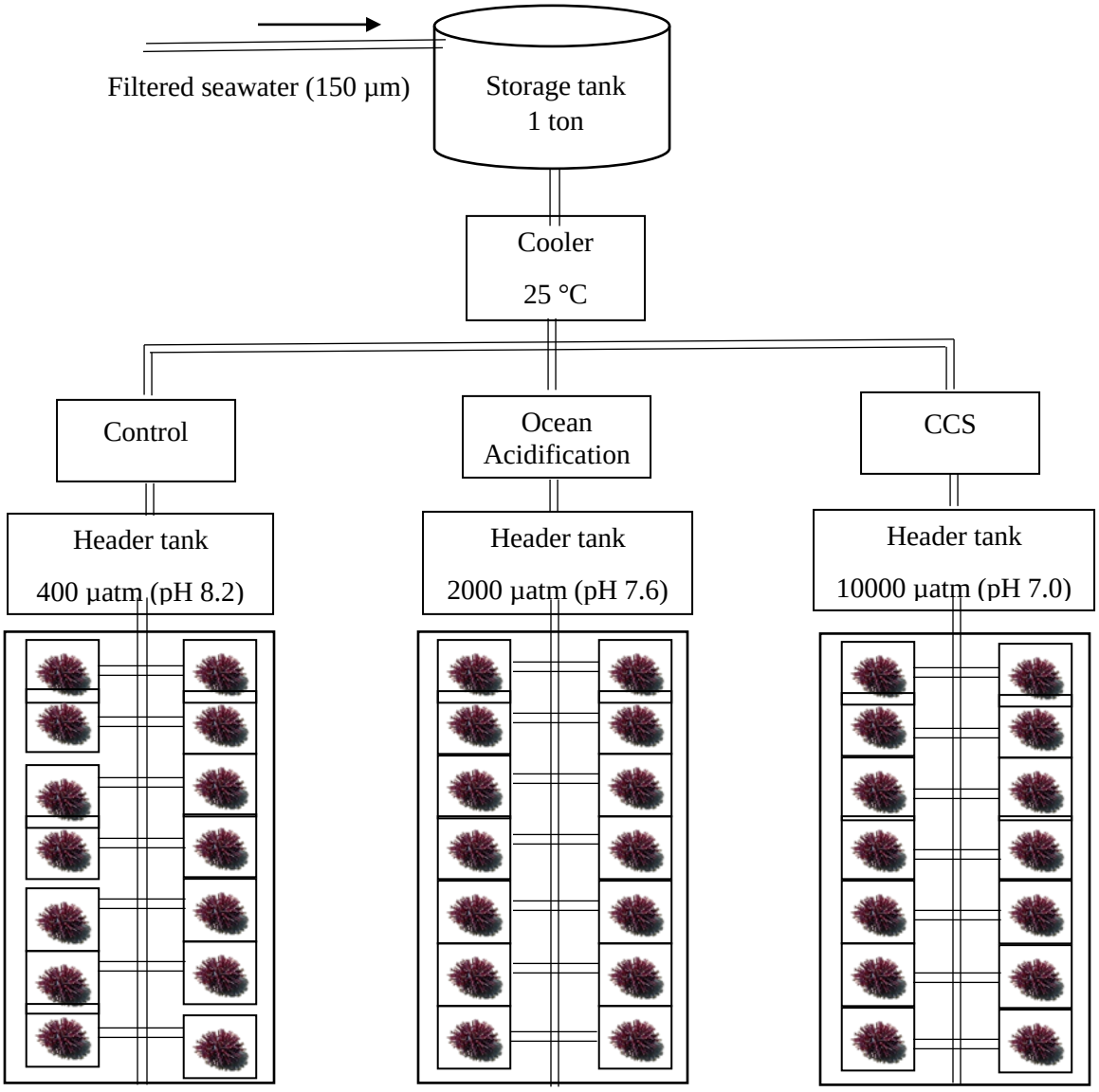


Fig III-A-1 Experimental set-up of sea urchin study

2.3.2 Histology and measurement of cross-sectional area

Three sea urchins of each treatment were used for histology observation. Once isolated the retractor muscles of each sea urchin were immediately frozen in chilled isopentane and liquid nitrogen and stored at -80°C until analysis. Sections of retractor muscles (5 μm) were fixed in ice-cold acetone. The sections were stained with hematoxylin and eosin (H. E.). Images were acquired with a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) using a camera and processed using BZ-II analysis software. The sections of retractor muscle were photographed at ×20 magnification using a BIOREVO BZ-9000 microscope (Keyence). The cross-sectional areas (CSA) of myofibers (n = 40 (400 μatm); n = 46 (2000 μatm); n = 79 (10000 μatm) were measured. Data are expressed as fiber size distribution.

2.3.3 Protractor muscle contraction force measurement

The experiment with the protractor muscle was performed in August 2015 using the method modified from Wilkie et al. (1998). Five or six sea urchins, of each treatment, were used for the measurement of contraction force. The tests were cut off with about 5-10 mm left surrounding each lantern. The full set of ten protractor muscles were measured. The retractor muscles were totally removed from the lantern and the peristomial membranes were incised to separate the lantern from the test. The test was clamped horizontally with two acrylic plates in the air. A tungsten needle (0.5 mm diameter, 50 mm length) was inserted down the central axis of the lantern and its upper end was connected to the probe of isometric transducer (SB-1T, Nikon Kohden, Tokyo, Japan). A small acrylic stopper connected to the pointed end of the needle, was attached underneath the teeth to prevent the teeth from slipping out from the lantern. The lantern was then pulled upward into the resting position using a micromanipulator. The resting position was defined as the level in which the lantern corresponded to the normal spatial relationship with the test in the living animal. The lantern was continuously dripped with the seawater at the same PCO_2 level as during exposure by using a gravity-feeding apparatus. To induce maximal contraction, the perfusate was switched from seawater to seawater containing 10^{-4} M acetylcholine (ACh) (Florey and Cahill, 1980), and the

response was recorded until contraction force began to decrease. The maximal value was used as a contraction force in each measurement. The muscle contraction was recorded once per individual.

2.4 Statistical analysis

One sample t-test was applied to determine difference between measured and nominal PCO_2 values in each treatment. One-way ANOVA was applied to determine difference between group means and Tukey's HSD run to confirm group differences using PASW statistics 18. Hierarchical Bayesian Inference was applied to compare the percentage probability of posterior distribution between the control and experimental conditions for muscle contraction force. Bayesain modelling was computed with Stan code by using R software (Rstan package).

Table III-A-1 Seawater pH, percentage dissolved oxygen (DO), Temperature (Temp.), Salinity, Total alkalinity (TA), partial pressure carbon dioxide (PCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^-) (mean ± SD) of each treatment during acclimation in 2014 and 2015

Treatment (CO_2)	400 (µatm))		2000(µatm)		10000(µatm)	
Parameters (	2014 Mean ± SD)	2015 Mean ± SD)	2014 Mean ± SD)	2015 Mean ± SD)	2014 Mean ± SD)	2015 Mean ± SD)
$pH_{(NBS)}$	8.16 ± 0.02	8.13 ± 0.02	7.56 ± 0.03	7.62 ± 0.03	6.90 ± 0.03	6.98 ± 0.03
DO (%)	96.00 ± 1.55	98.47 ± 1.51	96.40 ± 1.68	98.95 ± 0.90	95.90 ± 1.96	98.51 ± 0.92
Temp. (°C)	24.58 ± 0.55	23.30 ± 0.54	24.59 ± 0.55	23.30 ± 0.55	24.58 ± 0.56	23.25 ± 0.56
Salinity (PSU)	34.63 ± 0.58	34.25 ± 0.43	34.63 ± 0.58	34.25 ± 0.43	34.63 ± 0.58	34.25 ± 0.43
TA (µmol/Kg-SW)	2121.3 ± 8.4	2122.2 ± 9.4	2128.1 ± 6.7	2126.1 ± 7.25	2130.2 ± 15.5	2132.46 ± 16.3
*PCO_2 (µmol/Kg-SW)	399.70 ± 21.60	414.41 ± 19.55	1911.70 ± 116.20	1604.82 ± 68.43	9307.00 ± 524.00	7764.43 ± 283.11
*HCO_3 (µmol/Kg-SW)	1677.70 ± 10.90	1692.22 ± 12.63	2009.00 ± 13.40	1973.69 ± 5.23	2128.60 ± 12.40	2087.44 ± 1.87
*CO_3 (µmol/Kg-SW)	196.80 ± 1.20	169.26 ± 4.16	61.50 ± 3.50	59.38 ± 1.68	14.20 ± 0.70	13.74 ± 0.36

*calculated from seawater pH, temperature, salinity, total alkalinity by using CO2SYS program

3. Results

3.1 General observation

On the second day, sea urchins had excreted over than normal, which found in CO_2 treatments and excrement amount related to CO_2 concentration. The spines of sea urchin in10000 µatm were very fragile and broke after exposure for 5 days. The sea urchins' spines started dropping after exposure over 12 days and continued dropping through the experiment. This was no appear in 400 µatm and 2000 µatm. Sea urchin appearance was observed and I found that sea urchins in PCO_2 10000 µatm were very weak. They had less power to attach the wall and tube foot stalks were turned white after exposure for 48 days (Fig. III-A-2). One sea urchin in PCO_2 10000 µatm even had no disc at the tip of tube foot (Fig. III-A-2).

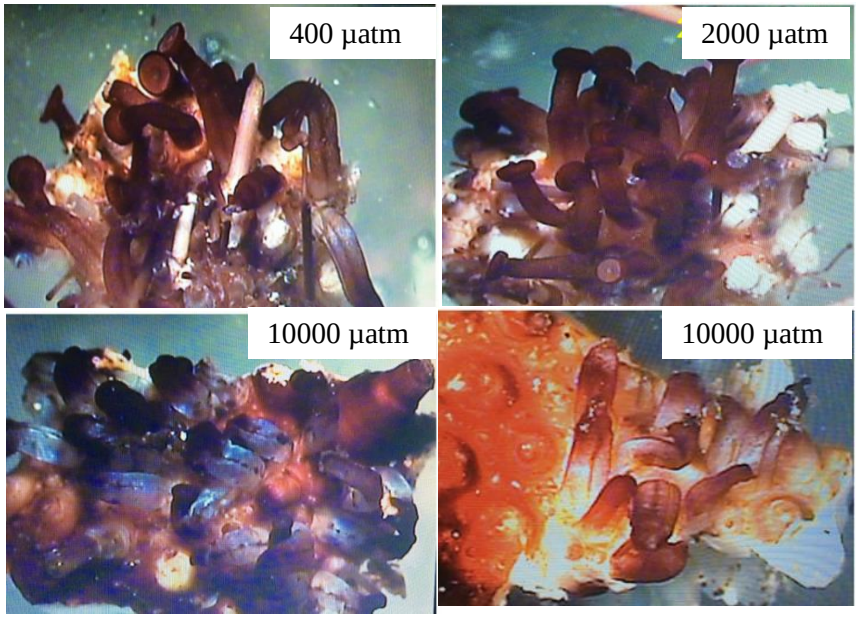


Fig. III-A-2. Sea urchin tube feet image after exposure in PCO_2 at 400 µatm, 2000 µatm and 10000 µatm

3.2 Feed intake

The feed intake of sea urchins at CO_2 concentrations of 400 µatm and 2000 µatm was found to be relatively stable through 48 days of acclimation, which had means of 0.37 ± 0.05 and 0.36 ± 0.04 g dry ind^{-1} day^{-1} (mean ± SD) respectively. For sea urchins at 10000 µatm CO_2 concentration, feed intake was very low in the beginning (0.025 ± 0.0003 g dry ind^{-1} day^{-1}, mean ± SD), but started increasing after 26 days of acclimation (0.075 ± 0.021 g dry ind^{-1} day^{-1}, mean ± SD) and then was stable until the end of experiment (Fig. III-A-3). One way ANOVA has significant difference in feed intake between treatments ($F_{2,60}$ = 457.37, P = 0.000). Tukey's test showed that feed intake was significantly lower in 10000 µatm (P = 0.000), but it was no significant different between 400 µatm and 2000 µatm (P = 0.405).

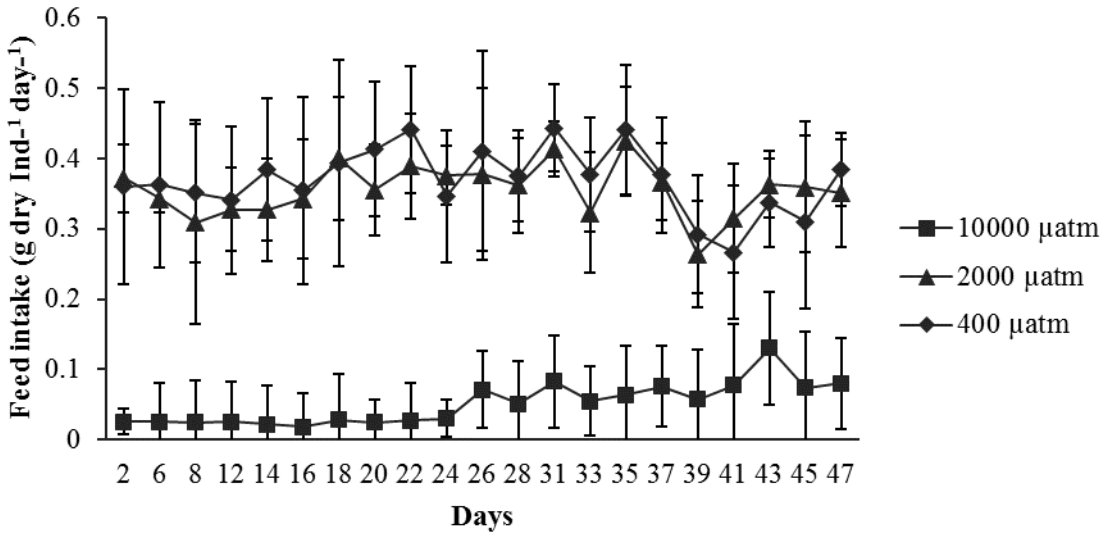


Fig III-A-3. Feed intake of *P. depressus* reared for 48 days at 400 µatm, 2000 µatm and 10000 µatm CO_2. Filled rhombus represent 400 µatm, filled triangle 2000 µatm and filled square 10000 µatm.

3.3 Histology of retractor muscles

The histology observations revealed that retractor muscle fibers were damaged in high CO_2 groups as seen by the atrophy of muscle fibers and proliferation of perimysium (Fig. III-A-4). The cross-sectional area of retractor muscle fibers of the masticatory apparatus was 334.18 ± 16.35, 264.42 ± 15.55 and 31.03 ± 1.53 μm^2 (mean ± SE) in 400 µatm, 2000 µatm and 10000 µatm respectively (Fig. III-A-5). One way

ANOVA had significant muscle area different between treatments ($F_{2,164}$ = 251.632, P < 0.000) and Tukey showed muscle area decrease in high CO_2 concentration (Tukey: P < 0.000).

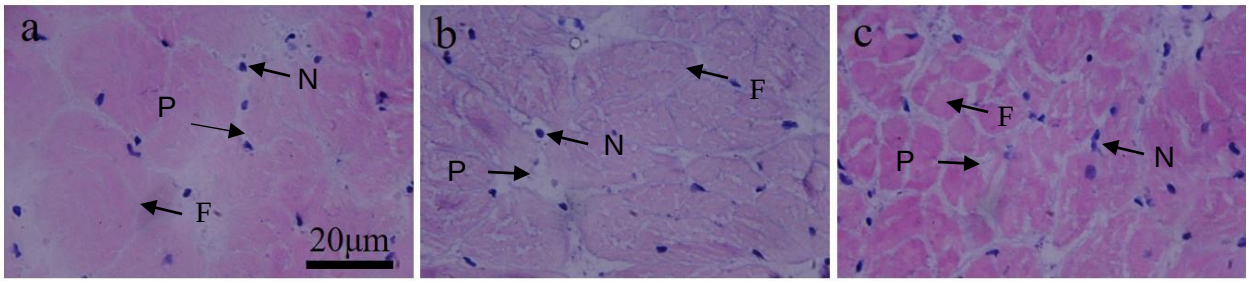


Fig III-A-4. Histology of retractor muscles of the Alistotle's lantern of *P. depressors* after exposure to elevated CO_2 for 48 days at 400 µatm (a), 2000 µatm (b) and 10000 µatm (c) using H.E. staining, N = Nucleus, P = Perimysium and F = Muscle fiber.

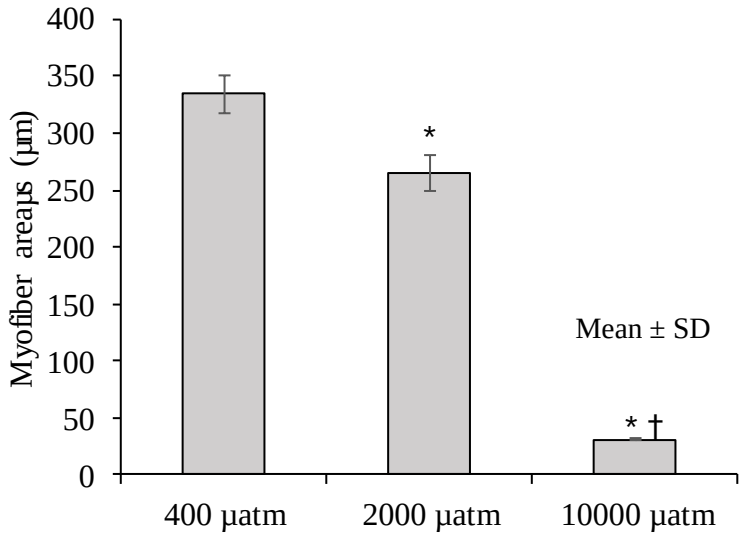


Fig III-A-5 Mean cross-sectional areas of retractor myofiber of *P. depressors* after exposure to three different CO_2 levels (400 µatm, 2000 µatm and 10000µatm) for 48 days. * significant different from 400 µatm, † significant different from 2000 µatm

3.4 Contraction force of protractor muscles

Ten protector muscles of five or six sea urchins of each treatment were used for measuring contraction force. The result showed that the mean contraction force of 400 μatm, 2000 μatm and 10000 μatm was 1.51 ± 0.19 N, 1.35 ± 0.09 N and 1.12 ± 0.16 N (mean ± SE) respectively (Fig. III-A-6). One way ANOVA was no significant difference in contraction force between treatments.

