GROWTH, BIOCHEMICAL, AND PHYSIOLOGICAL RESPONSES OF THE MUD CRAB *SCYLLA SERRATA* TO CO_2 DRIVEN OCEAN ACIDIFICATION

Thesis Submitted to Bharathiar University, Coimbatore, India

For the Award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Submitted by

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SAID HAMID THANGAL P

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1. GENERAL INTRODUCTION

1.1. Marine

The ocean is a massive salt body covering nearly 71% of the earth's surface and shares 97% of the total earth's water, which determines the climate and ecology. The diversity of the marine environment provides several ecological importance, such as balancing climates and providing vital foods in terms of seafood, medicines, employment and so on. Due to the greenhouse effect, the Ocean absorbs more than 90% of the excess heat trapped in the earth's atmosphere. This helps moderate the earth's temperature and prevent it from becoming too hot or cold. The ocean also helps distribute this heat worldwide through ocean currents. Ocean absorbs about 25% of the carbon dioxide released into the atmosphere and helps slow down the rate of climate change. The ocean is a major component of the earth's water cycle, which regulates the amount of water available for precipitation and evaporation. The biogeochemical cycle of the ocean regulates the movement of chemicals and nutrients between biotic and abiotic factors in the environment. Nearly 138 million km^2 are the exclusive economic zone (EEZ) of the world ocean, with an average harvestable potential of 115.2 million tons of total capture and culture fish (FAO, 2020). Moreover, India has an 8129 km^2 coastal line and an average of 2.02 million km^2 EEZ. The marine environment provides the most important ecological services and supports species' existence (Dixon, 2020). At least half of the oxygen was supplied by the ocean environment due to the presence of phytoplankton, macro algae, sea grass mangroves, etc. (NOAA, 2003). The ocean is the main source of food and nutrients for billions of people worldwide, providing nutritional security and employment opportunities.

1.2. Ecological and economic importance of ocean

The diversity of marine and land organisms differs in many ways due to differences in their physical environments. It is estimated that more than 250,000 known species have been identified so far, and at least two third of the marine species still need to identify (Palmer, 2017). However, scientists estimate that the ocean contains a wide range of organisms like a virus, bacteria, fungi, phytoplankton, zooplankton, macro algae, sponges, worms, corals, molluscs, crustaceans, fishes, mammals etc., which are being played

a critical role in ecosystem functioning such as nutrient cycle, carbon cycle, regulation of climate change, migration, etc. Besides, the marine ecosystem provides humankind with an excellent source of food, medicine, and chemicals (Goulletquer et al., 2014). Fisheries production and fishing play a critical role in the global economy, providing employment, food and income to millions worldwide. Fish and other seafood are important sources of protein and essential nutrients that provide livelihoods for millions of people, particularly in developing countries where they are a vital source of income and food security. As per the report of FAO (2022), the world's average harvestable potential of captured and cultured fish is 115.2 million tons in total. It is estimated that above three million species live in the ocean, and the coastal zone provides massive resources to humans, including nutritional safety and employment to the socioeconomic status of humans (Dixon, 2022). According to the latest data from the FAO (2020), the United Nations, the global fisheries and aquaculture sector produced a record 179 million tons of fish in 2018, with an estimated value of 401 billion USD, with maximum production by China followed by Indonesia, India, Vietnam, and Philippines.

1.3. Issues with the ocean environment

Although the marine environment offers enormous resources to humans, it has serious threats in terms of pollution. Currently, the marine environment is polluted by various anthropogenic releases of plastics, industrial chemicals, sewage wastes, pesticides, and heavy metals (Chitrakar et al., 2019), which harms the marine environment (Bashir et al., 2020). Plastic pollution is one of the emerging contaminants among the pollutants in terms of micro and nanoplastics. It become a major threat to the earth due to the mismanagement and poor recycling of plastics (Anandhan et al., 2022). The sewage contains harmful chemicals and microbes that negatively affect the health of an ecosystem (Wear et al., 2021). The excess usage of harmful pesticides is a reason to alter the physicochemical properties of freshwater and seawater, negatively affecting aquatic animals (Agarwal et al., 2010). The interspace between the atmosphere and the seawater is a vibrant borderline of the earth, which control the inter changes of materials that stimulus the chemistry of the climate and atmosphere and the transfer of necessary components to the organism’s health. The earth system is facing extraordinary deviations in worldwide biogeochemical and physical processes caused by anthropogenic emissions

of greenhouse gases (Ciais et al., 2014). In addition, an excess of greenhouse gases in the atmosphere can disturb the earth's carbon cycle and create global warming, directly affecting the weather, climate, ecosystems, and economy. The specific effect of greenhouse gases is predicted to become more severe and has massive consequences for the world (Collins et al., 2013).

1.4. Greenhouse gases

Greenhouse gases trap heat in the earth's atmosphere. The major greenhouse gases are carbon dioxide, methane, nitrous oxide, fluorinated gases (hydrofluorocarbons and perfluorocarbons), and water vapour. When the levels of greenhouse gases exceed a certain threshold, it leads to several issues, including climate change, global warming, ocean acidification, air pollution, and economic impact. According to the global carbon budget report 2021, the top tent greenhouse gas emitting countries are China (28.3%), Unites states (14.5%), India (6.6%), Russia (4.9%), Japan (3.6%), Germany (2.4%), Iran (2.2%), South Korea (1.8%), Canada (1.5%) and Saudi Arabia (1.5%) (GCP, 2021). CO_2 is one of the major greenhouse gases, with 60%, and the burning of fossil fuels and deforestation are significant sources of CO_2 in the atmosphere (IPCC, 2014). Global CO_2 emissions increased from 34.1 gigatons (GT) in 2010 to 37.9 GT in 2019 (EU JRC, 2020). China deserves first place for the contribution of CO_2 emission (11680.42 MT), followed by the United States (4535.30 MT), India (2411.73 MT), and Russia (1674.23 MT) (EUJRC, 2020). India's CO_2 emissions have steadily increased over the past few decades, primarily due to its rapid economic and population growth and increasing energy consumption. In 2019, India's CO_2 emissions were estimated to be around 2.4 billion tonnes, an increase of 5.2% from the previous year (Friedlingstein et al., 2021). The elevated CO_2 levels increase beyond a certain threshold. It can cause many issues in both the land and the ocean environments. Climate change is one of the main effects of excess carbon dioxide emissions, which can lead to increased temperatures and other harmful environmental impacts. Moreover, high levels of CO_2 can also contribute to air pollution, like poor air quality and respiratory problems in animals.

1.5. Ocean acidification

Among the variety of pollutants, CO_2 has a significant stand in marine pollution in terms of ocean acidification (OA), which changes seawater property and affect the biology and physiology of marine animals, followed by detrimental variations in food webs (Halpern et al., 2008; Brown et al., 2010; Hoegh-Guldberg, 2011). The oceans can absorb 30% of carbon dioxide emissions (Gruber et al., 2019). The atmospheric CO_2 enters the ocean by air-to-sea equilibration and is circulated by ocean movement (Abas and Khan, 2014). When CO_2 is absorbed into seawater, it reacts with water molecules to form carbonic acid (H_2CO_3) ($CO_2 + H_2O \rightarrow H_2CO_3$), which releases hydrogen ions (H^+) like all acid can make the ocean more acidic (Doney et al., 2009). The released hydrogen ions from the carbonic acid can react with other ions in seawater, such as carbonates (CO_3^{2-}) and bicarbonates (HCO_3^-). As the concentration of hydrogen ions increases, the concentration of carbonate ions decreases, making it more difficult for marine organisms to form their shells or skeletons (Fig.. 1). This increase in acidity and scarcity of carbonates ions can have negative impacts on survival, growth, physiology, immunology, and reproduction of marine organisms such as plankton, corals, molluscs, crustaceans, etc., that are rely on calcium carbonate to build their shells or skeletons (Hofmann et al., 2010; Long et al., 2013; Pansch et al., 2018; Turra et al., 2019; Zlatkin and Heuer, 2019) followed by the significant adverse impact on the food chain and ecosystem balancing. As per the forecasting of the Intergovernmental Panel on Climate Change (IPCC), the pH of seawater could be decreased to 7.8 or beyond by 2100 (IPCC, 2007). The excess emission of CO_2 might be an average decrease in seawater pH will become 0.2 to 0.4 units at the end of the 21st century (Hoegh-Guldberg, 2014).