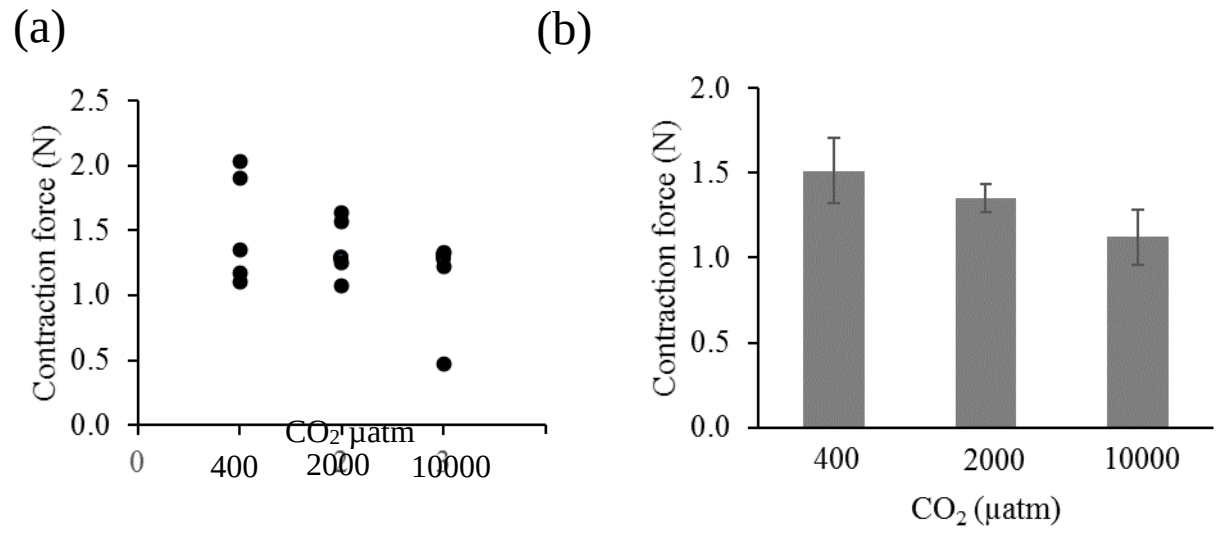


Fig III-A-6 Protractor muscles contraction force (N) of *P. depressors* at CO_2 level 400 μatm, 2000 μatm and 10000 μatm. a) individual contraction force, b) an average contraction force of each treatment (mean ± SE).

4. Discussion

4.1 Feed intake

The result of this study showed that feed intake of sea urchins at CO_2 concentration of 10000 μatm was significantly lower than 400 μatm and 2000 μatm. Previous studies reported that feed intake of *Strongylocentrotus droebachiensis* and *Anthocidaris crassispina* deceased after acclimation at 284 Pa (2800

μatm) over three weeks (Stumpp et al., 2012) or 3000 μatm over ninety days (Wang et al., 2013) respectively. In addition, Siikavuopio et al. (2007) found that feed consumption in adult *S. droebachiensis* reduced after being incubated in CO_2 concentration at 8000 μatm (pH 6.98) for about 2 months. Although Kurihara et al. (2013) found that the feeding rate was significantly suppressed at a CO_2 concentration lower than this experiment (1000 μatm), they stated that they were not confident with their result because this experiment only ran over a very short period of 16 days. However, the feed intake of 10000 μatm increased after exposure for 24 days. Moulin et al. (2015) suggested that sea urchins should be able to regulate their extracellular pH during long term acclimation. They did not find that CO_2 affected growth of *Echinometra mathae* when acclimated in seawater pH 7.7 for seven months. The results of previous studies differed with this study and this may be attributed to differences in acclimation period, CO_2 concentration, species and/or habitat. It is suggested that the effect of elevated CO_2 on sea urchins should be studied in long term experiment at least over than 1 year.

4.2 Muscle function and structure

Cross sections of retractor muscle showed that the retractor muscles were damaged in the high CO_2 groups. This was observed as muscle fiber atrophy and infiltration of mononuclear as well as a significant decrease in the muscle fiber areas in the high CO_2 groups. Similarly, previous studies found that the muscle mass of starfish was decreased when the star fish was acclimated in seawater pH at 7.7, 7.3 and 6.8 for 40 days (Wood et al., 2008). Marine invertebrates have been extensively studied as bioindicators of environment stress (Jha, 2004). It has been demonstrated that oxygen consumption and metabolism depressed in adult sea urchins after exposure in acidified sea water (Siikavuopio et al., 2007; Stumpp et al., 2012; Wang et al., 2013; Padilla-Gamiño et al., 2013). Studies at the molecular level also confirmed that CO_2-driven seawater acidification caused down-regulation in metabolic genes of sea urchin larvae (Todgham and Hofmann, 2009). When metabolism is suppressed, the cells will conserve energy for survival and processes that require the high energy tend to shut down such as protein synthesis (Surks and Berkowite, 1971). On the other hand, it is possible that protein contributes energy during periods of long term starvation

(Berg et al., 2002). Sant et al. (2009) found that the diameter of muscle fibers decreased and the space between muscle fibers increased in *Hoplias malabaricus* fish after long period without food. Similarly, Medina et al. (1995) found skeletal muscle mass in rats decreased about 23% for soleus and 32% for extensor digitorum longus muscles after starvation for 2 days. However, I have no information about oxygen consumption, metabolism and gene expression to support this finding.

The result of protraction muscle contraction force showed that there was no significant difference in the results between different treatments. The result of this study differed with the study of Nasuchon et al. (2017) on tube feet contraction force in the same sea urchin species and CO_2 concentration. This may be because the two studies measured different muscles. Another reason is that the contraction of tube feet may affected from nervous system. Although, ANOVA analysis showed that there was no significant difference comparing protractor contraction force between treatments, Hierarchical Bayesian Inference indicated that contraction force of control was higher than at 2000 μatm and 10000 μatm with probability of 58.6 % and 68.3 %, while contraction force of 2000 μatm was higher than 10000 μatm with probability 61.2 % (Fig. III-A-7). The result of my study was not clear, this may because of low number of measured sea urchins (N = 5 – 6 individuals).

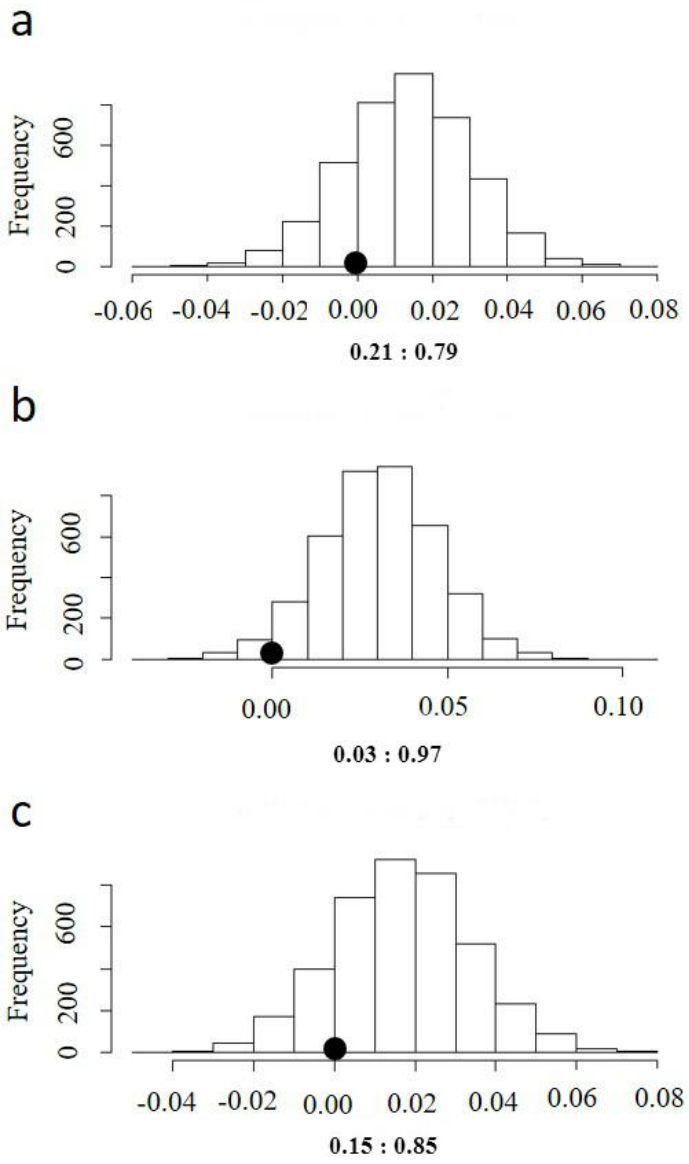


Fig III-A-7. The probability of tube feet contraction force of sea urchin *P. depressors* using Heirarchical Bayesian model. The solid circle on the x-axis of the histogram represents the proportion of contraction force between two treatments that were higher than another; (A) comparison between CO_2 concentration 400 µatm (control) and 2000 µatm, (B) comparison between CO_2 concentration 400 µatm (control) and 10000 µatm and (C) comparison between CO_2 concentration 2000 µatm and 10000 µatm.

III-B

Effects of elevated CO_2 on contraction force and proteome composition of sea urchin tube feet

(Nasuchon et al., 2017)

1. Introduction

There are two processes by which seawater is acidified by the addition of carbon dioxide (CO_2), ocean acidification and accidental leakage from a carbon capture and storage (CCS) site. Ocean acidification occurs by absorption of CO_2 across sea surface from the atmosphere, and now widely recognized as a serious threat to the structure and function of marine ecosystems in the coming decades. Reductions in surface ocean pH by ocean acidification have been confirmed at a number of observation sites in the world oceans (Orr, 2011). Accidental leakage of stored CO_2 through the seabed, which might impact benthic ecosystems near a leakage site, has been one of the major concerns with CCS technology (Taylor et al., 2014). The spatial extent of an acidification event will depend on the position of the CCS infrastructure and the nature of the leak. At the present time, we have little to no experience with CCS leakage, and therefore it is difficult to predict how and to what extent marine faunas and floras near the leakage site would be affected. If CO_2 leakage occurs, CO_2 concentrations in the surrounding seawater can reach much higher levels than those predicted from ocean acidification. Therefore, the CO_2 levels used in the recent risk assessment studies of leakage from CCS sites are as high as 18,325 µatm (Murray et al., 2013), 20,000 µatm (Restelli et al., 2015), 29,000 µatm (Ishida et al., 2013), or even up to 313,862 µatm (De Orte et al., 2014), one or two orders of magnitude higher than in ocean acidification studies (typically up to 1000 or 2000 µatm).

Effects of CO_2 on marine organisms have become a focus of marine biological research during the past 20 years, mainly because of the increasing concern with the impacts of ocean acidification (Gattuso and Hansson, 2011) and potential CO_2 leakage from a CCS site (Noble et al., 2012) on marine ecosystem structures and ecological services. It is now generally accepted that calcification (i.e., the formation of

$CaCO_3$ structures from Ca^{2+} absorbed from seawater and HCO_3^- generated by metabolism and originated from seawater carbon pool, Furla et al., 2000) is one of the most sensitive biological processes negatively affected by seawater acidification (Kroeker et al., 2013). For example, Li et al. (2016) recently demonstrated that the tubes of serpulid worm *Hydroides elegans* had lower hardness and a smaller radius when subjected to the projected acidic conditions for 2100 (pH 7.8), causing the worm to be mechanically weaker, although different organisms may show different sensitivities and response patterns (Ries et al., 2009). In addition, an increasing amount of data have become available on CO_2 effects on early development, growth, metabolism, photosynthesis, and survival of marine organisms (Kroeker et al., 2013). By comparison, very little is known on how CO_2 affects the integrity and functionality of muscular systems among marine animals. Wood et al. (2008) reported that the muscle mass in the arms of the brittlestar, *Amphiura filiformis*, decreased in lowered pH (7.7, 7.3 and 6.8) but without structural changes in muscles, while in another species *Ophiura ophiura* muscle density remained unaffected in lowered pH (7.7 and 7.3; PCO_2 1300–1400 and 2300–2500 μatm, Wood et al. 2010). Schalkhausser et al. (2013) found significant declines in the force generated by the adductor muscle in the king scallop, *Pecten maximanus*, collected in Norway and reared under PCO_2 of 1120 μatm at 10°C. However, the effect was not detected in the same species from France under the same experimental conditions (Schalkhausser et al., 2014).

The purpose of this study was to examine the effect of CO_2 on contraction force and protein composition of the tube feet of the sea urchin, *Pseudocentrotus depressus.* Tube feet are unique hydraulic mechano-sensory adhesive organs found in echinoderms. Tube feet have a variety of functions including light sensitivity, respiration, chemoreception and locomotion (Lesser et al., 2011). They consist of a basal extensible cylinder, the stem, which bears an apical flattened disc that makes contact with and adheres to the substratum. The stem wall of a tube foot consists of an outer epidermis, a basiepidermal nerve plexus, a connective tissue layer (mutable collagenous tissue), a myomesothelium (retractor muscle) and an inner epithelium that surrounds the water-vascular lumen (Santos, 2005). Recent proteomic characterization of tube feet from the sea urchin *Paracentrotus lividus* identified 328 non-redundant proteins, including 44 phospho-proteins and 18 glycoproteins, and revealed that the organs are composed of sensory-perception-

related proteins, nerve-related proteins, muscle-related proteins, development/regeneration-related proteins, immunological-response-related proteins, and temporary-adhesion-related proteins (Santos et al., 2013). While in-depth proteomics has been reported in sea urchin tube feet, comparative analysis of the tube feet proteomes in response to an environmental change, such as ocean acidification, remains to be elucidated. I examined the contraction force and proteome composition of tube feet under three CO_2 concentrations based on the present-day level (400 µatm), the prediction by IPCC in the year 2300 (2000 µatm) and extreme conditions (10000 µatm) predicted as the model of CCS leakage. Adult sea urchins (*P. depressus*) were acclimated to these CO_2 concentrations for 48 days. The contraction force was measured using isolated tube foot preparations. Proteomic analysis was applied to elucidate the response of muscle contraction to elevated CO_2 at the molecular level.

2. Materials and methods

2.1 Animal collection and maintenance

Wild adult sea urchins were collected by a local fisherman in Saga Prefecture, Japan (33°32'55.82" N; 129°50'56.74" E). Their shell diameter and wet body weight were 54.26 ± 2.26 mm and 56.12 ± 6.24 g (Mean ± SD, n = 42) respectively. The sea urchins were transferred to the Institute for East China Sea Research of Nagasaki University, Japan and stocked for 10 days in a 100 L tank supplied with a continuous flow of filtered seawater at 2 l/min at ambient pH (8.17) and temperature (23.3 – 24.0°C). The sea urchins were fed with artificial food pellets prepared according to the recipe by Hiratsuka and Uehara (2007) every second day.

2.2 CO_2 exposure

The sea urchins were acclimated for 48 days (from 13 July to 27 August 2014) in seawater equilibrated with ambient air (CO_2 400 µatm, seawater pH 8.2, control), or CO_2-enriched air at a concentration of 2000 µatm (pH 7.6, ocean acidification) or 10000 µatm (pH 7.0, CCS leakage). The sea

urchins were reared individually in seven replicate containers (14×22×14 (depth) cm) per treatment placed in a water bath (120×75×20 (depth) cm) with a water depth of 12 cm. Seawater was double filtered (150 μm and with string-wound cartridges) before being supplied to the header tanks. The filtered seawater was gravity-fed from the header tank to each of the 7 rearing containers at a flow rate of 50 mL/min. Seawater in the header tanks was bubbled continuously with an outside air at flow rate of 10 L/min for the control condition, or with CO_2-enriched air (2000 or 10000 ppm), which was prepared with a gas blender (Kofloc, GB-2C, Japan) by mixing dried air and pure CO_2 for the two higher CO_2 conditions. In addition, seawater in the rearing containers was gently bubbled with the same gases. The seawater temperature was kept stable at 25°C. The water baths were covered with plastic sheet to avoid CO_2 exchange with room air. Seawater pH (NBS) and dissolved oxygen concentration were monitored every second day with a digital multiparameter meter (WTW multi 3420, Germany) calibrated with a standard buffer solution of pH 4.01, 6.86 and 9.18. Salinity and temperature were monitored daily throughout the experiment using a salinity refractometer (S/Mill-E, Atago, Japan) and a solar digital thermometer (SN-1200, Netsuken, Japan). Alkalinity was measured weekly with a total alkalinity titrator (Kimoto, ATT-05, Japan). Partial pressure of CO_2 (PCO_2) was calculated from the data of seawater pH, temperature, salinity and alkalinity, using the CO2SYS program (E. Lewis, Brookhaven National laboratory).