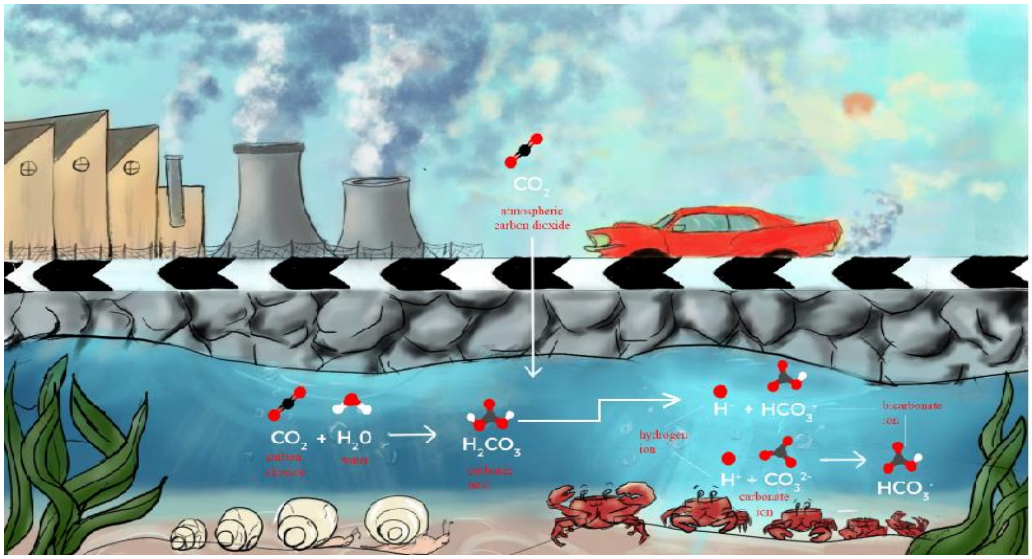


Figure 1: Ocean acidification and its impact on marine calcifiers (Thangal et al., 2022).

1.6. OA in the natural environment

The present-day average ocean pH is nearly 8.1. However, the average pH of the ocean was 8.2 before the industrial revolution. Decreasing one pH unit equals a ten-fold increase in ocean acidity, meaning the average acidity of seawater today is around 25% higher than in preindustrial times (USEPA, 2022). According to the report of the Air Pollution and Climate Secretariat (APCS, 2012), the Caribbean Sea was found to be the largest drop in seawater pH (3%) compared to the pre-industrial years. Also, calcification decreased by about 15% over the past century. Based on the APCS report (2012), the western Pacific Ocean calcification rate gradually decreased in past years. The report of NOAA (2015) predicted that the Pacific Northwest, Narragansett Bay, Long Island Sound, Gulf of Mexico, Chesapeake Bay and Massachusetts are the hotspot of the US shellfish industry, had a loss of one billion dollar loss in shellfish capturing due to the ocean acidification. The weakening of coral structures in the Caribbean, Scotland and Norway seas was observed earlier (NOAA, 2015). Moreover, reports revealed that the aragonite saturation due to OA was observed in the Indian Ocean at 240 and 300 meters in the Bay of Bengal and Andaman Nicobar Island, respectively (Sarma and Narvekar, 2001; Sarma et al., 2002). The calcite saturation was observed at 3900 to 4000 meters in the entire Northern Indian Ocean and 3000 to 3400 meters in Oman margins (Berger, 1978; Millero et al., 1998; Mintrop et al., 1999). Fig..2 shows the OA at the global level.

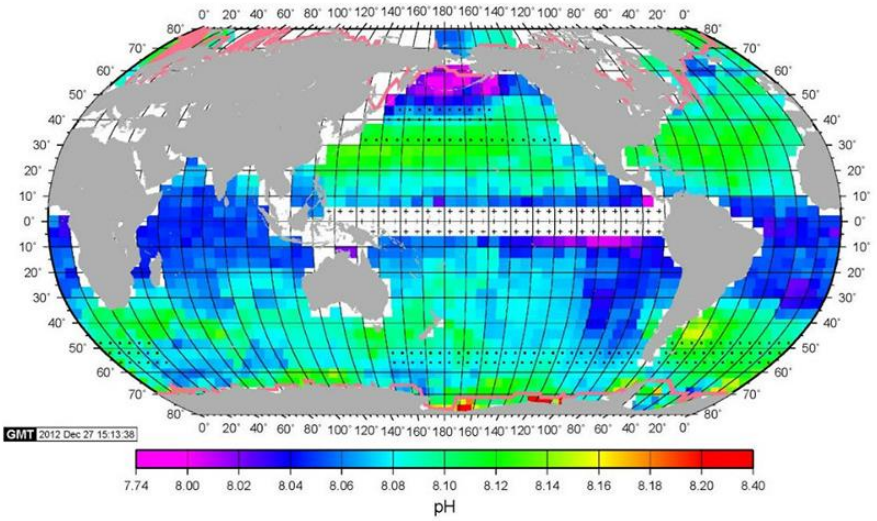


Figure 2: OA at the global level (Source: Columbia climate school (EPA 2015)

1.7. Impact of OA on marine organism

Change in seawater chemistry affects marine organisms in three different ways (1) decreased carbonate ion concentration that decreases the calcification process in marine organisms, (2) Influences the acid-base regulation, (3) High level of CO_2 alters the ability of primary photosynthesis producers (Logan 2010). In phytoplankton, OA increases the toxic phenolic compounds that could be transferred to higher tropic-level organisms (Jin et al., 2015). Several studies were conducted to analyze the effects of OA on larval, juvenile and adult invertebrates (Kurihara et al., 2008; Bierbower and Cooper, 2010; Carter et al., 2013). Increased CO_2-charged waters can lead to a transient increase in surface pCO_2 sufficient to cause widespread physiological shock, climatic shock, or both, resulting in mass mortality of marine animals (Knoll et al., 1996). Elevated internal CO_2 concentrations also contribute to physiological mechanisms that trigger metabolic slowing or arrest. Studies from Ries et al. (2009) show that some marine organisms like crabs, shrimp, lobster, limpets, sea urchins, etc., in which the net calcification process decreases with increasing pCO_2. In this context, animal species like blue mussels, pencil urchins, hard calm, conch, serpulid worm, periwinkle, bay scallop, oyster, whelk, and soft clam showed increasing calcification with increasing pCO_2 levels. Dissanayake and Ishimatsu (2011) found a synergistic effect of elevated CO_2 concentrations and

temperature, which can impair penaeids' breathing and swimming capacities. Organisms that produce $CaCO_3$ skeletons are particularly sensitive to hypercapnia because carbonate bio mineralization requires precise control of the acid-base balance. At high partial pressures, CO_2 binds directly with respiratory pigments, decreasing their ability to carry oxygen. Studies reported that the projected future rises of seawater pCO_2 and accompanying reductions of pH would lead to decreases in the $CaCO_3$ saturation state (Feely et al., 2004) to the extent that several marine calcifying organisms suffer from a reduction of calcification rate and an increase in $CaCO_3$ dissolution rate (Riebesell et al., 2000; Orr et al., 2005; Gazeau et al., 2007). Therefore, calcifying marine organisms are probably one of the earliest organisms to be impacted by the OA due to ever-increasing atmospheric CO_2 levels (Kurihara and Shirayama, 2004). Recently researchers identified that CO_2-driven ocean acidification negatively impacted coral reefs (Mongin et al., 2021). The declining ocean pH due to the CO_2 causes the decline of calcification and physiology of the sea urchin *Salmacis virgulata* (Anand et al., 2021). It is harder to form calcium carbonate shells and skeletons for some marine organisms, such as pteropods, corals, and oysters (Hoegh-Guldberg et al., 2017; Lemasson et al., 2017). Species like coccolithophores, crabs, sea urchins etc., are affected by the OA (Baumann et al., 2011; Dodd et al., 2015; Campbell et al., 2016; Tasoff and Johnson, 2019).

OA, along with other stressors such as temperature, hypoxia, heavy metals, salinity, and micro plastics, can have harmful impacts on the biology and physiology of marine animals, including fishes (Lopes et al., 2018), sea urchin (Zhan et al., 2020), bivalves (Shi et al., 2016), and crabs (Turra et al., 2019). The combined effects of ocean acidification (pH 7.3) and ocean warming (at temperatures of 18 and 25 °C) significantly influenced the foraging behaviour of the Japanese stone crab, *Charybdis japonica* (Wu et al., 2017). The effects of low pH and temperature negatively affect the survival, growth, and moulting of crabs *Acanthocyclus hassleri* and *Maguimithrax spinosissimus* (Swiney et al., 2017; Manríquez et al., 2020; Gravinese et al., 2022). The blue crab *Callinectes sapidus* exposed to low pH (7.16) along with low dissolved oxygen (3.74 mg L^{-1}) showed a significant decrease in survival was observed earlier (Tomasetti et al., 2018). A notable decrease in the levels of carbohydrates and calcium in the muscle of *A. hassleri* when exposed to high pCO_2 levels (1400 μatm) at the temperature of 20°C has been

reported (Manríquez et al., 2020). The Chinese mitten crab *Eriocheir sinensis* exposed to low pH levels (7.8, 7.3, and 6.5) along with the heavy metal cadmium (1 mg L^{-1}) showed an increased level of antioxidants like SOD and CAT has been reported previously (Zhao et al., 2021).

1.8. Marine crustaceans

More than 52,000 crustacean species have been identified, which includes crabs, shrimps, barnacles, lobster, brine shrimps, etc. Crustaceans are in high demand in the world aquaculture trade due to their rich nutrients, delicious taste and high market value. Marine crustaceans contributed 9.4 million tons (69.3 billion USD) to the world aquaculture and fisheries economy (FAO, 2020). Nevertheless, crustaceans are highly sensitive to biotic and abiotic (pH, temperature, dissolved oxygen, etc.) factors. Hence, they are used as bio-indicators (El-Kahawy et al., 2021). Moreover, crustaceans are vulnerable changing in ocean carbon chemistry (Whitely, 2011). Hence, the modifications in the carbon chemistry in the surrounding environment can produce fluctuations in the regulation of acid-base balance, moulting, calcification and immunity which cause poor survival and easily vulnerable to several biotic and abiotic factors (Roleda et al., 2012; Taylor et al., 2015, Rehman *et al*., 2021). In addition, OA disrupts the marine ecosystem function and seafood productivity, threatening various nations' nutritional security (Branch et al., 2012). In recent years, researchers proved that CO_2-driven OA harms the physiological and biological activities of marine crustaceans like crabs (McElhany et al., 2022), shrimps (Muralisankar et al., 2021), lobsters (Styf et al., 2013) brine shrimps (Gao et al., 2017).

1.9. Mud Crab *Scylla serrata*

The mud crab *S. serrata* is an Indo-Pacific species with high nutrients, minerals, and market values (Keenan et al., 1998; Paterson, 2011). They are short-living and fast-growing animals and biphasic. The mud crabs are also called mangrove crabs and giant crabs. *S. serrata* is used as a bio-indicators to monitor environmental changes (Flint et al., 2021). *S. serrata* has a diverse habitat range and is commonly found in coastal areas, particularly in mangrove forests, estuaries, tidal flats and other brackish water environments. Mud crabs are strongly associated with mangrove ecosystems,

where they can be found in the intertidal zones among the roots of mangrove trees. These habitats provide shelter, food, and protection for the crabs. The habit of the *S. serrata* species can be observed in shallow water and predominantly in mangrove areas and estuaries (Nurdiani and Zeng, 2007). They are opportunistic omnivores, feeding on various plant and animal matter, including detritus, algae, molluscs, and small fish. Moreover, *S. serrata* has an outstanding nutritional index than other invertebrates, which has high economic value and feasible seafood in the international market (FAO, 2020; Bhuiyan et al., 2022). The mud crabs are being cultured in many Asian countries such as Malaysia, Indonesia, the Philippines, Taiwan, Sri Lanka, Vietnam, India, and China (Azra and Ikhwanuddin, 2016). Southeast Asian nations such as Vietnam, the Philippines and Indonesia are the major mud crab production country in the world, with an average production of 65,463, 18,100, and 15,000 tons, respectively (Yxtung, 2020). The taxonomic position of the *S. serrata* is as follows

↪ Kingdom : Animalia

↪ Phylum : Arthropoda

↪ Sub Phylum: Crustacea

↪ Class : Malocostraca

↪ Order : Decapoda

↪ Infra Order: Branchyura

↪ Family : Portunudae

↪ Genus : *Scylla*

↪ Species : *serrata*

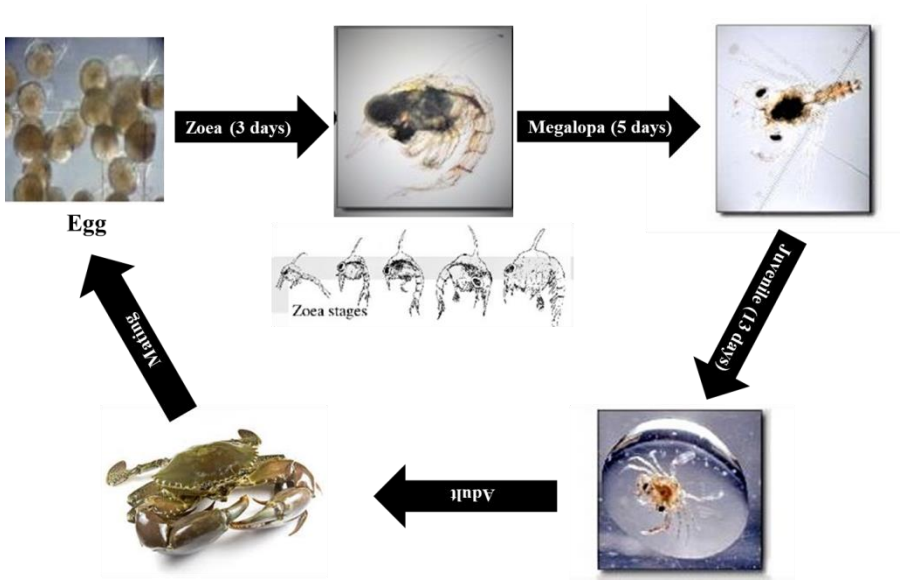


Figure 3: Lifecycle of *S. serrata*

The male and female identification of *S. serrata* is based on their external appearance. (1). Abdomen shape: Male crabs tend to have a narrow, triangular-shaped abdomen, while females have a broader, more rounded abdomen. This difference is much more noticeable in mature individuals. (2). Abdomen flap: Female mud crabs have a distinct flap-like structure on the abdomen known as the "apron" or "abdominal flap." The apron covers the abdomen's underside and is absent in males. (3). Claws: Male mud crabs usually have larger and more elongated claws than females. These claws are used for Fig.hting and courtship displays. (4). Coloration: The colour of *S. serrata* is a mixture of light brown and green. However, this characteristic can vary depending on habitat, age, and individual variation (Heasman, 1980; Fazhan et al., 2017). The mating period will happen mid-spring–early autumn (Heasman et al. 1980). Mud crabs will start to migrate themselves to oceanic waters for spawning purposes. During the breeding season, *S. serrata* produces a relatively large number of eggs. The exact number of eggs can vary based on the size and maturity of the female crab and environmental conditions. A female *S. serrata* can produce between 1 to 2 million eggs per brood. The eggs' size can also vary, but they are generally small and measure around 0.3 to 0.4 millimetres in diameter. The life cycle of *S. serrata* is depicted in Fig.. 3 (RGCA- Mud crab manua-2018).

1.10. Heavy metals

The metals that have a high molecular weight (63.5–200.6 g mol^{-1}) are categorized under heavy metals (Shadman and Mehrgardi, 2019). Recently, the accumulation of heavy metal contaminations in the land and water has been huge compared to earlier (Suamiet al., 2019; Jahan and Strezov, 2019; Pinzón-Bedoya et al., 2020). The major sources of heavy metals are mining, tailings, industrial wastes, agricultural runoff, battery industries, paint factories, etc. They are releasing a huge quantity of heavy metal waste into the environment. Copper, lead, zinc, cadmium, mercury and arsenic are heavy metals released into the seawater from various sources (Sun et al., 2020). Among them, some are trace, and some are highly toxic to the animals, which creates severe consequences. Due to bioaccumulation, toxicity and durable heavy metals cause more effects on aquatic animals (Zhou et al., 2007; Frid and Caswell, 2017). The toxicity of heavy metals (cadmium, copper, mercury lead) on marine crustaceans (crab, prawn) has shown detrimental effects on their physiology, growth, reproduction and overall health. Moreover, heavy metals with other stressors can produce more adverse effects on marine animals, including crustaceans (Roméo et al., 2000). Other stressors like OA, temperature and salinity also cause serious injury to marine crustaceans. The Mitten crab *E. sinensis* subjected to pH 7.8, 7.3 and 6.5 and cadmium showed a notable elevation in SOD and CAT activities (Zhao et al., 2021). The combined effect of heavy metal (cadmium, copper and zinc) temperatures (20 and 24 °C) showed inhibition of oxygen consumption in Juvenile crayfish *Orconectes immunis* has been reported (Khan et al., 2006). The isopods *Jaera albifrons* exposed to the heavy metal Cadmium, zinc and mercury along with low salinity shows a significant alteration in the osmoregulatory ability has been reported (Jones, 1975).