2.3 Measurement of tube feet contraction

Seven sea urchins, one from each rearing container, were used per treatment for the measurement of the contraction force of isolated tube foot. A small piece (0.5 cm^2) of the lateral side of test was snipped off with the tube feet, submerged in seawater at room temperature with the same PCO_2 level as during acclimation, and continuously superfused with the seawater using a gravity-feeding apparatus. The cut end of the test and the tip of a tube foot were each pinched using a pair of micro bulldog clamps. The clamp at the tip of the tube foot was connected to the probe of an isometric transducer (SB-1T, Nihon Kohden, Tokyo, Japan). The tube foot was gradually extended to a length of about 20-30 mm from its base, using a micromanipulator, until spontaneous rhythmic contractions occurred as seen in intact animals. To induce

maximal contraction, the perfusate was switched from seawater to seawater containing 10^{-4} M acetylcholine (ACh) (Florey and Cahill, 1980), and the response was recorded until contraction force began to decrease. ACh is the natural excitatory transmitter substance in sea urchin tube feet (Florey et al., 1975). The maximal value was used as a contraction force in each measurement. The number of successful measurements in each animal varied from 1 to 3 (i.e., 1 to 3 tube feet because each tube foot was used only once), depending on the animal's physiological conditions. I used average values of each sea urchin for statistical comparison (Table III-B-S1).

2.4 Collection of tube feet for proteomic analysis

Four sea urchins were used per treatment for this purpose. Approximately 10 to 60 mg of tube feet were isolated on ice under a microscope from each sea urchin. To prevent protein digestion, the tube feet were soaked in 1 mL protease inhibitor solution (one tablet of cOmplete™ Mini/10 mL seawater, Roche, Indianapolis, IN, USA) during collection, and then placed into a 2-mL screw-cap centrifuge tube (Sarstedt, Nümbrecht, Germany). The samples were centrifuged at 12000×g for 1 min to remove the seawater. Subsequently, the samples were weighed and kept at –80°C until analysis.

2.5 Protein extraction

To accomplish extraction, separation and identification of proteins from a small amount of tube feet, I applied a small-scale proteomic approach (Yamaguchi, 2011; Khandakar et al., 2013) One milliliter of Trizol reagent (Life Technologies, Carlsbad, CA, USA), 100 µg of zirconia beads (0.6 mm in diameter, BMS, Tokyo, Japan), and a stainless-steel bead (5 mm in diameter, BMS, Tokyo, Japan) were added to the frozen tube feet in a 2 mL screw-cap centrifuge tube (Sarstedt, Nümbrecht, Germany). The tubes were mounted in a Master Rack aluminium block (BMS, Tokyo, Japan) and agitated for 2 min at 25ºC in a ShakeMaster Auto ver 1.5 (BMS, Tokyo, Japan). The homogenates were incubated at 25ºC for 5 min and 0.2 mL of chloroform was added to each tube. The tubes were then shaken vigorously by hand for 15 s and incubated at 25ºC for 3 min. The mixture was centrifuged (12000×g, 15 min, at 4ºC) to separate it into a

lower organic phase, an interphase, and an upper aqueous phase. After removing the aqueous phase, 300 µL of ethanol was added to the tube. The sample was mixed by inversion 3–5 times, incubated at 25ºC for 3 min, and then centrifuged (12000×g, 1 min, at 4ºC) to remove the DNA pellets.

The resulting supernatant was transferred to dialysis tubing with a 3500 Da molecular weight cut-off (Spectra/Por RC dialysis membrane 3, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), and dialyzed against 400 volumes of Milli-Q water (once renewed) for 72 h at 4ºC. The dialysate was dehydrated with solvent-absorbent powder (Spectra/Gel Absorbent, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), suspended in 300 µL of IEF solution A without carrier ampholite (8 M urea, 50 mM DTT, 2% w/v CHAPS, 0.001% w/v bromophenol blue), incubated at 25ºC for 16 h, and centrifuged (15000×g, 15 min, 25ºC). The supernatant was transferred to a new tube and the remaining pellet was re-suspended with 100 µL of IEF solution A, centrifuged (15000×g, 15 min, 25ºC) and the supernatant was combined with the first supernatant (total 400 µL). The combined supernatant (4 aliquots of 100 µL in a 0.5 mL Safe-Lock Protein LoBind Tube, Eppendorf, Hamburg, Germany) was stored at –70ºC. Protein concentration was determined using RC DC™ Protein Assay Kit (Bio-Rad, Hercules, CA, USA), with bovine serum albumin (BSA) as a standard.

2.6 Two-Dimensional gel electrophoresis (2-DE)

The first dimensional IEF separation was carried out using a 7-cm ReadyStrip® IPG Strips (Linear pH gradient, pH 3–10, Bio-Rad). The IPG strips were passively rehydrated for 12 h at room temperature in 125 µL of resuspension solution (8 M urea, 50 mM DTT, 2% w/v CHAPS, 0.2% carrier ampholytes, 0.0001% bromophenol blue), containing 30 µg protein/strip. IEF was carried out at 20°C, for a total of 20000 Vh (15 min with a 0–250 V linear gradient; 2 h with a 250–4000 V linear gradient; and finally 4000V held constant until 20000 Vh had been reached). After IEF, the IPG strips were incubated in 2.5 mL of 2% DDT-containing equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol) for 20 min and then incubated with 2.5% iodoacetamide-containing equilibration buffer for 20 min at room temperature. The second dimensional electrophoresis was performed with a Mini-PROTEAN Tetra

electrophoresis cell (Bio-Rad, Hercules, CA, USA). The equilibrated IPG strips were placed on the top of a Mini-PROTEAN TGX® precast gels, sealed with ReadyPrep® overlay agarose (Bio-Rad) and electrophoresed at 200 V in running buffer (25 mM Tris base, 192 mM glycine and 0.1% w/v SDS), for 30 min at room temperature. Gels were fixed with a fixing solution (40% ethanol, 10% acetic acid) for 2 h. Following this, the gels were stained with Flamingo® fluorescent stain (Bio-Rad) and the gel images for figure presentation were captured using a GELSCAN® laser scanner (iMeasure, Nagano, Japan). For quantitative analysis of 2-DE patterns and spot volumes, a total of 24 gel images, consisting of four biological replicates (i.e., proteins individually prepared from four specimens per treatment) and two technical replicates for each protein sample, were compared using the Prodigy SameSpots® software package (Non-linear Dynamics, Newcastle, UK). Reproducible gel spots were counted using the same software during spot alignment and pre-filtering stages.

2.7 Protein in-gel digestion and MALDI-QIT-TOF mass spectrometry

Protein spots were manually excised from the 2D gels using a spot image analyzer (FluoroPhoreStar 3000®, Anatech, Tokyo, Japan) equipped with a gel picker (1.8 mm in diameter). The methods I used for in-gel digestion and peptide extraction were slightly modified from the previously described methods of Shevchenko et al. (2006) and Khandakar et al. (2013). To reduce self-digestion of trypsin and avoid contamination, I used Trypsin Singles (proteomics grade, Sigma-Aldrich, MO, USA) at a lower concentration (10 ng/µL trypsin in 25 mM ammonium bicarbonate containing 10% acetonitrile) instead of Trypsin (sequencing grade, modified, Promega Corp. 13 ng/µL trypsin in 10 mM ammonium bicarbonate containing 10% acetonitrile) used by Shevchenko et al. (2006). Expecting faster peptide dryness, I used an extraction solution of 0.05% TFA in 50% acetonitrile, instead of 5% formic acid in 50% acetonitrile (Yamaguchi, 2011; Khandakar et al., 2013). In brief, the samples were dehydrated in 100 µL of acetonitrile and agitated for 10 min. The supernatant was withdrawn. The residues were added with 8 µL of the Trypsin Singles and left on ice for 20 min. After removal of an excess amount of the solution, the gels were incubated at 37°C overnight. Twenty µl of the extraction solution was added to the tube,

incubated at 37°C in a shaker (100 r/min) for 15 min, and sonicated in a cup horn sonicator (Astrason Ultrasoinic Processor XL2020, Misoinix, at output level 4) for 1 min. The solutions were transferred to a new 0.5-mL centrifuge tube and then centrifuged with a Speed Vac (SPD 131 DDA, Thermo Scientific) at 200 rpm and at 45°C for 15 min. The dried samples were dissolved in 5 µL of 0.5 mg/mL of 2,5-dihydroxybenzoic acid (DHBA, Shimadzu, Japan) in 33% acetonitrile, 0.1% trifluoroacetic acid. The 1 µL samples of the solution were spotted onto a µFocus MALDI target plate (Hudson Surface Technology, NJ, USA) and dried at room temperature. MS and MS/MS spectra were obtained using a MALDI-QIT-TOF mass spectrometer (AXIMA Resonance, Shimadzu, Kyoto, Japan). MS/MS ion search was performed using MASCOT® version 2.3 (Matrix Science, London, UK) against SwissProt 2014_07 (5456000 sequences; 194259968 residues) and EST Echinidae 2015_04 (851028 sequences; 212054698 residues) in our own MASCOT server. Search parameters used were: enzyme, trypsin; maximum missed cleavages, 2; fixed modification; carbamidomethyl (C); variable modifications, oxidation (HW and M); peptide mass tolerance, ± 0.3 Da; fragment mass tolerance, ± 0.2 Da; mass values, monoisotopic. Mascot score was assigned to identify protein with significance threshold at $p < 0.05$ as shown in figure S1.

2.8 Determination of pI and MW

Theoretical isoelectric point (*pI*) and sequence mass of precursor protein were calculated using ProtParam (http://www.expasy.ch/tools/protparam.html). Observed *pI* was calculated from the horizontal migration of the spot. Observed mass was estimated from the vertical migration of the spot by the method of Weber and Osborn (1969).

2.9 Statistical analysis

One sample t-test was applied to determine difference between measured and nominal PCO_2 values in each treatment. One-way ANOVA was applied to determine difference between group means and Tukey's HSD run to confirm group differences using PASW statistics 18. For 2-DE pattern analyses, four biological replicates for each condition and two technical replicates for each protein sample were

compared between different CO_2 levels. Differences in spot volumes were statistically evaluated using ANOVA at $p < 0.05$, using Prodigy SameSpots® software package (Non-linear Dynamics, Newcastle, UK). Differentially accumulated proteins were defined as significant at fold ratios ≥ 1.4.

3. Results

3.1 Seawater chemistry

Daily seawater temperature fluctuated slightly as air temperature varied between 23.4–25.2°C. Throughout the experiment, salinity remained at 34–35 PSU and the dissolved oxygen saturation was always above 90 % (Table III-B-1). Seawater pH varied slightly but remained relatively stable throughout the experiment (Fig. III-B-1). The seawater pH, PCO_2, and HCO_3^- and CO_3^{2-} concentrations all differed significantly between treatments (one-way ANOVA, Table III-B-1). Measured PCO_2 values were not significantly different from corresponding nominal values at 400 or 2000 µatm, whereas it was not the case at 10000 µatm (one-sample t-test, $t_{400\mu atm} = 0.035$, P = 0.975; $t_{2000\mu atm} = 2.039$, P = 0.111; $t_{10000\mu atm} = 3.548$, P = 0.024).

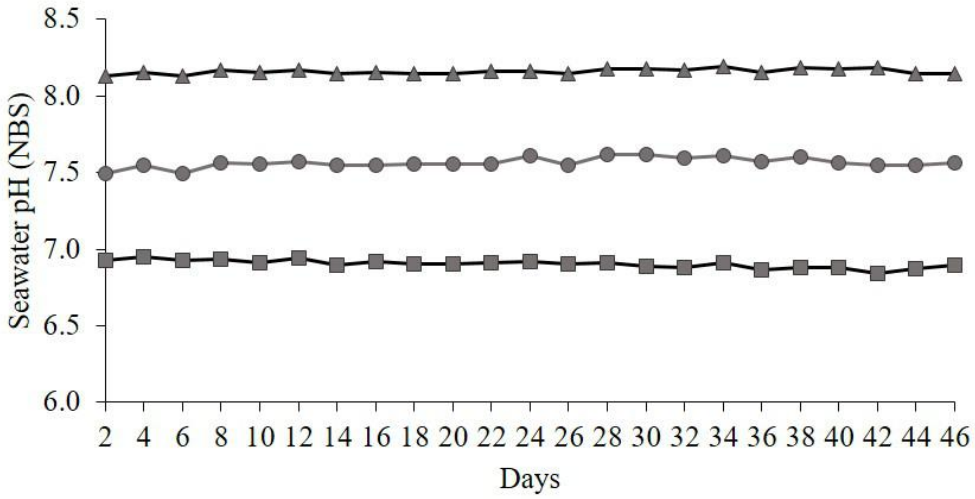


Fig. III-B-1 Temporal changes in seawater pH during 48-day exposure experiment. Filled triangles represent 400 µatm, filled circles 2000 µatm, and filled squares 10000 µatm treatments.

Table III-B-1 Seawater pH, percentage dissolved oxygen (DO) saturation, temperature (Temp.), salinity, alkalinity (TA), partial pressure of carbon dioxide (PCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) concentrations (mean ± SD) of each treatment

Treatment	400 (µatm)	2000 (µatm)	10000 (µatm)
$pH_{(NBS)}$	8.16 ± 0.02	7.56 ± 0.03[a]	6.90 ± 0.03[a,b]
DO (%)	96 ± 2	96 ± 2	96 ± 2
Temp. (°C)	24.6 ± 0.6	24.6 ± 0.6	24.6 ± 0.6
Salinity (PSU)	34.6 ± 0.6	34.6 ± 0.6	34.6 ± 0.6
TA (µmol/Kg-SW)	2121.3 ± 8.4	2128.1 ± 6.7	2130.2 ± 15.0
[1]PCO_2 (µatm)	400 ± 22	1912 ± 116[a]	9307 ± 524[a,b]
[1]HCO_3^- (µmol/Kg-SW)	1677.7 ± 10.9	2009.0 ± 13.4[a]	2128.6 ± 12.4[a,b]
[1]CO_3^{2-} (µmol/Kg-SW)	196.8 ± 1.2	61.5 ± 3.5[a]	14.2 ± 0.7[a,b]

[1]Calculated from seawater pH, temperature, salinity, alkalinity and total carbon by using CO2SYS program

[a]Significantly different than 400 µatm, [b]significantly different than 2000 µatm

3.2 Tube feet contraction force

There was no difference in test diameters or body weights of the sea urchins between treatments (one-way ANOVA, $F_{2,18} = 1.127$; $P = 0.346$ and $F_{2,18} = 0.475$, $P = 0.63$, respectively). Tube feet contraction force was significantly lowered with increasing CO_2 levels in a PCO_2-dependent manner (one-way ANOVA, $F_{2,18} = 13.89$, $P = 0.000$; Fig. III-B-2). Multiple comparison analysis revealed that contraction force differed significantly between all pH treatments (Tukey's HSD, $P < 0.05$; Table III-B-2).

Table III-B-2 Contraction force of tube feet in individual sea urchin and statistics analysis

Sea urchin No.	Contraction force (Newton)								
	400 µatm			2000 µatm			10000 µatm		
	1	2	3	1	2	3	1	2	3
1	0.0451	0.0569	0.0332	0.0114	0.0164	0.0212	0.0012	0.0051	0.0028
2	0.0259	0.0577	0.0644	0.0314	0.0189	0.0140	0.0049	0.0072	0.0062
3	0.0550	0.0393	0.0192	0.0347	0.0180	0.0359	0.0226	0.0291	0.0029
4	0.0442	0.0373	0.0209	0.0312	0.0383	0.0283	0.0336	0.0226	0.0229
5	0.0228	0.0285		0.0199	0.0331	0.0324	0.0015	0.0012	0.0002
6	0.0192	0.0671		0.0412			0.0091	0.0061	0.0021
7	0.0320	0.0438		0.000			0.0122	0.0048	

One-way ANOVA

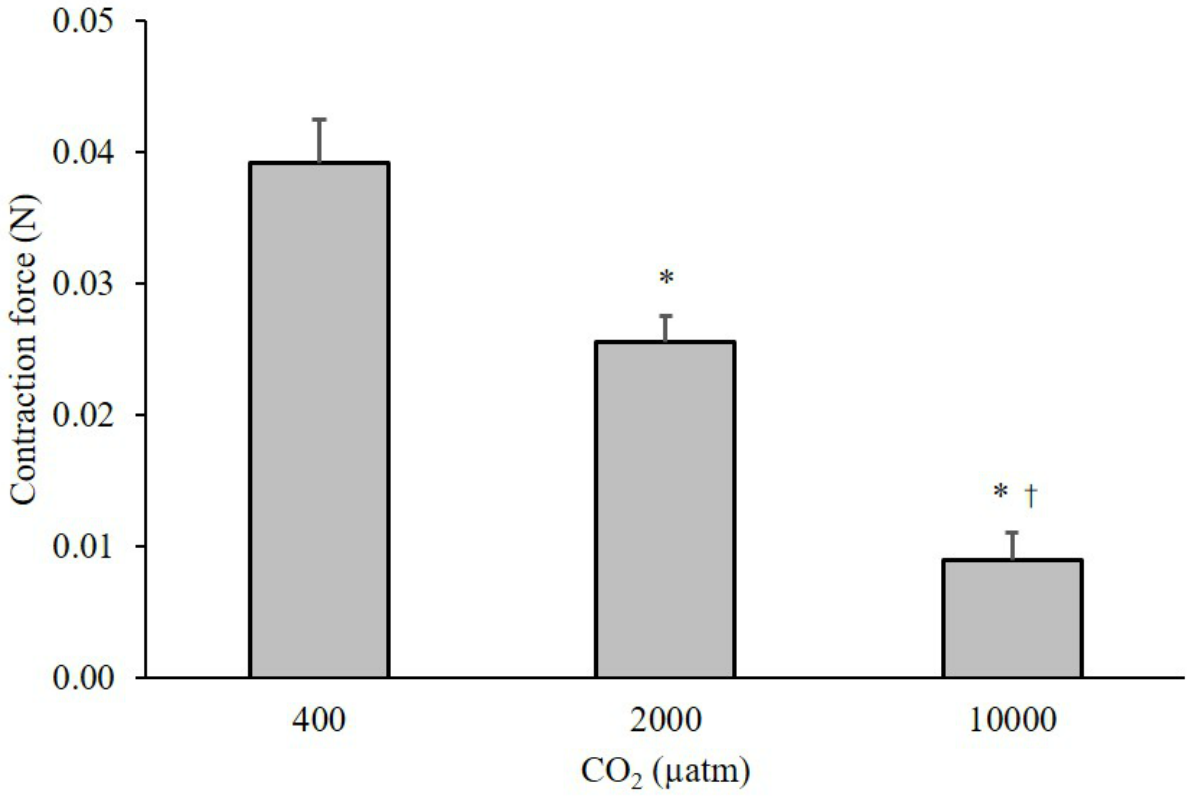
	Sum of squares	df	Mean square	F	P
Between groups	0.003	2	0.001	13.885	0.000
Within groups	0.002	18	0.000		
Total	0.005	20			

Multiple comparisons (Tukey HSD)

(I)Treatment	(J)Treatment	Mean difference (I-J)	Std. Error	P
1	2	0.01478	0.00553	0.039
	3	0.02915	0.00553	0.000
2	1	-0.01478	0.00553	0.039
	3	0.01437	0.00553	0.046
3	1	-0.02915	0.00553	0.000
	2	-0.01437	0.00553	0.046

Treatment 1 = 400 µatm, Treatment 2 = 2000 µatm, Treatment 3 = 10000 µatm

Fig III-B-2. Contraction force of the isolated tube feet of *P. depressus* induced by 10^{-4} M acetylcholine (Mean ± SE). The sea urchins were acclimated to 400 µatm (control), 2000 µatm and 10000 µatm PCO_2 for 48 days at 25°C.