1.11. Cadmium

Cadmium (Cd) is one of the top metal pollutants in the world (Su et al., 2020), having an atomic number of 48 and a mass number of 112. According to the WHO and EPA reports, the Cd's permissible limit in seawater is 2.1 and 1.0 µg $L^{-1,}$ respectively. The toxic properties of Cd are well documented in animals. Entering of Cd reasons to the increase of ROS, which influences the oxidization of biological macromolecules and

results in several physiological damages to tissues and organs (Thevenod, 2009; Revathi et al., 2011). The effects of Cd in humans are well documented, and entered through diets (Gaspic et al., 2002). It causes morphological deformities, physiological dysfunctions and even death (Sun et al., 2016). Itai-itai is a disease recognized in the 1960s in Japan that is caused by the excess exposure of cadmium and carelessness released into the environment (Friberg et al., 1971; Nogawa et al., 1996). Loss of membrane function and DNA damage in various tissues due to the cadmium was perceived earlier. Cadmium heavy metals lead to genetic alterations in several crustaceans like crab *E. sinensis* (Tang et al., 2019) and freshwater crab *Sinopotamon henanense* (Sun et al., 2016). Recent studies show that cadmium causes the weakening of reproductive activity and dislocates endocrine function in *Enchytraeus albidus* worms (Novais et al., 2012).

Review of Literature

2. REVIEW OF LITERATURE

2.1 Introduction

The ocean is a massive salt water of the earth with 138 million km^2 of the exclusive economic zone (EEZ) and an average harvestable potential of 177.8 million tons of total capture and culture fisheries for humankind (FAO, 2022). In recent years, pollution like industrial, sewage, pesticide, chemical, pharmaceutical, micro plastics wastes etc., due to anthropological activities making unfavor to the ocean environment (Ansari and Matondkar, 2014). Likewise, ocean acidification (OA) is becoming one of the global severe, dangerous issues due to continues emission of carbon dioxide (CO_2) (NOAA, 2020). The ocean surface absorbs approximately one-third of the carbon dioxide that has already been emitted to the atmosphere over the last 10 decades (Caldeira and Wickett 2003), and this resulted in the increase of partial carbon dioxide (pCO_2) and increased the acidity of seawater (Denman et al., 2007). Briefly, OA is the atmospheric CO_2, when combined with the seawater surface, causes ocean pH reduction and forms carbonic acid (H_2CO_3). The H_2CO_3 release the hydrogen (H^+) ions and form bicarbonate (HCO_3^-) ions (Fig.uerola et al., 2021). These changes in the seawater cause calcium carbonate ($CaCO_3$) reduction in seawater. Thus the reduction in CO_3^{2-} reducing the $CaCO_3$ saturation in seawater resulted in a negative impact on the calciferous animals (Kleypas et al., 2006). Reports are insight that the calcification rate, survival, moulting, biochemical, hypercapnia, reproduction, feeding habit, calcite and aragonite of marine animals such as corals, Echinoidea, Gastropoda, Annelida, foraminiferans, coccolithophores, bivalves, and crustaceans are decreasing due to the increasing pCO_2 level in the seawater (Fabry et al., 2008, Andersson and Gledhill, 2013, Thomsen et al., 2015, Duquette et al., 2017, Verkaik et al., 2017, Guevara et al., 2019, D'Amario et al., 2020, Muralisankar et al., 2021). Also, the survival, growth, moulting, food indices, and reproduction capacity can affect marine calciferous animals (Kurihara et al., 2008, Kroeker et al., 2012, Thangal et al., 2022).

Marine crabs are a diverse group of crustaceans that play a significant ecological and economic role in marine ecosystems. They are found in various habitats, ranging from intertidal zones to deep-sea environments. Ecologically, marine crabs are being served as important predators, prey and scavengers, contributing to maintaining a healthy

environment and balancing the marine food webs. They also play a crucial role in nutrient cycling and ecosystem functioning, as they help to break down organic matter and recycle nutrients (Xie et al., 2022); as they feed on decaying organic matter and help to break it down and recycle it back into the ecosystem. In addition to their ecological importance, marine crabs have significant economic value as a seafood resource. Crab fisheries provide significant income and employment opportunities for coastal communities, especially in developing countries where they are a major source of protein for local populations. Southeast Asian nations such as Vietnam, the Philippines and Indonesia are the major crab production countries, with an average production of 65,463, 18,100, and 15,000 tons, respectively (Yxtung, 2020). Crabs help to maintain the balance of marine ecosystems by controlling the populations of other marine organisms, such as small fish, molluscs, and other crustaceans. Despite their importance, marine crabs are threatened by various anthropogenic stressors, including overfishing, habitat destruction, and pollution. As such, it is important to manage these resources sustainably and protect their habitats to ensure the continued ecological and economic benefits that they provide. The effect of OA on marine crabs' survival, growth, physiology, and immunology (Long et al., 2017; Meseck et al., 2016; Turra et al., 2019) have been studied and reported earlier. The present review summarized the effect of ocean acidification and ocean acidification with other stressors (heavy metals, temperature, dissolved oxygen, and salinity) on survival, growth, food indices, moulting, physiology, immunology, reproduction and development of marine crabs. Besides this review has also discussed the research gaps and future required investigations.

2.2 Effect of OA on survival and growth of crabs

Survival and growth are the essential parameters to know the physiological status of an organism because these parameters can alter by biotic and abiotic stressors. It has been well documented that the decreasing ocean pH can harm the growth and survival of many marine organisms, including crabs. Many studies have investigated the effect of OA on the survival and growth of crabs. Seawater acidification caused decreased survival and growth of blue crabs *Callinectes sapidus* in both planktonic (at pH 7.8) and juvenile (at pH 7.2, 7.3, and 7.8) stages have been noticed (Giltz and Taylor, 2017; Tomasetti et al., 2018). The significant decreases in the survival of juvenile blue king crabs

(*Paralithodes platypus*) exposed to pH 7.5 for one year were studied by Long et al. (2017). Similarly, a notable reduction in the survival and growth of the mud crab *Scylla serrata* instars exposed to the CO_2-driven acidified environment (pH 7.6, 7.4, 7.2 and 7.0) for sixty days was observed (Thangal et al., 2022). Long et al. (2013) recorded the cent percent mortality and reduced growth in the juvenile red king crab (*Paralithodes camtschaticus*) and Tanner crab (*Chionoecetes bairdi*) exposed to the pH 7.5 on the 95^{th} day of the 200 days experiment. Whereas the hermit crab (*Pagurus criniticornis*) exposed to a pH of 7.7 for 120 days showed an insignificant reduction in survival and a significant decrease in growth (Turra et al., 2019). At the same time, a report published by McElhany et al. (2022) stated that a higher survival rate and poor growth were observed in the juvenile Dungeness crab *Metacarcinus magister* exposed to higher CO_2 (pH 7.2) than in ambient CO_2 for 300 days. The decreased larval development was recorded in the spider crab (*Hyas araneus*) exposed to the CO_2-driven acidic water with pH 7.81 and 7.33 (Walther et al., 2010) (Table 1). All these studies suggest that OA can significantly negatively impact the survival and growth of marine crabs. Crabs under acidified environments spend more energy to tolerate the acidic stress and regulate the internal pH, decreasing feeding and poor survival and growth. Moreover, OA could disrupt the sensory organs of the crabs, which can lead to a decrease in their ability to detect food particles, which leads to decreased survival and growth. The existing reports carry limited information on the detrimental effect of OA on marine crabs. Hence more studies must be focused on the different developmental stages of various crabs, including edible species. Molecular mechanisms related to poor growth in growth gene expression can also be focused on marine crabs under OA treatments.

2.3 Moulting and chitin quantity of crabs under OA

Moulting is a critical process for crustaceans, including crabs, as it allows them to grow and regenerate lost limbs. However, studies have shown that OA can affect the moulting process of crabs in several ways. One of the primary ways that OA affects crabs is by reducing the availability of calcium carbonate, which is a crucial component of the exoskeletons of crustaceans. As the pH of the ocean decreases, the concentration of carbonate ions in the water also decreases, making it more difficult for crabs to form and maintain their exoskeletons. This can lead to slower growth rates, smaller body sizes, and

increased vulnerability to predation and pathogens. OA can also affect the ecdysone hormone levels of juvenile horseshoe crabs (*Tachypleus tridentatus*) at pH 7.3 after seven days of the experiment (Liu et al., 2022). This disruption can lead to abnormal moulting cycles, increased susceptibility to disease, and reduced overall fitness. Reports showed that the red king crabs *P. camtschaticus* lived in the pH 7.5 for three weeks and showed a slower moulting process than the ambient pH 8.1 (Long et al., 2019). The mud crab *S. serrata* exposed to the acidified (pH 7.8, 7.6, 7.4, 7.2, 7.0) seawater for 60 days showed an abnormal moulting rate when compared to the ambient pH (Thangal et al., 2022). According to the report of Turra et al. (2019), the hermit crab (*P. criniticornis*) exposed to pH 7.7 for 120 days showed a slower moulting frequency than the crabs in ambient pCO_2 (Table 1). In this context, Glandon et al. (2018) revealed that the juvenile blue crab (*C. sapidus*) exposed to the high pCO_2 for an interval of the complete moult did not affect intermoult and growth moult periods. These studies suggest that OA can significantly impact crabs' moulting process, ultimately affecting their physiological activities, followed by growth and survival. Liu et al. (2022) stated that under acidified environment level of ecdysone, hormone production could affect crab *T. tridentatus* (pH 7.3), which is responsible for moult regulation. Above studies indicated that crabs' decreasing moulting and chitin levels are due to the changes in seawater chemistry during the OA process. Nevertheless, the specific molecular mechanism is still unclear. Therefore, future studies need to be focused on these aspects.