3.3 Protein profile under changing in CO_2 concentration

The total protein amounts soluble to CHAPS-based IEF solution obtained from the tube feet in 400 µatm, 2000 µatm and 10000 µatm treatments were 0.65 ± 0.27, 1.60 ± 0.48 and 1.40 ± 0.33 mg g^{-1} fresh weight, respectively. The number of protein spots that were resolved from protein extracts from 400 µatm, 2000 µatm and 10000 µatm treatments were 120 ± 2, 120 ± 2 and 119 ± 1 respectively (Fig. III-B-3). Comparative quantitative image analysis of 2-DE patterns showed that a total of eight protein spots changed significantly in spot volume (i.e. spot intensity); two spots were down-regulated and six spots were up-regulated under increasing CO_2 conditions (Figs. III-B-3 and III-B-S1). Eight differentially accumulated protein spots (spots 1–8) and four constantly expressed protein spots (spots a-d, prominent spots selected for easier protein extraction/identification) were excised from the 2-DE gels (Figs III-B-3 and III-B-S1),

digested in-gel with trypsin, and the extracted tryptic peptides were subjected to MALDI-QIT-TOF mass spectrometry. Although nucleotide/protein sequences reported from *Pseudocentrotus depressus* number less than one thousand (56 proteins and 941 ESTs), 9 of the 12 protein spots were positively identified by cross-species searches (Tables III-B-3 and III-B-S1). Four constantly expressed proteins were identified as actin-1 (spot a), tropomyosin (spot b), voltage dependent anion channel 2 (spot c), and calmodulin-A (spot d). Of the 6 up-regulated proteins, three were identified as tubulin beta chain (spot 2), tropomyosin (spot 3) and actin (spot 4). The other three (spots 1, 5, and 6) were not identified. Two down-regulated proteins were identified as myosin light chain (spot 7) and actin (spot 8). The observed masses of actins, spots 4 (25.4 kDa) and 8 (17.4 kDa), were significantly lower than the theoretical masses of actin (41.8 kDa) (Table III-B-3). In contrast, the observed mass of constantly expressed actin (spot a, 39.0 kDa) was close to the theoretical mass. These observations and the peptides assigned to the actin sequence by MS/MS ions searches indicated that spots 4 and 8 were N-terminal and C-terminal fragments of actin, respectively (Tables III-B-3 and III-B-S1). In a similar way, spot b (34.7 kDa) was considered to be intact tropomyosin, and spot 3 (28.3 kDa) a fragment of tropomyosin (Table III-B-3).

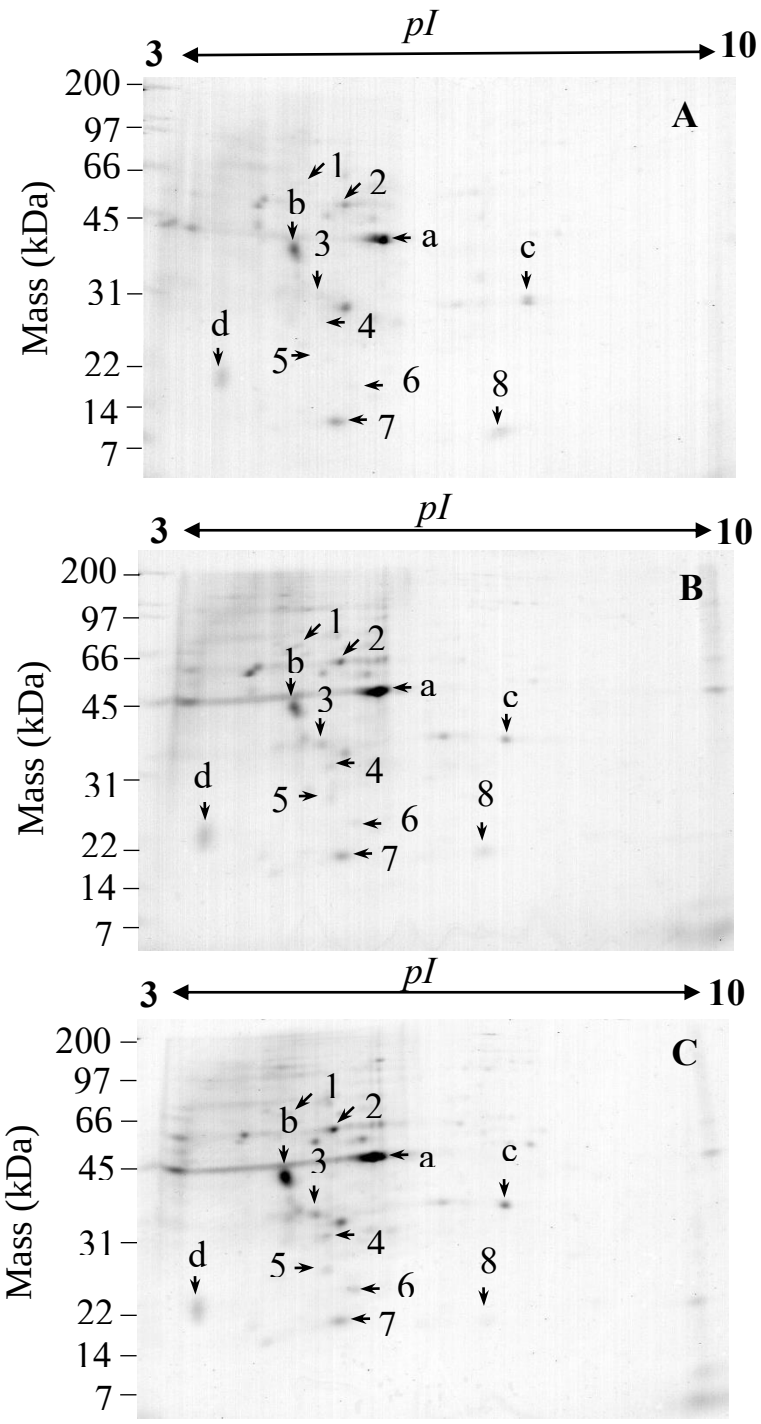


Fig. III-B-3. Two-dimensional gel electrophoretic (2-DE) separation of *P. depressus* tube feet proteins extracted from the specimens treated in three different CO_2 levels; (A) 400 μatm, (B) 2000 μatm and (C) 10000 μatm. The first dimensional isoelectric focusing of 2-DE was performed using a pH range of 3–10. The characters a–d represent the constantly expressed protein and the number 1-8 represent the differentially accumulated protein.

Table III-B-3 MS/MS identification of the protein spots of sea urchin tube feet that showed constant (a-d) or significantly different volumes (1-8) in response to elevated CO_2 conditions

Spot No.	Protein name	Species	Accession no (NCBI)	Theoretical mass (kDa)/pI	Observed mass (kDa)/pI	Fold 10000µatm/400µatm
Constant proteins						
a	Actin-1	*Strongylocentrotus purpuratus*	ACTA_STRPU	41.8/5.3	39.0/5.7	NA
b	Tropomyosin	*Paracentrotus lividus*	AM197503	32.7/4.7	34.7/4.7	NA
c	Voltage-dependent anion channel 2	*Paracentrotus lividus*	AM187061	31.1/8.8	29.4/7.2	NA
d	Calmodulin-A	*Strongylocentrotus intermedius*	CALM_STRIE	17.6/4.1	18.9/3.8	NA
Differentially accumulated proteins						
1	NF					+1.5
2	Tubulin beta chain	*Paracentrotus lividus*	TBB_PARLI	50.1/4.7	47.9/5.2	+1.4
3	Tropomyosin fragment	*Paracentrotus lividus*	AM197503	32.7/4.7	28.3/5.0	+1.5
4	Actin N-terminal fragment	*Strongylocentrotus purpuratus*	ACTA_STRPU	41.8/5.3	25.4/5.1	+1.4
5	NF					+1.4
6	NF					+1.6
7	Myosin light chain	*Paracentrotus lividus*	AM573676	17.0/4.8	17.4/5.3	-1.7
8	Actin C-terminal fragment	*Strongylocentrotus purpuratus*	ACTA_STRPU	41.8/5.3	17.4/6.8	-1.5

NF = Not found

NA = Not available

4. Discussion

This study demonstrated that the contraction force of the tube feet was significantly reduced by 48-day exposure to 2000 and 10000 μatm CO_2 in the sea urchin, *P. depressus*. The mechanisms underlying this functional impairment are yet to be elucidated, but the proteomic analysis demonstrated alterations of proteome composition, mainly a consequence of post-translational processing and/or proteolysis of muscle-related proteins, which could give a clue for a mechanistic understanding of the physiological disorder. Presently, very little is known about the effects of ocean acidification and CO_2 leakage from CCS sites on muscular systems of marine invertebrates (see Introduction). We have recently found that CO_2 significantly reduced the locomotion speed of the sea urchin, *Hemicentrotus pulcherrimus*, reared under 1000 μatm PCO_2 both at ambient and at an elevated temperature (+ 2°C) for seven months (Yin, Lee, Kurihara and Ishimatsu, in preparation). Similarly, some studies showed negative impacts on muscle systems in fish. Chambers et al. (2014) and Frommel et al. (2016) showed subtle histological alterations in skeletal muscle of the fish larvae (*Paralichthys dentatus* and *Thunnus albacares*) under elevated CO_2 conditions (1800 and 4700 μatm, and 2000 – 9600 μatm, respectively). Bignami et al. (2014) reported that maximum swimming velocity was reduced in pelagic larvae of *Coryphaena hippurus*, reared under 1460 μatm PCO_2 for 21 days, but this effect was absent in larvae of another pelagic fish, *Rachycentron canadum* (Bignami et al., 2013) reared under 2100 μatm for 22 days. On the other hand, there are studies demonstrating that fishes were relatively insensitive to elevated CO_2. Maneja et al. (2013) found swimming activity of larval Atlantic cod, *Gadus morhua*, was robust to high CO_2 exposure, and Melzner et al. (2009) also found that critical swimming speed of adult *G. morhua* was unaffected by exposure to 5800 μatm PCO_2 for 12 months or 3100 μatm PCO_2 for 4 months. Furthermore, we have recently published the data on the effect of CO_2 and temperature on the escape response of the Japanese anchovy, *Engraulis japonicus* to mechanical stimuli, and found no significant difference in various parameters of the response except turning rate, which was elevated at a higher temperature (Nasuchon et al., 2016).

One possible interpretation of the observed negative effects of CO_2 on locomotive behaviors in marine invertebrates (and fish) is that elevated CO_2 or resultant acidification of body fluids had direct negative impacts on the integrity and/or functionality of muscular system in these animals, as suggested by my proteome analysis. However, different hypotheses are also possible. Recent papers on the effect of ocean acidification on fish have demonstrated that increasing CO_2 can disrupt sensory and brain functions

of fish (Nagelkerken and Munday, 2016). There is some evidence for the involvement of a neurotransmitter, gamma-aminobutyric acid (GABA) in these phenomena in fish under elevated CO_2 (Nilsson et al., 2012; Chivers et al., 2014; Hamilton et al., 2014). The current hypothesis for the involvement of GABA assumes decreased chloride ion concentrations in extracellular fluid, as a result of acid-base restoration through ionic exchange with bicarbonate ions, to be responsible for the reversed (from inhibitory to excitatory) response to GABA released from a nerve terminal, as known under some pathological conditions (Nilsson et al., 2012). GABA was detected in tube foot extract of sea urchins, and known to cause excitation of cholinergic motoneurones but have no direct effect on muscle fibers (Florey et al., 1975). However, since ion concentrations of perfusate were held constant during the measurement, the reduced contraction force of the tube feet seen in this study was more likely attributable to the effect of pH/CO_2 per se, rather than through some GABA-related process triggered by lowered chloride concentrations. Sea urchins have a lower capacity for acid-base regulation of both extracellular (Spicer et al., 2011; Stumpp et al., 2012; Kurihara et al., 2013) and intracellular fluids (Stumpp et al., 2012) than fish (Ishimatsu et al., 2005), it is unlikely that elevated CO_2 levels, at least those predicted in the context of ocean acidification, will cause substantial decreases of chloride ion concentrations in the body fluids of intact sea urchins. Another possibility is that 48-day exposure to CO_2 resulted in some irreversible alterations in the functions of GABA receptors. Further, low pH per se could have reduced neuromuscular transmission (Landau and Nachchen, 1975; Takahashi and Copenhagen, 1996). Clearly, further studies are needed for mechanistic understanding of the observed reduction in the contraction force of tube feet under elevated CO_2 conditions.

Comparative quantitative analysis of 2-DE profiles detected eight protein spots that had changed in spot volume. Of six up-regulated spots, three were identified as tubulin beta chain, tropomyosin fragment and actin N-terminal fragment, while two down-regulated ones were identified as myosin light chain and actin C-terminal fragment (see Results). These proteins are all involved in muscle contraction. An interaction between actin and myosin generates movement relative to each other. Troponin and tropomyosin are involved in regulating on actin site (Szent-Györgyi, 1975). Myosin light chain of 17 kDa is an essential light chain (ELC), which has the role of stabilizing the lever arm. The interaction between the C-terminal domain of ELC and N-terminal sub-domain of the heavy chain of the myosin may be involved in coupling ATP hydrolysis and rotation of the lever arm (Ushakov, 2009). My data showed that the majority of actin was constantly expressed as the intact protein under all the CO_2 conditions tested, whereas minor fragments

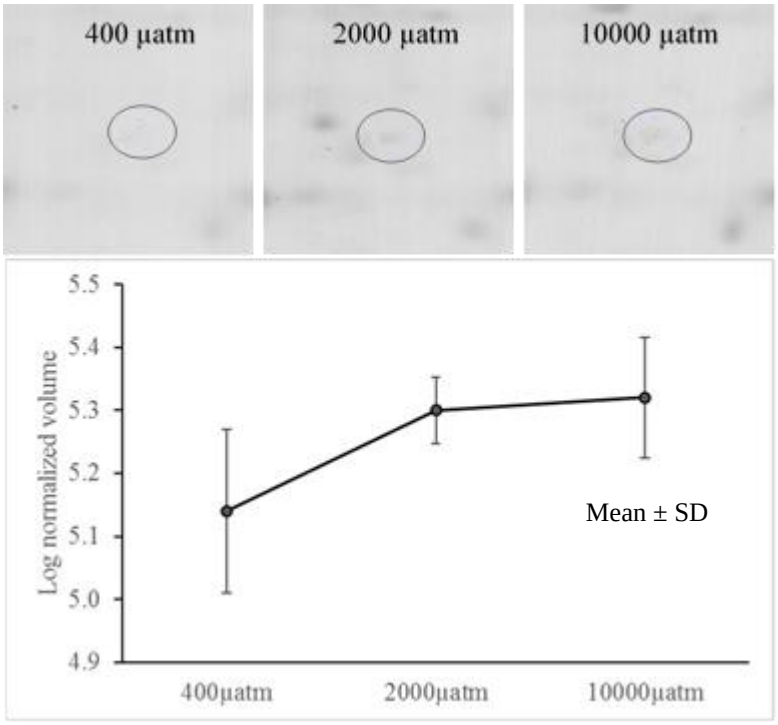
of actin were differentially expressed: the actin C-terminal fragment decreased, while the actin N-terminal fragment increased at higher CO_2 levels. Actin has been shown to be cleaved by caspases into N-terminal 32-kDa (Fractin) and 15-kDa (tActin) fragments. The site of this proteolytic cleavage at the C-terminal side of ^{240}**YELPD**^{244} is conserved from yeast to humans, implicating it as an important regulatory sequence (Gourley and Ayscough, 2005). N- and C-terminal fragments of actin (spots 4 and 8) identified in this study are unlikely generated via caspase-mediated processing/degradation because both of fragment contained sequence S**YELPD**GQVITIGNER that was uncleaved at the conserved site (Figs III-B-S2 and Table III-B-S1). Accumulation of the N-terminal fragment of actin may be a product of caspase-independent proteolysis of intact actin in response to elevated CO_2. Whereas, the C-terminal fragment could be a product of leaky ribosomal scanning (i.e., alternative translation) of actin gene, which is expressed in a low abundance in the normal condition, and the C-terminal fragment may decrease via a caspase-independent proteolysis in response to high CO_2. For tropomyosin, a coiled-coil dimer bonds molecules winding around the actin helix (Li et al., 2002) and has the role of blocking myosin binding sites on actin molecules, thus preventing cross-bridge formation, which inhibits muscle contraction without nervous input (Geeves and Holmes, 1999). Tropomyosin associated with troponin plays an essential role in the regulation of muscle contraction. Thus, the increased abundance of tropomyosin fragment may induce the change in the troponin-tropomyosin conformation and alter myosin binding sites on actin filament. A comparative proteomic analysis of the oyster *Crassostrea gigas* found up-regulated two isoform gene products of tropomyosin (33.06 kDa, *pI* = 4.57, and 26.91 kDa, *pI* = 4.49) in mantle tissue at 2000 µatm CO_2 (Wei et al., 2015). In contrast, my study showed that the majority of tropomyosin was constantly expressed, while up-regulation was found in a short form (Table III-B-3,), derived from the same tropomyosin gene. Thus, the increased short form, most likely a fragment, of tropomyosin may also have affected muscle contraction. Voltage dependent anion channel 2 (VDAC2, spot c) and calmodulin-A (spot d) were identified as unchanged abundant proteins (Fig. III-B 3 and Table III-B-3). Unlike actin or tropomyosin, neither fragment of VDAC2 nor calmodulin-A was detected. Therefore, these proteins were probably not responsible for the reductions in muscle contraction force at high CO_2 conditions in the sea urchins.