Chitin is a natural polymer widely distributed in the animal kingdom, playing a fundamental role in structural support, defence, and other biological functions (Lavall et al., 2007; Abdou et al., 2008; Hendriks et al., 2015). It is the primary constituent of the exoskeletons of crustaceans, such as crabs, lobsters and shrimp. A notable decrease in the chitinase enzyme was observed earlier in horseshoe crab *T. tridentatus* exposed to pH 7.3 for 28 days (Liu et al., 2022). A negative reduction in the chitin level and quality changes in the mud crab *S. serrata* exposed to pH 7.4, 7.2 and 7.0 for two months was observed (Thangal et al., 2022). About 38% of the reduction in the microhardness of the claw of the Tanner crabs *Chionoecetes bairdi* exposed to pH 7.5 for two years was observed. It might be due to reduced pH altering the carapace's elemental contents, such as low calcium and high magnesium (Dickinson et al., 2021). Dissolution of the carapace

of the larval Dungeness crabs *M. magister* due to the OA (pH 7.48) along the United States west coast and nearly 10% of the dissolution arose over the last two decades due to the excess emission of the atmospheric CO_2 (Bednaršek et al., 2020). The loss of chitin in the crab species may result due to the loss of Chitin synthetases and chitinolytic enzymes under acidified seawater environment (Merzendorfer and Zimoch, 2003) (Table 1). Nonetheless, the studies on the impact of OA on the chitin production and quality of marine crabs are fragmentary. Hence more studies have to be focused on various crab species at different growing stages. Moreover, the molecular mechanism of OA on the crab chitin and moulting must also be focused on in future investigations.

2.4 Effect of OA on food indices of crabs

OA's effect on crabs' food indices has been investigated in some studies. A study investigated the impact of OA is reduce feeding behaviours like feed intake, food handling time and lasting predator capture time in mud crab *Panopeus herbstii* exposed to the high pCO_2 (9274 µatm) during the 71 days experiment (Dodd et al., 2015). Another study by Manríquez et al. (2021) examined the reduction in the feeding rates and claw pinching strength of the crab *Acanthocyclus hassleri* exposed to high pCO_2 (1400 µatm) for 10-16 weeks. This study also found that acidification reduced the amount of energy available to the crabs, which could affect their ability to reproduce and maintain their normal physiological functions. The food consumption ratio, such as feed intake and feed conversion ratio, was reduced in the mud crab *S. serrata* exposed to pH 7.2 and 7.0 (Thangal et al., 2022). The brown crab *Cancer pagurus* exposed to the high pCO_2 (1200 and 2300 µatm) for two weeks showed a negative impact on the food consumption rates, foraging behaviour like time to break the prey, searching time, food consuming time, and handling time than control crabs (Wang et al., 2018). The shore crab *C. maenas* showed a 41% decrease in food consumption rates at high pCO_2 (1250 and 3000 µatm) exposed for 10 weeks (Appelhans et al., 2012). Significant damage in the antennular flicking rate and long duration to identify the food was observed in deep-sea hermit crabs *Pagurus tanner* exposed to pH 7.1 for 20 weeks (Kim et al., 2016). In this context, the green crab *Carcinus maenas has* positively responded to predation behaviour in acidified conditions (Landes and Zimmer, 2012) (Table 1). The overall available literature reveals that OA can significantly impact the food indices of crabs, leading to poor growth,

survival, and other physiological activities. Nonetheless, the existing reports OA the food index parameters are very limited to crab species. Therefore, more studies are essential in various species, including edible crabs, for understanding the effect of OA on various marine crabs.

2.5 Biochemical constituents of crabs exposed to OA

Crabs are fascinating creatures that inhabit various aquatic environments. As with many other living organisms, crabs rely on a complex network of biochemical elements such as proteins, amino acids, carbohydrates, lipids, nucleic acids, and numerous secondary metabolites to carry out their essential biological functions. In this context, studying crabs' biochemical constituents has become an increasingly important area of research for understanding the fundamental biology of these animals. Moreover, several studies have shown that crab biochemical elements possess unique properties that make them attractive for various biomedical applications. For example, certain proteins and peptides isolated from crab hemolymph have demonstrated antimicrobial, antifungal, and anticancer activities (Shan et al., 2016; Tornesello et al., 2020; Chen et al., 2021; Yang et al., 2022). Animals under stressful conditions need more energy to overcome the stress. Under this circumstance, the available elements like protein, carbohydrates, and lipids in the body can undergo for the catabolic process to produce more energy, which leads to decreases in these biochemical elements in the tissues (Michaelidis et al., 2005; Rosa et al., 2014). Few investigations have revealed the alterations in biochemical elements of crabs under an acidified environment. A study by Thangal et al. (2022) showed that OA (pH 7.8, 7.6, 7.4, 7.2 and 7.0) could affect the biochemical constituents like protein, amino acid, and carbohydrates of crabs, which can have negative consequences for their growth, development and overall physiological activities. The outcome of this study reveals that crabs under acidified environments did not accept adequate feed, followed by poor growth and survival. A stronger reduction in the carbohydrate levels of the crab *A. hassleri* reared for 16 weeks in high pCO_2 (1400 µatm) was observed earlier (Manríquez et al., 2020). Also, a reduction in intracellular osmolytes such as glycine and proline amino acids was observed in the green crab *C. maenas* maintained at pH 7.4, 6.6 and 6.3 for four weeks (Hammer et al., 2012). Further, OA can reduce lipids in the crab's tissues, leading to health consequences as lipids play a vital role in various physiological

processes, including energy storage, membrane structure, and cellular signalling. A notable drop (42%) in the lipid content of hermit crab *P. criniticornis* treated at low pH of 7.7 for 120 days experiment was reported previously (Turra et al., 2020). A significant decrease in the lipid level was noted in mud crab *S. serrata* under the low pH (7.4, 7.2 and 7.0) for two months (Thangal et al., 2022). Further, a study on the Dungeness crab *C. magister* exposed to a pH of 7.5 showed that the heat maps of 94 metabolites and 127 lipids reveal that numerous molecules respond to low pH (Wanamaker et al., 2019) (Table 1). Based on the above information, it indicates the detrimental effect of OA on the biochemical elements of some marine crabs. Nevertheless, the studied crab species are limited in these aspects. Furthermore, to our knowledge, no one report is available on the impact of OA on marine crabs' amino acids and fatty acids composition. Therefore, forthcoming studies are required on these gaps to know the marine crab species' physiological state and muscle quality.

2.6 Antioxidants and lipid peroxidation status of crabs treated under OA

The antioxidant system has a major function in the animal body to prevent cell damage caused by environmental stresses (Jia et al., 2018). Superoxide dismutase (SOD) is one of the main operative antioxidant enzymes in the cells. SOD catalyzes O_2^- free radicles and H^+ ions to O_2 and H_2O_2, respectively. Further, Catalase (CAT) decays H_2O_2 into H_2O and O_2. This way, the interactions between the SOD and CAT successively eliminate the reactive oxygen species (ROS) produced by external stress (Ighodaro and Akinloye, 2018). Lipid peroxidation (LPO) acts as a free radical-mediated chain of reaction (Hampel et al., 2016) that can lead to the oxidative breakdown of polyunsaturated fats and cell membranes are the primary target parts of a biological system. Many studies proved that OA could alter animals' antioxidants and LPO status, including crabs (Lin et al., 2020; Liu et al., 2022). The juvenile horseshoe crab *T. tridentatus* exposed to pH 7.3 for 28 days duration showed notable increases in the ROS and LPO levels that indicate the juvenile horseshoe crab was under stress and oxidative damage of cells. In addition, elevation of SOD, CAT and Glutathione peroxidase (GPx) activity was noted in the juvenile horseshoe crab at low pH (pH 7.3) (Liu et al., 2022). A sudden substantial elevation was observed in SOD and Glutathione S-Transferases (GST) activities, as well as the rapid regulation in the mRNA expression of ecCuZnSOD and cMnSOD in the

hepatopancreas of the chines crab *Portunus trituberculatus* reared in high pCO_2 (750 and 1500 µatm) for four weeks, which indicate a significant elevation in the antioxidant capability of this crab (Lin et al., 2020) (Table 1). Furthermore, the mud crab *S. serrata* exposed to the acidified seawater (pH 7.8, 7.6, 7.4, 7.2 and 7.0) for 60 days showed a significant elevation in the SOD, CAT and LPO activities observed recently by Thangal et al. (2022). However, the studied crab species are very limited in these biomarkers aspects. Hence, more information is required to understand the status of biomarkers in the different life stages of crab species under OA.