5. Conclusion and future study

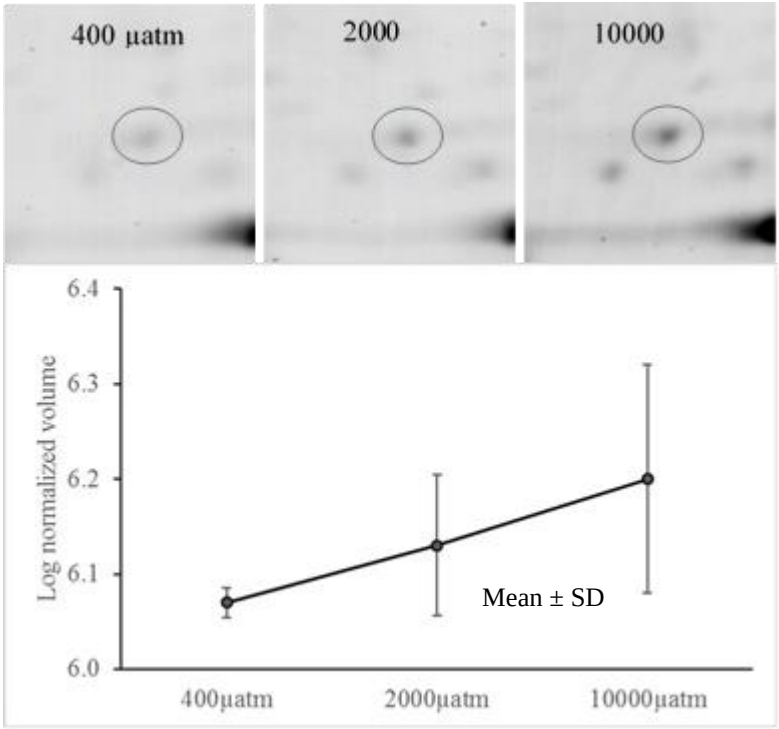
The results have shown that elevated CO_2 reduced the contraction force of sea urchin tube feet in a concentration-dependent manner. Further, 2-DE based proteomics showed that proteins involved in muscle contraction changed their spot volumes under high CO_2 conditions. These results suggest that elevated CO_2 possibly affects muscular system in sea urchins through alterations in proteome composition via a post-translational proteolysis, although other interpretations are also possible. For example, disruption of neuromuscular transmission or coordination at higher neuronal levels by elevated CO_2 or lowered pH in nervous system is a possibility. On the other hand, although this study pointed out the importance of proteomic approaches to understand how sea urchin tube feet respond to elevated CO_2 in the molecular level, correlation between transcriptomic and proteomic changes in response to elevated CO_2 remain to be elucidated. In addition, the muscular systems in various organs of sea urchins and other marine invertebrates need to be examined for their CO_2 sensitivity to understand how future oceanic environmental changes will affect physiological functions driven by muscle contraction.

Figure III-B-S1. Magnified images of protein spots that showed significantly different changes in sea urchin tube feet between 400 µatm, 2000 µatm and 10000 µatm treatment ($P < 0.05$).

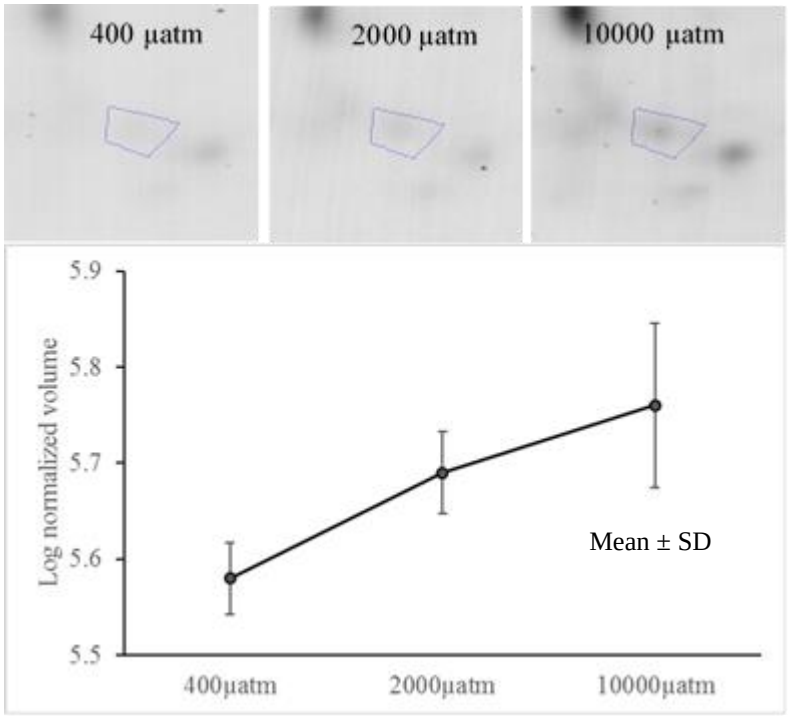
Spot No. 1



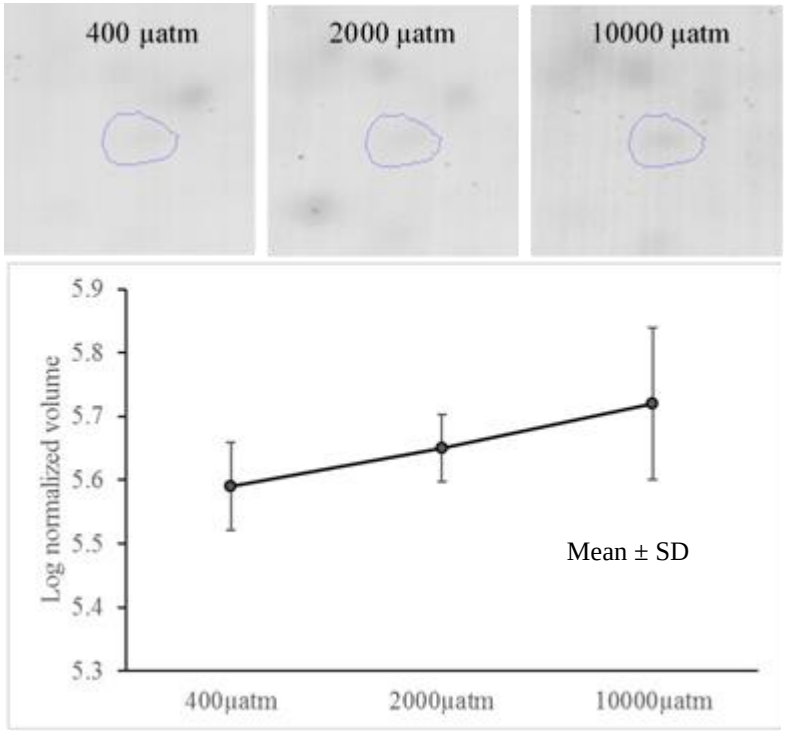
Spot No.2



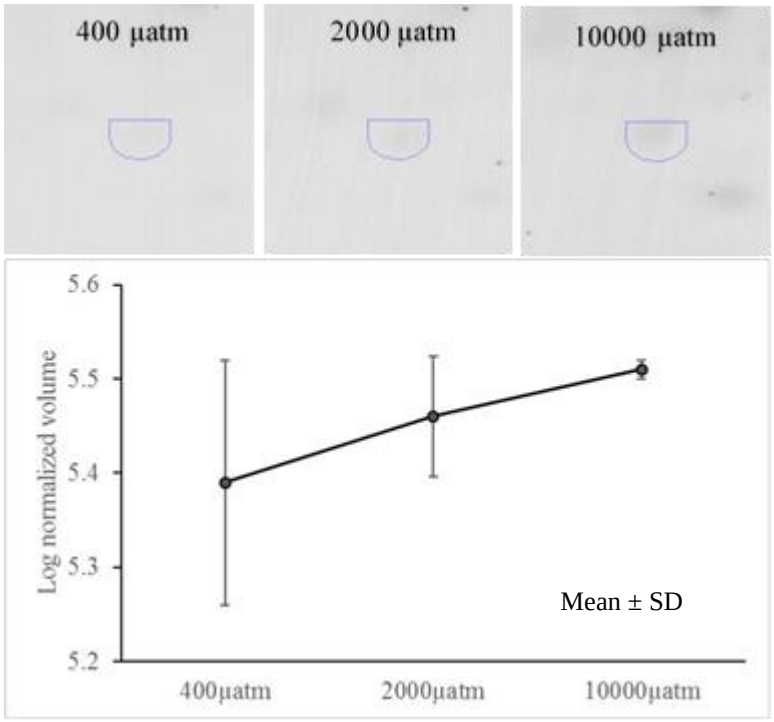
Spot No.3



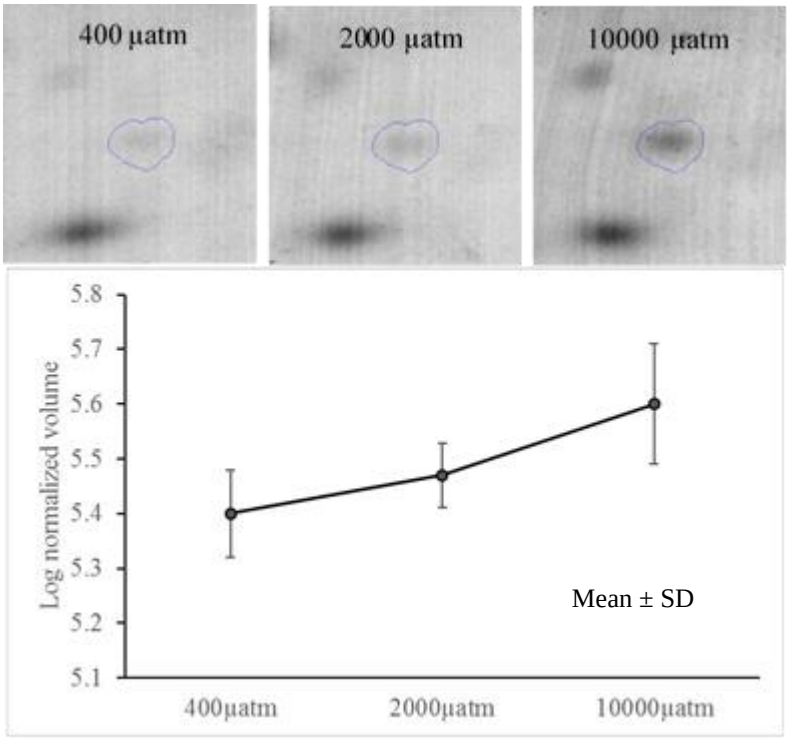
Spot No.4



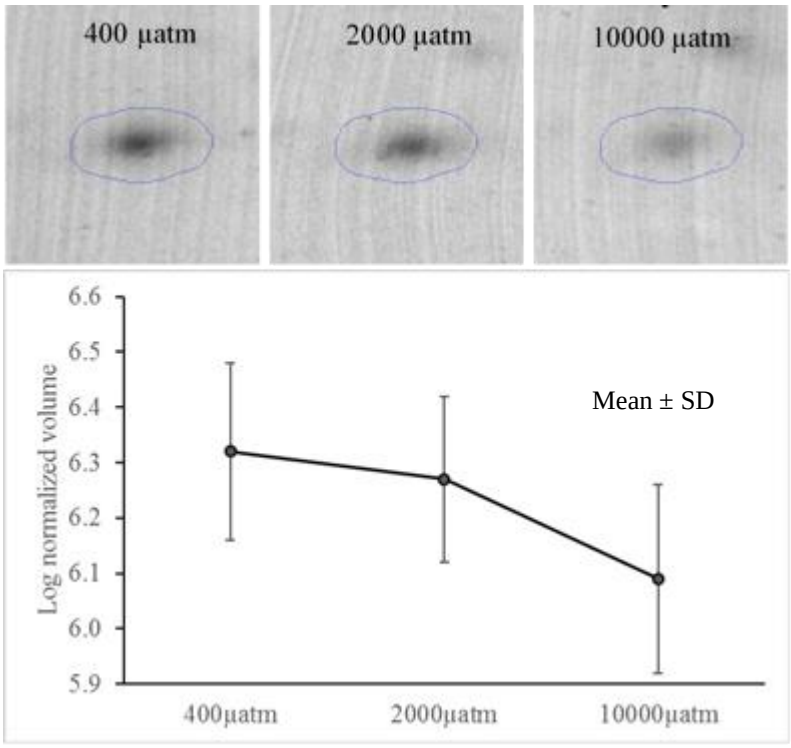
Spot No.5



Spot No.6



Spot No.7



Spot No.8

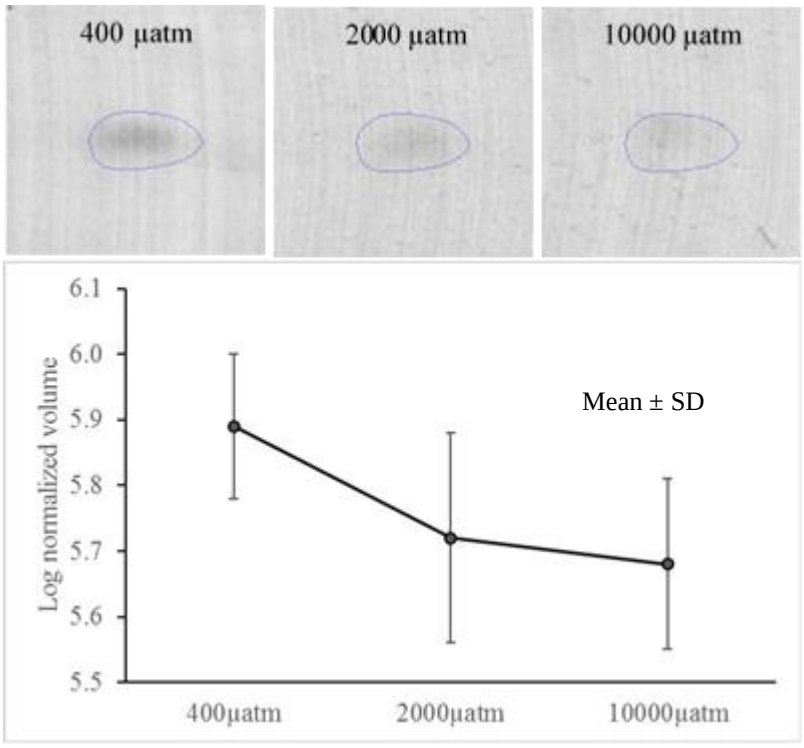


Figure III-B-S2 Full peptide sequence of actin of sea urchin *Pseudocentrotus depressus*: Bold texts are peptide sequences of actin N-terminal fragment (above) and actin C-terminal (lower). Highlighted is the amino acid sequence around the caspase-cleaved site and the arrow indicated the site of cleavage by caspase.

MCDEEVAALVVDNGSGMCKAGFAGDDAPRAIFPSIVGRPRHQG
VMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKI
WHHTFYNELR**VAPEEHPVLLTEAPLNPK**ANREKMTQIMFETFN
TPAMYVAIQAVLSLYASGRTTGIVMDTGDGVTHTVPIYEGYALPH
AILRLDLAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYV
ALDFEQEMATAASSSSLEKSYELPDGQVITIGNERFRCPEALFQPA
FLGMESPGIHETTYNSIMKCDIDIRKDLYANTVLSGGTSMYPGIA
DRMQKEITSLAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQM
WISK**QEYDESGPSIVHR**KCF

Table III-B-S1 Results of MS/MS ion search and BLAST search for protein identification

Spot No	Protein ID	EST (Accession No.)	Homolog (Accession No.)	Species	m/z	Sequence	Delta	Miss	Score	Expect
Constant proteins										
a	Actin-1	ND	ACTA_STRPU	*Strongylocentrotus purpuratus*	1790.12	SYELPDGQVITIGNER	0.19	1	83	5.1e-006
					1954.31	VAPEEHPVLLTEAPLNPK	0.25	0	42	0.024
b	Tropomyosin	gi|89438467	AM197503	*Paracentrotus lividus*	1686.96	RLETIEVEADENLR	0.10	1	92	7.8e-007
					1762.12	KLQMTEQQLEVAEAK+oxidation	0.21	1	39	0.11
c	Voltage Dependant Anion Channel 2	gi|89444236	AM187061	*Paracentrotus lividus*	2531.16	TADFQLHTAVNEGSDFSGSIYQK +oxidation	-0.01	0	68	0.0001
d	Calmodulin-A	ND	CALM_STRIE	*Strongylocentrotus intermedius*	1738.68	VFDKDGNGFISAAELR	-0.2	1	77	1.9e-005
Differentially accumulated proteins										
1	NF									
2	Tubulin beta chain	ND	TBB_PARLI	*Paracentrotus lividus*	1159.61	LAVNMVPFPR+oxidation	-0.02	0	52	0.0074
					1636.67	LHFFMPGFAPLTSR+oxidation	-0.16	0	42	0.059
					1959.00	GHYTEGAELVDSVLDVVR	0.02	0	60	0.00096
3	Tropomyosin fragment	gi|89438467	AM197503	*Paracentrotus lividus*	1686.88	RLETIEVEADENLR	0.01	1	63	0.0006
					1761.94	KLQMTEQQLEVAEAK + oxidation	0.03	1	51	0.01
4	Actin N-terminal fragment	ND	ACTA_STRPU	*Strongylocentrotus purpuratus*	1954.18	VAPEEHPVLLTEAPLNPK	0.11	0	72	5.4e-005
5	NF									
6	NF									
7	Myosin light chain	gi|139327852	AM573676	*Paracentrotus lividus*	1011.70	HVLSTLGER	0.14	0	43	0.08
					1271.72	LEEAEVDIIIK	0.01	0	45	0.05
8	Actin C-terminal fragment	ND	ACTA_STRPU	*Strongylocentrotus purpuratus*	1516.69	QEYDESGPSIVHR	-0.01	0	43	0.059
					1790.91	SYELPDGQVITIGNER	0.02	0	67	0.00031

CHAPTER IV

EFFECTS OF ELEVATED TEMPERATURE AND CO_2 ON EARLY LIFE STAGE OF GIANT TIGER SHRIMP *Penaeus monodon*

1. Introduction

The anthropogenic CO_2 emission does not only increase hydrogen ion (H^+) concentration in the ocean but it also altered the concentration of carbonate system species in seawater. The dissolved CO_2 leads to increase in bicarbonate ion (HCO_3^-) concentration as well as a reduction of carbonate ion (CO_3^-) concentration and then saturation state of calcium carbonate (Ca_2CO_3) become lower (Orr, 2011). Hence, the calcifying organisms are probably one of the earliest organisms to be impacts by ocean acidification due to the increasing of anthropogenic CO_2 emission. The studies in the past twenty years indicated that ocean acidification affected the physiological process in marine animals. Kroeker et al. (2013) reviewed that survival, calcification, growth, development and abundance of marine organisms decreased in response to ocean acidification. Rising in global temperature leads ocean temperature increasing. Warming occurs at surface down to 700 m water depth. Thus, warming could affect coastal marine animals more than deep sea animals. Temperature is one of the most powerful environmental factors to modulate biological activities (Willmer et al., 2005). Reported that temperature reduced timing of development from hatching to adult of a marine copepod *Pseudocalanus newmani* (Lee et al., 2003). Wyban et al. (1995) addressed that temperature affected growth and feed intake of a whiteleg shrimp *Litopenaeus vannamei* and caused growth slow and ovarian maturation delay in a red cherry shrimp *Neocaridina heteropoda* (Tropea et al., 2014).

Shrimp is an important commercially species. Globally consumed about 4.1 million metric tonnes in 2014, and it demand tends increasing year by year. Southeast Asia is one of major producer in the world, and produces about 32% of world's production. Major production countries of this region are Indonesia, Vietnam and Thailand (FAO, 2014). Up to date, it is little known about how OA and temperature or their combination affect shrimp, particularly the tropical shrimps (e.g. *Fenneropenaeus merguiensis*, *Penaeus.*

monodon, *Litopenaeus vannamei*). It has been reported that OA affected the hatching rate, growth, survival and gene expression in brine shrimp when exposed in pH 7.8 and 7.6 (Zheng et al., 2015). Acidified seawater could reduce exoskeleton mineralization in red rock shrimp and European lobster (Arnold et al., 2009; Taylor et al., 2015). Kurihara et al. (2008) stated that CO_2 was significantly suppressed survival and egg production of *Palaemon pacific* in both of 1000 µatm and 1900 µatm, and frequency of molting in 1000 µatm for long term study (15 weeks for 1900 µatm and 30 weeks for 1000 µatm). I hypothesized that ocean warming and acidification affect early life stage of shrimp.

Giant tiger shrimp was selected for this study because it is a second-most widely cultured species in the world and widely cultures in southeast Asia, such as Thailand, Vietnam and Indonesia (FAO, 2016). Early life stages have been considered to be vulnerable or fragile in relation to environmental stresses than adult (e.g. Ishimatsu et al., 2005). Therefore, the aim of this study was to examine the effect of elevated temperatures and CO_2 or their combination on early stage of giant tiger shrimp. Measured parameters were hatching, survival, swimming performance and muscle system. Experiment was set in four temperature levels, 28°C (control), 30°C, 32°C and 34°C, and four pH levels 8.1 (control), 7.6, 7.0 and 6.8.

2. Materials and methods

2.1 Artificial seawater

Seawater used in this experiment was prepared by diluting high salinity seawater (80 ppt) which was purchased from a brine shrimp farm to a salinity level of 30 ppt. Chlorine at concentration 1 ppm was applied for water purification. The seawater was dried with sun light for three days and adjusted in alkalinity and pH by using calcium carbonate and acetic acid in the range of 0.08-0.12 mM/L and 8.2 respectively before used. The seawater was then transported to 1 L tanks inside the building and filtered with string wound filter cartridges.

2.2 Experimental animals

In March 2016, a gravid female was purchased from the private hatchery shrimp farm in Ca Mau province, Vietnam. It was then transferred to the Collage of Aquaculture and Fisheries, Can Tho University. The gravid female was stimulated ovary development by using the eyestalk ablation method. After that, the gravid female was reared in 200 L tank at seawater salinity 30 PSU until the eggs developed into the ripe stage. After that the females were transferred to 1000 L tank for allowing it lay eggs. Fertilized eggs were collected immediately after spawned by using scoop net and soaked in formaldehyde solution concentration of 200 ppm for 30 seconds and rinsed with artificial seawater.

2.3 Experimental Set-up

CO_2 experiment, artificial seawater pH was set at four different levels, pH 8.1 (control), 7.6, 7.0 and 6.8 units. These pH levels were set, based on the prediction in 2100 (pH 7.6) and my survey in hatchery farm (Table IV-1). Artificial seawater pH was reduced by bubbling with CO_2 gas until pH lower to 6.5, then adjusted to target levels by adding ambient artificial seawater to increase the water pH. For control treatment, seawater was bubbled with air. For temperature experiment, artificial seawater temperature was set in four different levels, 28°C (control), 30°C, 32°C and 34°C by using heater combination with sensor. Artificial seawater pH, dissolved oxygen and temperature were monitored daily with a digital multiparameter meters (WTW multi 3420, Germany) calibrated with a standard buffer solution pH 4.01, 6.86 and 9.18. Salinity was monitored throughout the experiment using a salinity refractometer (S/Mill-E, Atago, Japan) (See table IV-1).

Table IV-1 An average sea water pH of samples in the hatchery farm of tiger shrimp of each stage, which measured in every 6 hours on November 4, 2015 from Can Tho shrimp seed joint stock company

Time	Sea water pH of each stage				
	Zoea	Mysis 2	Mysis 3	Post larval 2	Post larval 8
06.00 h	6.98	7.02	7.05	6.93	6.93
12.00 h	NA	7.57	7.16	7.30	6.95
18.00 h	6.75	6.76	6.80	6.90	6.82
24.00 h	7.09	6.91	7.02	6.94	6.98

NA = not available

2.4 Measured parameters

2.4.1 Hatching success

Two hundred fertilized eggs were randomly collected and placed into 500 mL glass bottles of each pH and temperature. Each treatment was setup in three replicates. Bottles were closed and submerged about ¾ part of bottle into the 100 L tank. For pH experiment, the sample's bottles were submerged in the water with control temperature at 28°C. Number of hatched larvae and undeveloped eggs of each treatment were counted after exposure for 14 hrs. Hatching success was calculated in percentage.

2.4.2 Survival rate

One thousand fertilized eggs were randomly collected and placed into 10 L glass jars of each pH and temperature. The jars were closed with a plastic sheet for controlling gas exchanges surface. Each treatment was set up in three replicates. Artificial seawater was exchanged about 60% daily through experimental period. Larvae were fed in every 3 hours from zoea-1 to post larval-15 (PL-15) (Zoea-1 were fed with mixed *Skeletonema* and *Chaetoceros* at density 60 cell/mL in every 3 hours until developed to zoea-2, Zoea-2 were fed with *Skeletonema* and *Chaetoceros* switching with shrimp larval diet-ZM (Lansy-shrimp, size 10 - 80 µM) until developed to Zoea-3, Zoea-3 were fed with shrimp larval diet-ZM until

developed to mysis-1, mysis-2 to post larvae-15 (PL-15) were fed with shrimp larval diet-PL (Lansy-shrimp, size 150 – 400 µM, New Zealand) switching with brine shrimp in every 3 hours). The number of remained larvae at PL-15 were counted and survival rate was calculated in percentage.

2.4.3 Growth

Ten of zoea-1, mysis-1, post larvae-1 (PL-1) and post larvae-15 (PL-15) were randomly sampled from each rearing jar (thirty in total of each treatment). Total length of larvae of each stage were measured in millimeter, which is the distance between base of eyes and the tip of telson.

2.4.4 Histology

Five larvae of each treatment were observed histology of muscle. The larvae were preserved in 10% of formaldehyde until ready for histology analysis. Samples were fixed in 5% EDTA·4Na at pH 6 for 3 days for histology determination of muscle fiber in cross-sections of the body trunk. Paraffin sections 5 µm thick were prepared using an HM325 Micron and stained with hematoxylin and eosin. The sections of body muscle were photographed at ×20 magnification using Olympus FX380, FLVFS-LS version 1.12. At least hundred cross-sectional muscle fiber areas of each treatment were measured by using ImageJ 1.48v (National Institute of Health, USA). Data were expressed as fiber size distribution.

2.4.5 Swimming speed

Swimming performance was observed only for the temperature experiment. A plastic bowl (capacity 4 L, diameter 20 cm, height 10 cm) was used for recording swimming performance. The bowl was filled with artificial seawater to a depth of 3 cm. Water temperature was set at the same level with the rearing jars of each treatment. A video camera (Sony HDR-PJ760V) was installed 34 cm above the observation bowl. Seventeen to nineteen PL-15 were observed the swimming performance in a plastic bowl. The larvae were allowed to habituate themselves in the observation bowl for about 10 minutes until they started to swim in a normal manner. A video was recorded for 5 minutes of each treatment. The videos were converted

to AVI files using the MOVAVI video converter software. ImageJ 1.48v was applied for tracking x-y coordinator. Swimming speed was calculated using a following formula:

$$swimming\ speed = \frac{Distance}{Time}$$

Statistical analysis

The PASW statistics 18 was applied for all data analysis. Independent t-test was applied to compare mean of survival rate and muscle fiber areas between pH 7.6 and 8.1. One-way ANOVA was applied to compare mean of all measured parameters for pH and temperature treatments. Then Tukey's test was run to confirm where the differences occurred between groups.

3. Results

3.1 Hatching success

The percentage hatching success of pH 8.1 (control), 7.6 and 7.0 were 93.77 ± 1.1 (mean ± SD, n = 3), 91.17 ± 3.8 and 75.73 ± 1.7 respectively, while no hatch found for pH 6.8. For temperature experiment, percentage of hatching success for temperature 28°C (control), 30°C, 32°C and 34°C were 93.25 ± 2.6, 85.93 ± 3.6, 75.71 ± 7.1 and 0.53 ± 0.9 respectively (Fig. IV-1). Hatching success was significant difference between group means as determined by one-way ANOVA in both of pH and temperature treatments ($F_{3,8}$ = 1992.26, $P < 0.000$ for pH and $F_{3,8}$ = 307.71, $P < 0.000$ for temperature). Tukey's test was significant hatching success of control (pH 8.1) higher than of pH 7.6 ($P < 0.000$) but it was no significant difference between control and pH 7.6. ($P = 0.316$). For temperature experiment, Tukey's test was significant hatching success of control (temp. 28°C) higher than temperature 32°C and 34°C, but it was no significant difference between control and temperature 30°C ($P = 0.226$).

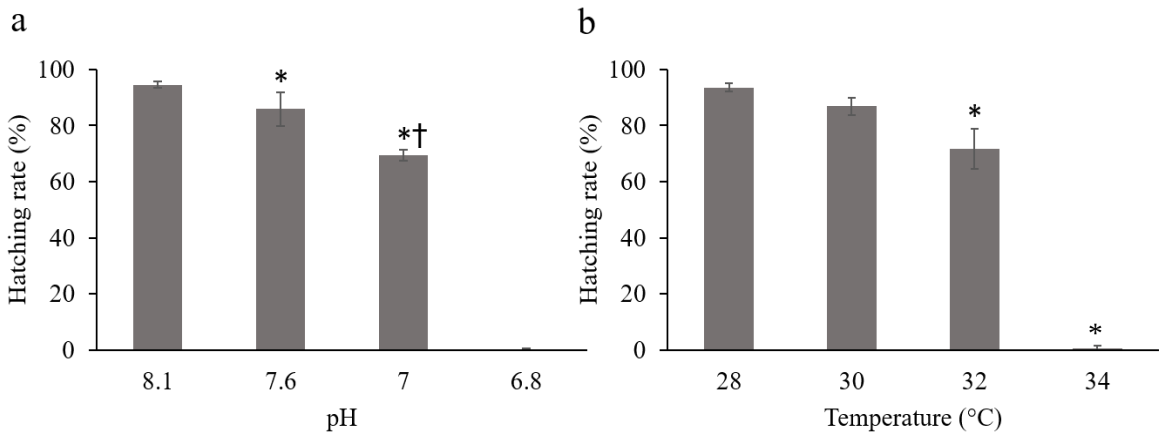


Fig. IV-1 Percentage of hatching rate of the giant tiger shrimp *P. monodon* (mean ± SD); a = pH experiment, b = temperature experiment; * significant different from control (pH 8.1, Temp. 28°C), † significant different from pH 7.6

3.2 Survival rate

The result showed that larvae could not survive in the seawater pH 7.0 and temperature 34°C, which all of larvae died at zoea stage (4-5 days after hatched). The percentage of survival rate of pH 8.1 (control) and 7.6 were 44.6 ± 5.5 (mean ± SD, n = 3) and 29.7 ± 6.5 respectively and 58.0 ± 9.9, 43.9 ± 5.4 and 29.7 ± 5.7 for temperature 28°C (control), 30°C and 32°C respectively (Fig. IV-2). T-test was showed that survival rate was significantly decrease in seawater pH 7.6 compared with control condition. ($T_{2,4}$ = 3.040, P = 0.038). The temperature was a significant effect survival of a giant tiger shrimp (One way ANOVA: $F_{2,6}$ = 11.29, P = 0.009). Tukey's test showed that survival rate was significant lower at temperature 32°C compared with control condition (P = 0.008) and no different was found between temperature 28°C and 30°C (P = 0.121) and between 30°C and 32°C (P = 0.118).

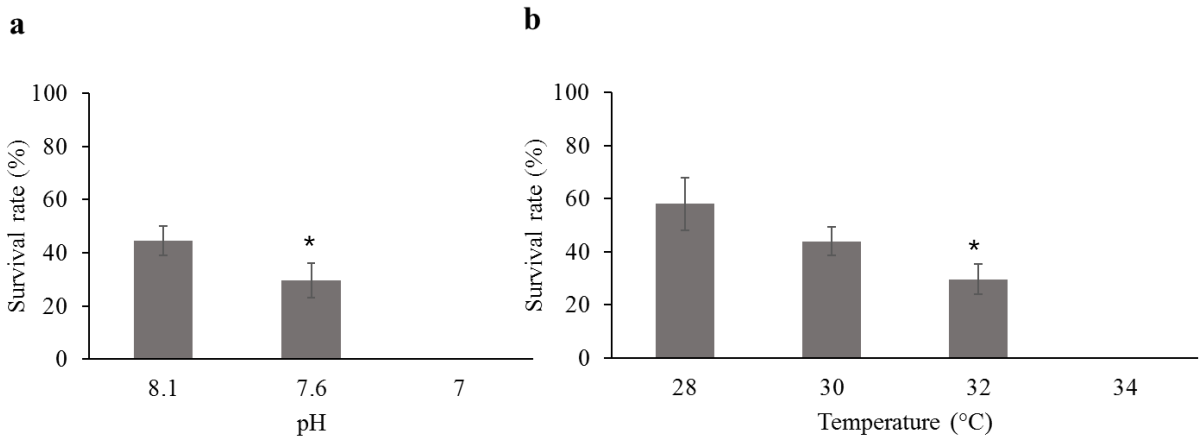


Fig. IV-2 Percentage of survival rate of the giant tiger shrimp *P. monodon* at post larval-15; a = pH experiment, b = temperature experiment; * significant difference from control (pH 8.1 and Temp. 28ºC).

3.3 Growth

The result showed that the mean total length of PL-15 in seawater pH 8.1 was larger than at pH of 7.6 (Table IV-2). T-test showed a significant total length of PL-15 of seawater pH 8.1 which was larger than pH 7.6 ($t_{2,58}$ = -3.665, P = 0.001). For the temperature experiment, the mean total length of PL-15 of temperature 32°C was larger than at 30°C and 28°C (Table IV-2). One way ANOVA analysis showed that the total length of PL-15 was significantly different between treatments ($F_{2,44}$ = 3.872, P = 0.028). Tukey test was showed a significant total length of PL-15 at a temperature 28ºC larger than at 32ºC (P = 0.025), while no difference was found between 28ºC and 30ºC (P = 0.745).