2.7 Metabolic enzymes and digestive enzymes levels in crabs under OA experiments

Metabolic enzymes such as Glutamic Oxalic Transaminase (GOT) and glutamic pyruvic transaminase (GPT) are vital to the organism for their digestion, cell respiration, transcription and energy storage. Variations in the metabolic enzymes may cause liver damage. Earlier studies noticed that crabs under acidified seawater showed significant changes in metabolic enzymes. A study by Kim et al. (2016) recorded significant elevations in the metabolic activities of the hermit crab *Pagurus tanneri* exposed to acidified seawater (pH 7.1) for 20 weeks. A significant reduction in the metabolic rate of intertidal porcelain crab *Petrolisthes cinctipes* exposed to a pH of 7.58 for six days was recorded by Carter et al. (2013). A notable increase in the level of GOT and GPT enzymes was identified in mud crab *S. serrata* exposed to the acidified seawater (Thangal et al., 2022). Alkaline phosphatase (ALP) is one of the membrane-bound metalloenzymes that have a key role in the metabolite and biomineralization process and is used as an indicator to identify the physiological situation of an animal under acidic stress (Zambonino-Infante et al., 2008; Szabo and Ferrier, 2014). ALP, a hydrolase in the innate immune system, can kill extracellular attackers directly. Alterations in ALP level was observed earlier in marine invertebrates, including crustaceans such as crabs and brine shrimp due to the acidic stress (Zheng et al., 2015; Wang et al., 2020; Liu et al., 2022). A notable decrease in the level of ALP was observed in the mud crab *S. serrata* exposed to the acidified seawater at pH 7.2 and 7.0 for 60 days of experimental duration (Thangal et al., 2022). It was observed to increase first and then decrease in the activity of ALP in the juvenile horseshoe crab *T. tridentatus* exposed to pH 7.3 for 28 days, which denoted the adverse effect of OA on ALP under chronic exposure (Liu et al., 2022).

Digestive enzyme activities are critical to nutrient digestion, overall health and survival of crustaceans. Studies have investigated the effects of OA on the digestive enzyme activities of crabs and found a significant detrimental impact, which can affect their ability to digest food and obtain the necessary nutrients for survival. The Chinese crab *P. trituberculatus* exposed to the elevated pCO_2 (750 and 1500 µatm for 20 weeks) showed a significant decrease in lipase and amylase activities when compared to the crabs in the ambient environment (Lin et al., 2020) (Table 1). The reduction in the lipase activity might be due to the switch from the lipid and protein metabolism in the hepatopancreas (Carter et al., 2013). Nonetheless, no more reports are available on the effect of OA on marine crabs' metabolic enzymes and digestive enzyme activities. Since marine crabs are being served as good sources of exogenous enzymes in the food chain, more investigations need to be conducted to explore the state of the metabolic and digestive enzymes of different developmental stages of various crab species in acidified seawater environments.

2.8. Effect of OA on minerals of crabs

Marine crustaceans, such as shrimp, crabs, and lobsters, are excellent sources of minerals essential for human health. These minerals play important roles in numerous biological processes and contribute to the overall nutritional value of crustaceans. Calcium (Ca), phosphorus (P), magnesium (mg) and potassium (K) are considered macro minerals, and Iron (Fe), zinc (Zn), copper (Cu) and selenium (Se) consider trace elements in marine crustaceans. Marine crustaceans are rich in Ca, P, Mg and K and have crucial roles in developing and maintaining strong bones, nerve and muscle functions, and fluid balance. Also, marine crustaceans are rich in Fe, Zn, and Cu, with significant roles like oxygen transport, immune function, and antioxidant defence. Reductions in the mineral levels affect marine crustaceans in various ways. OA alters the availability of carbonate ions, disrupts mineral homeostasis, and impairs the formation and maintenance of calcified structures such as calcium carbonate dissolution, impaired shell formation, and mineral homeostasis in marine animals (Hofmann et al., 2010; Kroeker et al., 2010; Guttaso et al., 2015). OA has been shown to significantly impact the mineral content of various marine animals, including crabs. In particular, seawater acidification has been found to cause a reduction in Ca, Mg, and Fe concentrations in crabs (Coffey et al., 2017).

Lack of availability of essential minerals negatively affects the organism, such as hyponatremia, weakness and fatigue, and skeleton strength (Karppanen et al., 2005). The red king crab *P. camtschaticus* and tanner crab *C. bairdi* exposed to the low pH (pH 7.8 and 7.5) seawater showed a significant decrease in the carapace Ca condition index and Ca contents (Long et al., 2013). Reduction in the Ca content was observed in the snow crab *C. opilio* exposed to pH 7.5 for two years (Algayer et al., 2023). The snow crab *C. bairdi* reared at pH 7.5 for two years showed a significant reduction of Ca content (Swiney et al., 2016). Thangal et al. (2022) observed a reduction in levels of Na, K and Ca in the carcass of mud crab *S. serrata* exposed to the acidified seawater (pH 7.8, 7.6, 7.4, 7.2, and 7.0) for 60 days indicates the OA may inhibit the intake of the minerals from water and feed. The same studies discuss that the reduction of the content of calcium carbonate in seawater might be responsible for the poor calcification levels in crabs in an acidic environment. Also, changes in Na, K and Ca characteristics of the carapace of the crab *P. camtschaticus* and blue crab *P. platypus* at low pH (pH 7.8 and 7.5) have been reported (Coffey et al., 2017) (Table 1). This information indicates the adverse effect of OA on Ca and other minerals in crabs. However, the mechanism of reducing mineral content in crabs under a reduced pH environment is unclear; hence, more studies are required to understand this mechanism. Nevertheless, based on our best knowledge, the impact of OA on the mineral contents of marine crabs is fragmentary. Meanwhile, marine crabs are being used as a good source of mineral nutrition. So, more attention is needed to discover the status of essential and trace minerals of different parts of the crab's body under acidified seawater.

2.9 Ocean acidification with other stressors on crabs

The acidification of seawater and other stressors can harm the biology and physiology of crabs. Increasing sea surface temperatures, often associated with climate change, can worsen the impacts of OA that enhance the metabolic rates of crustaceans but can also reduce their physiological tolerance to acidification (Gao et al., 2020). Hypoxia combined with OA can increase the physiological stress on crustaceans, affecting their growth, reproduction, and survival. In addition, pollution and contaminants such as heavy metals and pesticides can interact synergistically with OA to worsen the negative impacts on crustaceans. These interactions can impair their physiological processes and

compromise their overall health. Some investigations recorded the synergetic effect of OA with other stressors like temperature, salinity, heavy metals, and hypoxia condition of marine crabs. The combined treatments of OA (pH 7.3) and ocean warming (18 and 25 °C) for two days significantly affected the foraging behaviour of the Japanese stone crab *Charybdis japonica*. The observations revealed that the crabs exhibited prolonged durations in various foraging activities, including feed searching, breaking time, food eating, and handling time. Moreover, the study showed a decrease in prey profitability and reduced the predation of food by the crabs under these combined stressors (Wu et al., 2017). The shell-crushing crab *Acanthocyclus hassleri* exposed to the high pCO_2 **(**1400 μatm) along with temperature (25°C) for a period of 10 to 16 weeks showed a decreased survival (Manríquez et al., 2020). Also, the Caribbean king crab *Maguimithrax spinosissimus* showed a decreased survival and moulting rate exposed to the low pH (7.7) at 31°C (Gravinese et al., 2022). Swiney et al. (2017) revealed that the long time exposure (184 days) of the red king crab *P. camtschaticus* subjected to the low pH 7.8 along with 4°C temperature showed a significant reduction in survival, growth and taking more time to complete their moult. The multiple combined effects of OA along with the temperature and salinity treatment of the fiddler crab *Leptuca thayeri* showed a complete death at a high temperature (40 °C) experiment has been recorded earlier by Andrade et al. (2022). The blue crab *C. sapidus* exposed to low pH (7.16) along with low dissolved oxygen (3.74 mg L^{-1}) for 14 days showed an 87% decrease in survival (Tomasetti et al., 2018). Recently, a study reported that the mud crab *S. serrata* subjected to pH 7.7 along with multiple stress temperatures (34°C) and oil (5 mg L^{-1}) for one-month exposure showed a significant decrease in ingestion and absorption and increases in excretion at pH 7.7 +34 °C + 5 mg L^{-1} of oil (Baag and Mandal, 2022). Further, the shell-crushing crab *A. hassleri* reared in the high pCO_2 (1400 μatm) with high temperature (20°C) for 10 to 16 weeks experiment showed a noteworthy decrease in the carbohydrate level in claw muscles (Manríquez et al., 2021). The same study also found a decreased calcification rate at the high pCO_2 and warm temperature (1400 μatm + 25 °C). Also, a significant decrease in the Ca contents of the spider crab *H. araneus* subjected to the high pCO_2 seawater (710 and 3000 ppm) along with high temperatures (9 and 15 °C) has been observed previously (Walther et al., 2010) (Table 2).