Table IV-2 Mean **t**otal length (mm) of zoea-1, mysis-1 post larvae-1 (PL-1) and post larvae-15 (PL-15) of the giant tiger shrimp in different seawater pH and temperature

Conditions	Zoea 1	Mysis 1	PL1	PL15
Seawater pH				
8.1	0.97 ± 0.05	3.81 ± 0.05	5.52 ± 0.05	12.71 ± 0.29
7.6	0.93 ± 0.05	3.65 ± 0.06	5.55 ± 0.05	11.42 ± 0.21
7.4	0.87 ± 0.01			
Temperature				
28ºC	0.99 ± 0.01	3.62 ± 0.08	5.09 ± 0.11	11.60 ± 0.21
30ºC	0.96 ± 0.10	3.34 ± 0.05	4.93 ± 0.09	11.97 ± 0.52
32ºC	0.95 ± 0.09	3.29 ± 0.04	5.14 ± 0.09	12.81 ± 0.29
34ºC	0.79 ± 0.01			

3.4 Histology

Cross-section image showed that the muscle structure of the giant tiger shrimp larvae was no affected by seawater pH (Fig. IV-4) or temperature (Fig. IV-5). Cross section of muscle fiber areas were significantly decreased with seawater pH reductiion (t_{213}= -3.094, P = 0.002) (Table IV-3, Fig. IV-4). For temperature experiment, one way ANOVA analysis showed that muscle fiber areas were significant difference between three temperature levels ($F_{2,340}$ = 83.48, P = 0.000). Tukey's test showed that muscle fiber areas of temperature 28°C smaller than 30°C or 32°C (P = 0.000), while it was no significant difference between temperature 30°C and 32°C (P = 0.47) (Table IV-3, Fig. IV-5).

Table IV-2 Total length (mm) of zoea-1, mysis-1 post larvae-1 (PL-1) and post larvae-15 (PL-15) of the giant tiger shrimp

Conditions	Muscle fiber areas (μm^2) (Mean ± SD)
Seawater pH	
8.1	0.97 ± 0.05
7.6	0.93 ± 0.05*
Temperature	
28ºC	0.99 ± 0.01
30ºC	0.96 ± 0.10*
32ºC	0.95 ± 0.09*

*significant difference with control

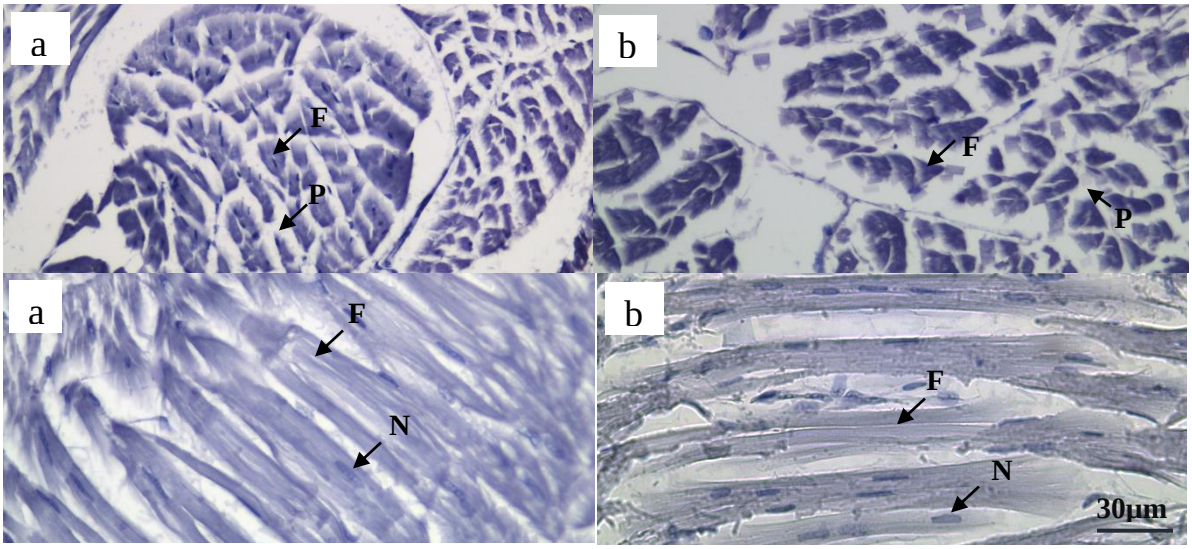


Fig. V-4 Cross section of flexor muscle of giant tiger shrimp using H.E. staining, a) seawater pH 8.1 and b) seawater pH 7.4 (F = muscle fiber, N = Nucleus, P = Perimysium)

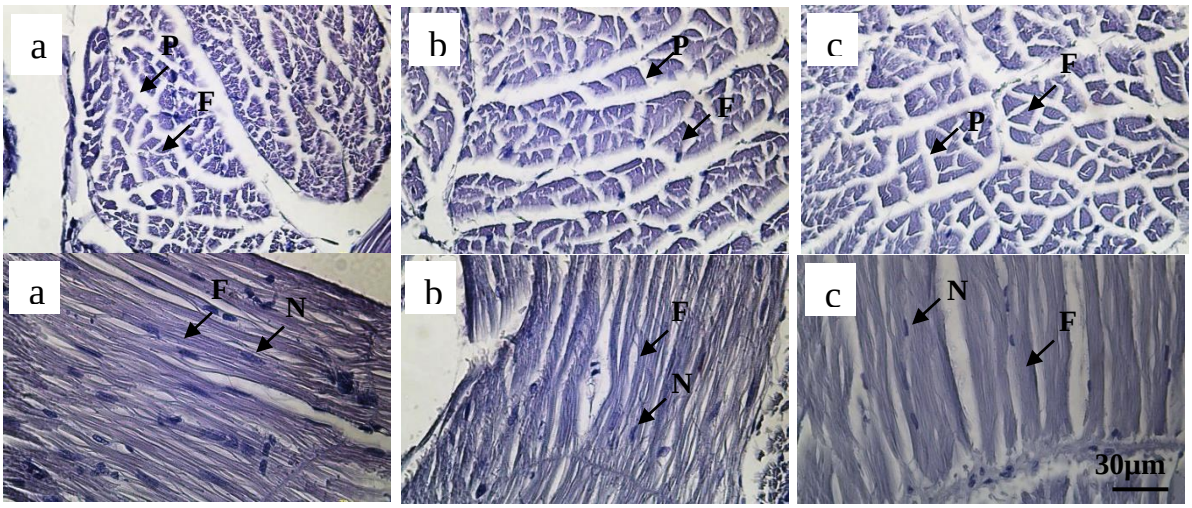


Fig. V-5 Cross section of flexor muscle of the giant tiger shrimp using H.E. staining, a) seawater temperature 28°C, b) seawater temperature 30°C and c) seawater temperature 32°C (F = muscle fiber, N = Nucleus, P = Perimysium

3.5 Swimming speed

Average swimming speed of PL-15 were 2.19 ± 1.32 (mean ± SD, N = 19), 3.47 ± 1.27 (N = 17) and 2.36 ± 1.32 cm/s of seawater temperature 28°C, 30°C and 32°C respectively. One way ANOVA was significantly swimming speed different between treatment ($F_{2,52}$ = 4.992, P = 0.01). Tukey's test was significantly swimming speed of seawater temperature 30°C higher than 28°C and 32°C (P = 0.014 and 0.037 respectively) (Fig. IV-6).

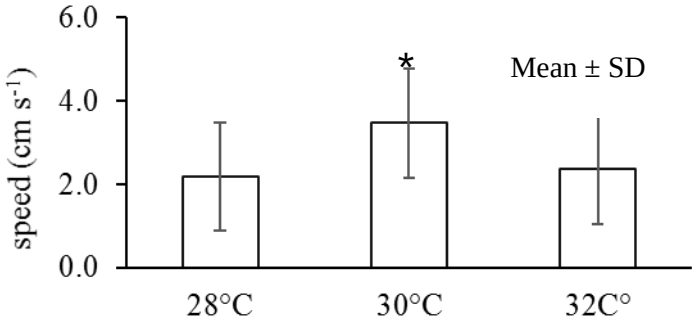


Fig IV-6 Mean **s**wimming speed of giant tiger shrimp PL-15 of seawater temperature 28ºC, 30ºC and 32ºC, * significant difference from control (28ºC)

4. Discussion

4.1 Hatching success, survival and growth

This study was a preliminary observation of the changes on the decreasing pH and the rising temperature of seawater which had effects on three parameters: hatching success, survival, and growth of the giant tiger shrimp. The decreasing pH below 7.6 and the rising temperature above 30°C in seawater were the causes affecting those three parameters as stated above. The experiment also indicated that seawater at pH 7.6 was the critical level for larvae survival and while the best temperature at 30°C was the most suitable for the giant tiger shrimp's growth.

The result of hatching success of this study similar with hatching success of the copepods *Acartia spinicauda* and *Centropages tenuire*, which was decreased when PCO_2 concentration was lower than 2000 µatm (pH 7.3) (Zhang et.al., 2011). Zheng et al. (2015) also found that hatching rate of a brine shrimp (*Artemia sinica*) was decreased after exposure in seawater pH 7.8 and 7.6. In addition, Kawaguchi et al. (2013) stated that CO_2 concentration above 1000 µatm (pH 7.8) would decrease hatching success of the Antarctic krill. However, McConville et al. (2013) indicated that hatching success of the copepods *Centropages typicus* and *Temora longicornis* had no effect when CO_2 concentration was lower than 750 µatm (pH 7.78). The reduction of hatching success of the giant tiger shrimp in acidified seawater has occurred may be because of CO_2 disrupted embryonic development and resulting embryos die before developing to the last stage. Forsgren et al. (2013) stated that the coral reef fish have increased the number of embryonic abnormalities when exposed to PCO_2 at 1400 µatm. Similarly, Kurihara et al. (2004) said that CO_2 concentration over 500 µatm disrupted development of embryos of the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei*. CO_2 also caused slow development of embryos of an amphipod *Echinogammarus marinus* (Egilsdottir et al., 2009).

Survival rate was similar with the study of the marine shrimp, *Palaemon pacificus*, which found survival rate was suppressed when exposed in CO_2 concentration of 1000 µatm and 1900 µatm (Kurihara et al., 2008. However, Cao et al. (2015) reported that survival of a copepods *Tigripus japonicas* decreased at

very low seawater pH 6.5. The study of Kurihara and Ishimatsu (2008) also stated that CO_2 concentration of 2000 µatm has no effect to the survival of a copepod *Acartia tsuensis*. In the same way, CO_2 had no effects on the survival of a European lobster *Homarus gammarus* at concentration of 1200 µatm (Arnold et al., 2009). The result of this study indicated that the giant tiger shrimp larvae have lower tolerance in low seawater pH than another species in the same group. High mortality was found in high CO_2 groups may caused by the CO_2 possibly altered property physiologically and chemically process and neuron system of the giant tiger shrimp larvae. The study in a coral reef fish stated that CO_2 has significantly affected the phototactic response of newly hatched larvae (Forsgren et al., 2013). Phototactic plays an important role in the vertical migration of zooplankton and invertebrate larvae (Johnson and Rhyhe, 2015). Takahashi and Ohno (1996) suggested that the high mortality rate during early stages of the nauplii was due to the difficulty in shifting energy source from yolk-related endogenous food to an exogenous one.

This experiment also showed that an average total length of PL-15 of seawater pH 8.1 (control) larger than pH 7.6. A review paper of Kroeker et al. (2013) indicated that PCO_2 decreased survival, calcification, growth, development and abundance of marine animals. Kurihara et al. (2007) reported CO_2-related disruption of shell formation in the oyster *Crassostrea gigas* at pH 7.4. The giant tiger shrimp larvae grew slowly in high CO_2 concentration, may because CO_2 altered acid-based status in the shrimp body. The decreased of intracellular pH disrupted a number of cell properties (e.g. membrane permeability) and decreased metabolism, which are important in normal development and growth (Roos and Boron, 1981).

Temperature is a powerful factor which always modulates biological activities in the animals. This study found that hatching success and survival was decreased when temperature was above 32ºC, while total length of PL-15 seemed to be low at temperature 34ºC. However, my study differed in the studies of other animals in the same group. This was probably each species had its own temperature optimum. Temperature could induce growth and development of the animals until the thermal limit of each species had been reached (Lee et al., 2003; Handeland et al., 2008). Tropea et al. (2015) had stated that a red cherry shrimp has optimum temperature for growth and survival at 28°C. Best percentage of hatch was obtained at 33°C and 35 ppt (87%) followed by 29°C and 35 ppt (82%). Wyban et al. (1995) addressed that the

temperature optimum of the small size (< 5 g) of a whiteleg shrimp *Litopenaeus vannamei* may be greater than 30ºC, while for the large shrimp, the temperature optimum is about 27ºC. The study in a banana shrimp *Penaeus merguiensis* showed that during nauplius stages, high survival rate was obtained at 33°C and development from zoea to PL-1 was faster at 33°C as compared to 29°C (Zacharia and Kakati, 2004). Thus, the results of this study suggested that the temperature optimum of the giant tiger shrimp is likely to be between 30ºC and 32°C.

4.2 Histology

The cross section of body muscle of the PL-15 showed that muscle structure was not affected by temperature or seawater pH. The result of this study was different from the studies of Nasuchon et al. (2017), which addressed that CO_2 damaged the retractor muscle fibers (muscle atrophy, infiltration of mononuclear and muscle fiber areas decreased) of the sea urchin *Pseudocentrotus depressus* when exposed to CO2 concentration 2000 µatm (pH 7.6) and 10000 µatm (pH 7.0). Chambers et al. (2014) and Frommel et al. (2016) showed subtle histological alterations in skeletal muscle of the fish larvae (*Paralichthys dentatus* and *Thunnus albacares*) under elevated CO_2 conditions (1800 and 4700 µatm, and 2000–9600 µatm, respectively). Wood et al. (2008) also found that the arm muscle mass in a starfish decreased under acidified seawater pH lower than 7.7. At this moment, due to insufficient data, I could not conclude whether CO2 and temperature would have any effect on the giant tiger shrimp.

The result of muscle fiber areas experiment showed that the areas decreased with rising temperature or declining seawater pH. This might have occurred due to the PL-15 size in higher temperatures or seawater pH being larger than in lower temperatures or seawater pH. Willmer et al. (2005) stated that temperature led metabolism rate increased and it also induced protein synthesis in animal. This probably caused shrimps in higher temperature uptake to receive more food than in lower temperature. For CO_2 experiment, elevated CO_2 could decrease feed intake as seen in sea urchins under acclimated CO_2 concentrations of 3000 µatm (Wang et al., 2013). Weinert (2009) reported that nutrition was an important source of protein synthesis.

Thus, bigger shrimps received more food than smaller sized shrimps, causing muscle fibers to become larger.

4.3 Swimming speed

The result of this study showed that swimming speed increased when temperature rose from 28°C to 30°C then the swimming speed decreased at temperature 32ºC. Swimming speed pattern of this study was similar with the study of *Schizothorax prenanti* juvenile, which was found that critical swimming speeds significantly increased between temperature 15 and 23ºC and decreased at temperature 27ºC (Cai et al., 2014). Kent and Ojangoren (2015) studied on the effects of temperature on routine swimming speed of *Poecilia reticulate* with set temperature lower and higher than normal habitat of this species (17, 20, 23, 26, 29 or 32°C). The result showed that swimming speed increased until temperature at 29°C and then was constant. Other studies indicate that temperature affected swimming speed of marine animals. Podolsky and Emlet (1993) found that the swimming velocity of larvae of the sand dollar *Dendraster excentricus* was reduced by about 40% when the temperature was reduced from 22 to 12°C. In the same way, Özbilgin (2002) stated that swimming velocity of *Artemia salina* nauplius, the rotifer *Brachionus plicatilis*, and the copepod *Acartia tonsa* was reduced 37%, 26% and 4% respectively when the temperature was reduced from 22ºC to 10ºC. This information indicated that temperature was an important factor effectively swimming speed of marine animal and each animal has their own critical temperature point, which already explained in the heading 4.1. This could confirm that the optimum temperature of the giant tiger shrimp should be in between 30ºC and 32ºC.

Chapter V

GENERAL DISCUSSION AND FUTURE DIRECTION

1. How ocean warming and acidification would affect muscular system and behavior of marine animals.

This thesis had made a progress on our knowledge of coastal marine animals, specifically on the effects of climate change related to stressors. In this thesis, it was the first study in Japan to consider the effect of ocean acidification and warming on the behavior and muscles of the marine animals. The muscles are essential component of animal behavior such as feeding, reproducing, movement, breathing etc. If seawater temperature or PCO_2 affected muscle systems, it would reduce the fitness of the animals through changes in behavior resulting in animal population's reduction. This experiment showed that the elevated CO_2 damaged the masticatory muscle and reduced the tube foot contraction force of sea urchins, resulting in the changed abundance of eight proteins related to contraction force (Chapter 3).

In addition, the preliminary study of the giant tiger shrimp showed that acidified seawater and warming would affect hatching success, survival and growth of the giant tiger shrimp. The measurement of swimming speed under elevated temperature showed that swimming speed at temperature 30ºC was faster than 28ºC and 32ºC, while there was no difference between 28ºC and 32ºC (Chapter 4). In contrast, neither CO_2 nor temperature significantly had any effect on kinematic parameters of the escape response in the Japanese anchovy, except for the turning rate, which was significant higher at 19°C than 15°C (Chapter 2).

The results of this study also suggested that anchovies have ability to acclimate in future acidification and temperature conditions better than other animals being experimented in this study. Pörtner (2002) pointed out that sessile epifauna species were in general characterized by lower critical temperatures in comparison with mobile species from the same latitude/habitat. Acid-base regulation in fish was generally more developed than in marine invertebrates. The gills function to regulate acid-base balance in fish, with the kidney play supporting role. The gills use cytosolic Carbonic anhydrase that catalyses the hydration of CO_2 to H^+ and HCO_3^- for export to the water. In the kidneys, cytosolic and membrane bound carbonic anhydrase isoforms have been implicated in HCO_3^- reabsorption and urine

acidification (Gilmour & Perry 2009). In the sea urchin, the composition of their coelomic fluid depended on the surrounding seawater (Stumpp et al., 2012). Miles et al. (2007) reported that the compensation of the pH of the coelomic fluid was absent in the sea urchin. Furthermore, gas exchange in sea urchins was very limited due to the lack of respiratory pigment and active respiratory mechanism (see Moulin et al. 2014 for review). Crustaceans extracellular pH-regulation occurs in the posterior gills by electroneutral ion exchange between the extracellular fluids and the surrounding seawater. Although gill has a role in acid-base regulation in shrimp, it was vulnerable to OA because many critical functions depended on their calcified exoskeleton (for example, growth, structure, mineralization and animal cryptic coloration) (Taylor et al. 2015).