A notable elevation in the level of antioxidants like SOD and CAT of the mitten crab *E. sinensis* subjected to the low pH (7.8, 7.3, and 6.5) with heavy metal cadmium (Cd) (1 mg L^{-1}) for three weeks of exposure has been reported (Zhao et al., 2021). In a 96 hours study conducted by Adeleke et al. (2020), it was found that exposure to low pH seawater (pH 7.2, 7.4, and 7.6) in combination with heavy metal Cd (0.5, 0.75, 1.00 mg/l), lead (Pb) (6.50, 8.50, 10.50 mg/l), and a mixture of Cd and Pb (4.50, 5.75, 7.00 mg/l) resulted in the accumulation of heavy metals in the exoskeleton of the crab *Dotilla fenestrate*. The accumulation order for cadmium was in pH 7.4 > 7.6 > 7.2 exposures. Also, the great spider crab *H. araneus* subjected to the high pCO_2 seawater (1120 and 1960 μatm) with the alterations in seawater temperature (5 and 10°C) showed an upregulation of metabolic and stress response-related genes at 1120 μatm + 10°C seawater (Harms et al., 2014). The fiddler crab *L. thayeri* reared at pH 7.0 and 6.3 along with multiple stresses like temperature (20, 25, 30, 35, 40 °C) and salinity (10, 20, 30, 40, and 50 psu) detected hyper osmoregulation at pH 6.3 and low oxygen consumption in all combined temperature and salinity treatments (Andrade et al., 2022). The Porcelain crab *Petrolisthes cinctipes* subjected to pH 7.6 and 7.15 with temperatures 25 and 30 °C for 2.5 weeks showed significant decreases in respiration rate and heat tolerance capacity at pH 7.15 + 30°C (Paganini et al., 2014). Besides, notable increases in respiration and decreases in critical thermal maxima of the mud crab *S. serrata* subjected to the pH 7.7 along with multiple stress temperature (34°C) and diesel oil (5 mg L^{-1}) has been reported earlier (Baag and Mandal, 2022) (Table 2). Most studies investigated the combined effect of OA and temperature on crabs, and minimal reports are available on the combined effect of OA with other stressors like salinity, hypoxia, and heavy metals. Therefore, more research is required to combine the effect of OA and other stressors like microplastics, hydrocarbons, pesticides, etc.

2.10 Conclusion and future perspectives

The present review explores how the crabs exposed to acidified environments negatively affect survival, growth, moulting, chitin level, food indices, tissue biochemical elements, antioxidants, metabolic enzymes, digestive enzymes, and minerals. Besides, the stressors like temperature, hypoxia, heavy metal, and salinity combined with OA have a

severely detrimental effect on the biological and physiological indices of the crab species. However, based on the view of the present review, future investigations have to focus on the following perspectives to address the research gaps.

a. The existing literature provides limited information on the detrimental effects of OA on marine crabs. Therefore, further studies should focus on different developmental stages of crabs, including edible and ecologically important species, and the molecular mechanisms underlying poor growth and altered gene expression under OA conditions need to be addressed.

b. Previous studies have indicated that the decline in moulting and chitin levels in crabs can be attributed to changes in seawater chemistry during the OA process; however, the specific molecular mechanisms remain unclear. Hence, the investigation should be prioritized on these aspects for a better understanding.

c. The available studies on the impact of OA on chitin production and quality in marine crabs are fragmentary. Therefore, more comprehensive studies are needed, encompassing various crab species at different growth stages. Additionally, future investigations should explore the molecular mechanisms underlying the effects of OA crab chitin production and the moulting process.

d. The available reports on food index parameters in crab species are very limited, particularly concerning edible crabs. Consequently, it is crucial to conduct further investigation on various edible crab species to understand the effects of OA on feed utilization conversion and growth.

e. No one report is available on the impact of OA on the composition of amino acids and fatty acids in marine crabs. Therefore, future studies should address these gaps to elucidate crabs' physiological state and muscle quality under acidified seawater conditions.

f. The studies conducted on biomarkers enzymes such as SOD, CAT, GPx, and GST in crab species are limited. More information is required to comprehend the biomarker status of crab species exposed to OA during different life stages.

g. There is a lack of reports on the effects of OA on metabolic enzymes and digestive enzyme activities in marine crabs. Since marine crabs serve as a valuable source of exogenous enzymes in the food chain, further investigations are necessary to explore the state of these bio enzymes in different developmental stages of various crab species under acidified seawater environments.

h. Current knowledge regarding the impact of OA on mineral content in marine crabs is fragmentary. Considering that marine crabs are an important source of mineral nutrition, more attention is required to assess the status of essential and trace minerals in crabs under OA.

i. The effect of ocean acidification on the specific and nonspecific immune response and susceptibility to pathogens is not yet addressed. Hence, more attention must be provided to these aspects. Also, the impact of OA on intestinal gut microflora is limited. Hence, more investigation needs to be conducted to explore the gut health status of crabs.

j. Most studies have investigated the combined effects of OA and temperature on crabs. At the same time, there is limited research on the combined effects of ocean acidification with other stressors such as salinity, hypoxia, and heavy metals. Therefore, further research is needed to examine the combined effects of OA and other stressors, including microplastics, heavy metals, hydrocarbons, pesticides, etc.

Marine crabs occupy an important place in ecological and economic sectors such as ecosystem engineers, nutrient cyclers and components of food webs. Based on the above views can be considered research gaps, and future research may be focused on these aspects to know the effect of ocean acidification on different stages of marine crabs.

Table 1: Effect of OA on marine crabs

Sl. No.	Species	pH/pCO_2	Parameters	Exposure duration	Observation	Reference
1.	Blue crabs (*C. sapidus*)	7.2, 7.3, 7.8	Survival (SR), growth (GR)	14 days	(↓) SR (pH 7.2, 7.3, 7.8) (↓) GR (7.8).	Giltz and Taylor (2017); Tomasetti et al., 2018;
2.	Blue king crab (*P. platypus*)	7.5	Survival	1 year	(↓) SR (pH 7.5)	Long et al., 2016
3.	Green crab *C. maenas*	7.7	Muscle length (ML) of crusher chela, predator pray behaviour	5 months	(↓) ML of chelated crusher legs (7.7). (↔) Predator-prey behavior (7.7)	Landes and Zimmer (2012)
4.	Mud crab *S. serrata*	7.8, 7.6, 7.4, 7.2, 7.0	Survivorship (SR), growth, moulting (M), chitin (CN), feed indices (FI), protein (P), carbohydrate (CHO), amino acids (AA), lipids (L). SOD, CAT, LPO, GOT, GPT, ALP, Minerals	60 days	(↓) SR (7.6, 7.4, 7.2, 7.0) (↓) GR (7.8, 7.6, 7.4, 7.2, 7.0) (↓) M (7.8, 7.6, 7.4, 7.2, 7.0), (↓) CN (7.4, 7.2, 7.0), (↓) FI and FCR (7.2, 7.0), (↓) PRO, CHO, AA (7.8, 7.6, 7.4, 7.2, 7.0), (↓) L (7.4, 7.2, 7.0). (↓)Ca, Na, K (7.8, 7.6, 7.4, 7.2, 7.0). (↑) SOD, CAT, LPO (7.8, 7.6, 7.4, 7.2, 7.0), (↓) ALP (7.2, 7.0), (↑) GOT, GPT (7.8, 7.6, 7.4, 7.2, 7.0),	Thangal et al., 2022
5.	Red king crab (*P. camtschaticus*), Tanner crab (*C. bairdi*)	7.8, 7.5	Survival, growth, calcium level	200 days	(↓) SR, GR (7.5), (↓) Ca (7.8 and 7.5)	Long et al., 2013
6.	Hermit crab (*P. criniticornis*)	7.7	Survival, growth, moulting, and lipid	120 days	(↔)SR (7.7), (↓) GR (7.7), (↓) Molting process (7.7), (↓) L (7.7).	Turra et al., 2019