Fish seem to robust in CO_2, it is possible that CO_2 alters fish behavior. The gamma-aminobutyric acid (GABAA) receptor was the major inhibitory mechanism in the central nervous system of vertebrate animals, and involved in reducing anxiety in humans, rodents and fish (Succol et al., 2012). The reduction of anxiety alters food intake, social behavior and activity levels in fish (Heuer and Grusell, 2014). Under ocean acidification, the concentration of HCO_3^- becomes elevated in the plasma of fish. This elevation of HCO_3^- in plasma alters the intracellular pH concentration. The cells compensate acid-base status by the exchanging Cl^-/HCO_3^- between extracellular and intracellular regions. This process causes reduction chloride concentration in the intracellular (Heuer and Grusell, 2014). The polarity of GABAA response depended on the intracellular chloride concentration (Succol et al., 2012). The GABAA receptors were found to be distributed throughout the Mauthner cell (Sur et al., 1995). It is therefore possible that increased CO_2 levels may have an intrinsic effect on the sensory performance and neural which control by the Mauthner cells. If elevated CO_2 altered the processing of sensory information, then this would directly influence behavior. The studies on the effect of OA in the past had shown that CO_2 could affect sensory function and behavior in both of marine vertebrates and invertebrates. Fish is only one group is a vertebrate in the ocean. The study in larval stage of coral reefs fish indicated that OA disrupted olfactory discrimination of chemical cues (Munday et al., 2009), visual perception of a predator fish (Ferrari et al., 2012b) which may be related to impaired retinal function (Chung et al., 2014) and auditory ability to discriminate sounds appropriate for settling (Simpson et al., 2011). Pimental et al. (2016) demonstrated that CO_2 concentration of 1400 µatm reduced the swimming

duration and resulted in lesser both attack and capture rates of prey in comparison with the control (350 µatm) of *Sparus aurata* (gilthead seabream) and *Argyrosomus regius* (meagre) larvae, while neither temperature (+4°C) nor interaction between temperature and PCO_2 (+4°C + 1400 µatm) affected the behavior of these fish. However, the study of *Atherina presbyter* (sand smelt fish) larvae behavior showed that PCO_2 of 2000 µatm did not affect routine swimming speed of a sand smelt larvae fish, whereas, it affected group cohesion, which presented a more random distribution when compared with control fish (Lopes et al., 2016). Silva et al. (2016) also found that elevated CO_2 (600 µatm, 1000 µatm and 1800 µatm) had no significant effect on swimming speed of *A. presbyter* larvae. The study in the sea bass larvae (*Dicentrarchus labrax*) showed that sea bass was resilience to future CO_2 concentration (Duteil et al., 2016). However, the results of the previous studies were very various which were depended on species. To understand the effects of OW and OA on muscular and behavior of fish, it may need to examine the integration between sensory system and nervous system for future study.

For marine invertebrates, it has no reported on how OA affects sensory system and nervous system concerned to behavior of this group. However, there were some studies indicated that elevated CO_2 altered acid-base status of invertebrates. Michaelidis et al. (2005) addressed that the levels of Ca^{2+} in the haemolymph and HCO_3^- in extracellular of adult mussels *Mytilus galloprovincialis* increased under elevated CO_2 conditions. In sea urchin, it has been reported that the concentration of Ca^{2+} and Mg^{2+} in the coelomic fluid was altered after exposure in elevated CO_2 (Spicer et al, 2011; Kurihara et al., 2013; Wang et al., 2013). Ca^{2+} and Mg^{2+} are involved in muscle contraction. Muscles are essential component of animal behavior such as feeding, reproducing, movement, breathing etc. Muscle contraction occurs when Ca^{2+} is released from the sarcoplasmic reticulum and binds on troponin on actin. When triggering was over, the concentration of Ca^{2+} became high, and then the Mg^{2+} was fluxed into the cell and binded on troponin that pushed the Ca^{2+} back to the sarcoplasmic reticulum (Potter and Gregely, 1975). The previous study had found that CO_2 significantly reduced the locomotion speed of the sea urchin, *Hemicentrotus pulcherrimus*, reared under 1000 µatm PCO_2 both at ambient and at an elevated temperature (+ 2°C) for seven months (Yin, Lee, Kurihara and Ishimatsu, in preparation). Schalkhausser et al. (2013) found significant declines in the force generated by the adductor muscle in the king scallop, *Pecten maximanus*, reared under PCO_2 of 1120 µatm at 10°C. Moreover, muscle fitness

was one factor that affected animal behavior. Wood et al. (2008) reported that the muscle mass in the arms of the brittlestar, *Amphiura filiformis*, decreased in lowered pH (7.7, 7.3 and 6.8) but structure in muscles did not change. Chapter III-A of this study found that feed intake lower in 10000 µatm. Wang et al. (2013) also found feed intake in sea urchin decreased under acclimation to CO_2 concentration 3000 µatm. It is possible that elevated CO_2 disrupt protein synthesis. On the other hand, protein had contributed energy during periods of long term starvation (Berg et al., 2002). Thus, elevated CO_2 probably could affect behavior of marine invertebrates.

Proteins were the most versatile macromolecules in living systems and serve crucial functions in essentially all biological processes. They were molecules made of amino acids and most abundant in the body. They functioned as catalysts, provide mechanical support and immune protection, generate movement, transmit nerve impulses, and control growth and differentiation. Indeed, the functions of individual proteins had varied due to their unique amino acid sequences and complex three-dimensional physical structures (Nelson and Cox, 1993). Thus, the study of proteomics had become the powerful way to investigate the response of environment on marine animals. Presently, the study on the effects of OA pay more attention on protein expression to interpret the response of marine animal to OA. For example, Wei et al. (2015) stated that PCO_2 affect energy and primary metabolisms, stressed response and calcium homeostasis of *Crassostrea gigas*, Dineshram et al. (2013) found that proteins involved in larval energy metabolism and calcification appeared to be down-regulated in response to low pH of *C. hongkongensis*. Tomanek et al. (2011) reported that 12% of protein (54 out of 456) in mantle tissue was different expression under elevated CO_2 compared with control condition. The results of this study in chapter III-B also found that muscle contraction force reduced in elevated CO_2 groups with eight proteins were changed in protein volume and four proteins involved in muscle contraction.

Therefore, marine vertebrate and/or invertebrates possibly were affected from OA. Fish seemed robust from ocean warming and acidification more than invertebrates. Generally, fish are related to other animals in the ecosystem food web that stay in the middle or top level of the ecosystem food web supported by lower levels (plankton and larvae and juvenile stage of aquatic animals) in the ecosystem. A removal or reduction of species in the food web will affect the balancing between prey and predator in the food web, particularly, a keystone of the ecosystem. The keystone species' disappearance can

affect other species that rely on it for survival. The studies on ocean acidification in the past 20 years indicated that ocean acidification had negative effects on early development, growth, metabolism, photosynthesis, and survival of marine organisms, particularly invertebrates (Kroeker et al., 2013). This can be interpreted that marine animal species vulnerable to CO_2 may have high mortality resulting in reduction of their population.

2. Future studies

Seafood is a critically important source of protein for global consumption. In 2014 around 167.2 million tonnes was consumed globally. Thailand is the fourth largest of the world's fishery exporters which is worth US$ 6565 million. For fisheries production, Thailand is ranked 11th in the world. Thailand produced about 2493 thousand tonnes of fish in 2014, with the major species being shrimp, mackerel, clam and anchovy (FAO, 2016: Department of Fisheries, 2016). Thailand is located in between two oceans and has a coastline of approximately 3219 km (ChartsBin, accessed 20 November 2016). The geology of Thailand makes the country susceptible to effects of El Niño. El Niño causes seawater surface temperature to be higher than average as seen in 1998 and 2010 (McPhaden, 2003). Average seawater temperature recorded in the inner Gulf of Thailand by data logger on 8 April 2010 was 31.06 ± 0.23ºC and it continued to rise to the maximum on 10 May 2010 (32.70 ± 0.31ºC) (GCRMN, 2010). This phenomenon caused severe coral bleaching in the Gulf of Thailand in 1998 and in the Andaman sea in 2010 (GCRMN, 2010). In addition, ocean warming also caused massive mortality of green mussel in Trang Province, which found about 800 tonnes of (Manager newspaper, accessed 2 February 2017). It has been reported that the mortality of green mussel was occurred by temperature induces a flat worm outbreak in the coastal zone (PEMSEA, accessed 21 November 2016). At the present time, study in Thailand on the effects of global climate change has only focused on ocean warming. There has not been any study conducted on the effects of ocean acidification on marine animals.

I work with the Marine Fisheries Research and Development Division, Department of Fisheries in Thailand. It is my division's role to achieve sustainable utilization of fisheries resources using marine

resource management. It is recommended from this study that the impacts of OW and OA are studied by conducting an experiment to ensure that marine resources are used sustainably. It is my intention to work with my colleagues on this issue, focusing on major species of the fisheries either from capture or culturing. Shrimp is major production species of Thailand, producing about 320 thousand tonnes in 2014, which most of comes from aquaculture. Thailand exports frozen shrimp around the world. The major importers of frozen shrimp are United States (26,801 tonnes; 10,085,981 US$ billion), Japan (13,324 tonnes; 5,189,986 US$ billion) and Canada (4,842 tonnes; 1,889,829 US$ billion) (Department of Fisheries, 2016). In addition, I found that ocean warming and acidification could affect the early stage of the giant tiger shrimp from my study in Vietnam, and therefore giant tiger shrimp should be a priority species for additional experimentation on the combination effects of ocean warming and acidification in Thailand. In addition, Indo-Pacific mackerel should be another important species for additional study. It is the most important commercial species in the Gulf of Thailand, and the fish stock is shared among countries around the Gulf of Thailand (Vietnam, Cambodia, Thailand and Malaysia). It is ranked first in Thailand's marine production and about 203 thousand tonnes were caught in 2014.

Most studies on the effects of OA in the past 20 years employed the experiment of a short period. Dupont et al. (2013) reported that sea urchins could acclimated after expose to CO_2 concentration for 16 months. It is indicated that some animals may have ability acclimate to ocean acidification. Thus, I plan to perform the study of the effects of OW and OA in those species for long period.

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Appendix A. List of Papers

Published papers

Nasuchon N., Yagi M., Kawabata Y., Kunshan G., Ishimatsu A. 2016. Escape response of Japanese anchovy *Engraulis japonicus* under elevated temperature and CO_2 conditions. Fisheries Science 82(3): 435-444.

Nasuchon N., Hirasaka K., Yamaguchi K., Okada J., Ishimatsu A. 2017. Effects of elevated CO_2 on muscle contraction and proteome composition of sea urchin tube feet. Comparative Biochemistry and Physiology, Part D 21: 10-16.

Appendix B. Curriculum vitae

NOPPARAT NASUCHON

Nationality: Thai

Date of Birth: October 28, 1973

Place of Birth: Udorn Thani Province, Thailand

Education:

Ph.D. in Marine Science (5-year doctoral course)

Nagasaki University, Graduate School of Fisheries and Environmental Science (Graduated March 2017), Nagasaki, Japan

Dissertation “The impacts of ocean warming and acidification on behavior and muscular system of the marine animals”

Bachelor of Science (Fisheries management) (Graduated March 1995)

Kasetsert University

Bangkok, Thailand

High School (Graduated March 1991)

Mooddindeang Demonstration School, Khonkean University

Khonkean, Thailand

Elementary School (Graduated March 1985)

Seangsawang Elementary School

Udorn Thani Province, Thailand

Employment:

Fisheries Biologist (June 1996 – present)

Department of Fisheries, Thailand

Regional Fisheries Policy Network (January – December 2011)

Southeast Asia Fisheries Development Center

General secretarial office, Bangkok, Thailand

List of papers and articles:

Nasuchon N., Nakrobrue J., Rochanaratana T.,1999. Reproductive biology of goldstripe sardinella (*Sardinella gibosa* (Beeker, 1849)) in the middle Gulf of Thailand (Prachuab Kirikhan, Chumphon and Surat-Thani Provinces) Technical paper No. 1/1999. Department of Fisheries, Thailand. In Thai

Puntuleng P., **Nasuchon N**. 2005. Reproductive biology of Indo-Pacific mackerel enclicling gill net in conservation area Prachuab Kirikhan, Chumphon and Surat-Thani Provinces. Technical paper No. 18/2005. Department of Fisheries, Thailand. 40 p. In Thai

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Nasuchon N., Suppanirun T., Bunluedaj C. 2007. Fishermen's attitude on closed season measure in Prachuap Khirikjan, Chumphon and Surat-Thani provinces. Technical paper No. 3/2007. Department of Fisheries. 28 p. In Thai

Phuttharaksa K. **Nasuchon N**., Kongchai T., Pinputtasin J. 2008. Reproductive Biology of Bigeye Scad (*Selar crumenopthalmus* (Bloch, 1793)) in the Gulf of Thailand. Technical paper No. 16/2008. Department of Fisheries. 22 p. In Thai

Nasuchon N. Phuttharaksa K., Srithakon T., Hussadee P. 2010. Reproductive biology of goldstripe sardinella (*Sardinella gibosa* (Beeker, 1849)) in the Gulf of Thailand. Technical paper No. 16/2010. Department of Fisheries. 28 p. In Thai

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Nasuchon N. 2009. Coastal management and community management in Malaysia, Vietnam, Cambodia and Thailand with a case study of Thai fisheries management. United Nation, New York. 91 p.

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Amadi, Mohamed H., Houng N.T.T., **Nasuchon N**., Oo A.N., Phomsouvanh A., Sitha H., Velasco P.E.L. 2011. Fisheries human resource: Gaps and requirement of Southeast Asia. Fish for the people 9(2): 115-125

Prompoj W., Songsangjinda P., **Nasuchon N**. 2011. Benchmarking of Thai national shrimp certification scheme against the FAO aquaculture certification guidelines. Fish for the People 9(3): 20-38

Saikliang P., **Nasuchon N**., Torell M. 2012. Port state measures and port monitoring in Southeast Asia. Fish for the People 10(1): 13-19

Nasuchon N., Yagi M., Kawabata Y., Kunshan G., Ishimatsu A. 2016. Escape response of Japanese anchovy Engraulis japonicus under elevated temperature and CO_2 conditions. Fisheries Science 82(3): 435-444. doi 10.1007/s12562-016-0974-z

Nasuchon N., Hirasaka K., Yamaguchi K., Okada J., Ishimatsu A. 2017. Effects of elevated carbon dioxide on contraction force and proteome composition of sea urchin tube feet. Comparative Biochemistry and Physiology : part D 21: 10-16.

Poster presentation

Nopparat Nasuchon, Jiro Okada, Kenichi Yamaguchi, Katsuya Hirasaka, Ryosuke Ono, Shohei Noma and Atsushi Ishimatsu. 2014. The effects of elevated CO_2 on the feeding muscular system of the red sea urchin *Pseudocentrotus depressus*. In "Human impacts on oceanic environment, ecosystem and fisheries symposium", 11-13 November 2014, Nagasaki, Japan

Do Van Buoc, Nopparat Nasuchon, Tran Minh Phu, Chau Tai, Tao, Tran Ngoc Hai, Nguyen Thanh Phuong, Atsushi Ishimatsu and Do Thi Thanh Huong. 2016. Development of embryo, larvae and post-larvae of black tiger shrimp (*Penaeus monodon*) rearing at different temperature. In the "International Fisheries Symposium – IFS2016, Phu Quoc Island, Vietnam, October 31-November 02, 2016.

Meetings and Symposiums attended:

- The United Nation – the Nippon Foundation of Japan fellowship programme: Asia – Pacific alumni meeting, 13-15 April 2009, Tokyo, Japan
- Fourth International Fishing Industry safety and health conference, 11-14 May 2009, Reykjavik, Iceland
- Inception workshop on bycatch management and reduction of discards in trawl fisheries, 3-6 November 2009, Samut Prakan, Thailand
- 43rd Meeting of the council of the Southeast GIANT Fisheries Development Center, 4-8 April 2011, Malacca, Malaysia
- ASEAN-SEAFDEC conference on sustainable fisheries food security towards 2020 "Fish for the People 2020: Adaptation to a changing environment, 13-17 June 2011, Bangkok, Thailand
- Inception workshop on follow up activities to the ASEAN-SEAFDEC conference on sustainable fisheries food security towards 2020, 4-6 July 2011, Bangkok, Thailand
- Special meeting on shark information collection in Southeast Asia, 15-17 September 2011, Bangkok, Thailand
- The 3rd Meeting of the Gulf Thailand Sub-region, 20-22 September 2011, Seam Reap, Cambodia

- The Japanese Society Fisheries Science symposium, 11-13 September 2013, Mie, Japan
- Human impacts on oceanic environment, ecosystem and fisheries symposium, 11-13 November 2014, Nagasaki, Japan
- The United Nation – the Nippon Foundation of Japan fellowship programme global meeting, 28 November – 3 December 2014, Tokyo, Japan
- The 7th world fisheries congress, 23 -27 May 2016, Busan, Korea
- The United Nation – the Nippon Foundation of Japan 2016 alumni meeting on Maritime Southeast Asia and South Asia: Mapping opportunities and challenges, 28 Nov. – 1 Dec 2016, Bali, Indonesia