Sl. No.	Species	pH/pCO_2	Parameters	Exposure duration	Observation	Reference
7.	Juvenile Dungeness crab (*M. magister*)	7.2	Survival, growth	300 days	↑SR (7.2), (↓) GR (pH 7.2)	McElhany et al., 2022
8.	Spider crab (*H. araneus)*	7.81, 7.33	Development of larvae and growth	-	(↓) LD, GR (7.81, 7.33).	Walther et al., 2010
9.	Red king crab (*P. camtschaticus*)	7.5	Moulting rate	3 week	(↓) M (7.5)	Long et al., 2019
10.	Juvenile blue crab (*C. sapidus*)	8000 µatm	Inter moulting periods (inter moult period (IMP) and growth moult period (GMP)	2 months	(↔) IMP and GMP (8000 µatm).	Glandon and Miller, 2017
11.	Horseshoe crab *T. tridentatus*	7.3	Ecdysone hormone production, Chitinase, ROS, LPO, SOD, CAT, GPx, ALP	28 days	(↓) Ecdysone hormone (7.3), (↓) Chitinase (7.3) (↑) LPO, SOD, CAT, GPx (7.3) (↑) ALP first and then (↓) (7.3).	Liu et al., 2022
12.	Tanner crabs *C. bairdidue*	7.5	Hardness of claw	2 years	(↓) the hardness of the claw (7.5).	Dickinson et al., 2021
13.	Dungeness crabs *M. magister*	7.48	Carapace dissolution		(↑) Dissolution of carapace	Bednaršek et al., 2020
14.	Mud crab *P. herbstii*	785 ± 154, 9274 ± 2243 µatm	Feeding behaviour	71 days	(↓) FI, FHT, PCT (785 ± 154, 9274 ± 2243 µatm)	Dodd et al., 2015
15.	Crab *A. hassleri*	1400 µatm	Feeding rate and claw strength, carbohydrate	10-16 weeks	(↓) FR, CPS(1400 µatm) (↓) CHO (1400 µatm)	Manríquez et al., 2020
16.	Brown crab *C. pagurus*	1200 and 2300 µatm	Feeding behaviour	2 weeks	(↓) FCR, FB, TBP, ST, FCT, HT 1200 and 2300 µatm)	Wang et al., 2018
17.	The shore crab *C. maenas*	1250 and 3000 µatm	Food consumption rate	10 weeks	(↓) FCR (1250 and 3000 µatm)	Appelhans et al., 2012
18.	Green crab *C. maenas*	7.4, 6.6 and 6.3	Intracellular osmolytes	4 weeks	(↓) Gly and Prol (7.4, 6.6, 6.3)	Hammer et al., 2012
19.	Dungeness crab *C. magister*	7.5	Metabolomic approaches	-	(↑) 94 metabolites and 127 lipids responded in a condition-specific Manner (7.5)	Wanamaker et al., 2019

Sl. No.	Species	pH/pCO_2	Parameters	Exposure duration	Observation	Reference
20.	Horseshoe crab *T. tridentatus*	7.3	Antioxidants	28 days	(↑) SOD, CAT, LPO, GPx	Liu et al., 2022
21.	Chines crab *P. trituberculatus*	750 and 1500 µatm	Antioxidants, Digestive enzymes	4 weeks	(↑) SOD, GST (↓) Lip, Amy (750 and 1500 µatm).	Lin et al., 2020
22.	Hermit crab *P. tanneri*	7.1	Food identification time and metabolic enzymes	20 weeks	Damage to the antennular flickering (↑) FIT (7.1) (↑) metabolic enzymes (7.1)	Kim et al., 2016
23.	Intertidal porcelain crab *P. cinctipes*	7.58	Metabolic rate	6 days	(↓) MR (7.58)	Carter et al., 2013
24.	Snow crab *C. opilio*	7.58	Calcium content	2 years	(↓) Ca (7.58)	Algayer et al., 2023
25.	Snow crab *C. bairdi*	7.5	Calcium content	2 years	(↓) Ca (7.5)	Swiney et al., 2016
26.	Great spider crab *H. araneus*	1120 µatm	Gene expression	10 months	Upwelling the gene expression profile (1120 µatm)	Harms et al., 2014

Alkaline phosphatase (ALP), Amino acids (AA), Amylase (Amy), Calcium (Ca), Carbohydrate (CHO), Catalyse (CAT), Chitin (CN), Claw pinching strength (CPS), Feed conversion ratio (FCR), Feed identifying time (FIT), Feed intake (FI), Feeding rate (FR), Food consuming time (FCT), Food handling time (FHT), Food handling time (FHT), Foraging behaviour (FB), Glutamic peroxidase (GPx), Glutamic pyruvate transaminase (GPT), Glutamix oxalate transaminase (GOT), Glysin (Gly), Growth (GR), Growth moult period (GMP), Inter moult periods (IMP), Lipid (L), Larval development (LD), Lipase (Lip), Lipid peroxidation (LPO), Lipids (L). Metabolic rate (MR), Molting (M), Muscle length (ML), Potassium (K), Predator capture time (PCT), Proline (Prol), Protease (Pro), Protein (P), Searching time (ST), Slow moulting process (SMP), Sodium (Na), Superoxide dismutase (SOD), Survival (SR), Survivorship (SR), Time to break the prey (TBP)

Table 2: Impact of OA with other stressors

Sl. No	Species	pH/ pCO_2	Other stress	Concentration	Parameter	Exposure duration	Observation	Reference
1	Mitten crab, *E. sinensis*	pH 7.8, 7.3, and 6.5	Cadmium (Cd)	1 mg/L^{-1}	Antioxidant	21 d	**(↑)** SOD and CAT (7.8, 7.3, 6.5 + 1 mgL^{-1}	Zhao et al., 2021
2	Crab *D. fenestrata*	7.2, 7.4, 7.6	Cd and lead (Pb)	Cd (0.5, 0.75, 1.00 mg L^{-1}), Pb (6.50, 8.50, 10.50 mg L^{-1}), Cd/Pb (4.50, 5.75, 7.00 mg L^{-1})	Accumulation in exoskeleton	96 h	**(↑)** Accumulation of Cd in 7.4 > 7.6 > 7.2. **(↔)** in Pb accumulation with varying pH. Accumulations in combined Cd and Pb (↑) in pH of 7.2 and 4.50, and 7.00 mg/l	Adeleke et al., 2020
3	Japanese stone crab *C. japonica*	7.3, 8.1	Temperature	18 and 25 °C	Foraging behaviour	2 days	(↑) FIT, FBT, FCT, FHT (pH 7.3 + 18 and 25 °C). (↓) PP, PPT (pH 7.3 + 18 and 25 °C)	Wu et al., 2017
3	Shell-crushing crab *A. hassleri*	pCO_2 (~500 and 1400 µatm)	Temperature	(~15 and 20 °C)	Oxygen consumption rate, survival, calcification rate, feeding rates, claw pinching strength, self-righting speed, sarcomere length of the crusher claw muscles, Carbohydrate, Gene expression (HSP70).	10-16 weeks	(↓) SR, CR, AL (1400 µatm + 25 °C). (↑) FI and OC (25 °). (↓) CPS (1400 µatm). (↓) CHO (1400 µatm). (↑) HSP70 level (25 °)	Manríquez et al., 2020
5	Porcelain crab *P. cinctipes*	7.6, 7.15	Temperature	25, 30 °C	Respiration rate and cardiac thermal limits	2.5 weeks	(↓) RR (pH 7.15 and 30 °C), (↑) HT (pH 7.15 and 30 °C)	Paganini et al., 2014.
6	Great spider crab *H. araneus*	1,120, 1,960 µatm	Temperature	5, 10°C	Gene expression profiling	10 weeks	Unregulated stress response genes (1,120 µatm, and 10°C).	Harms et al., 2014
7	Caribbean king crab *M.spinosissimus*	7.7	Temperature	31 °C	Survival, moult-stage duration, and morphology		(↓) SR (pH 7.7 + 31 °C), (↓) M (pH 7.7+ 31 °C)	Gravinese et al., 2022
8	Spider crab *H. araneus*	710 ,3,000 ppm	Temperatures	3, 9, 15 °C	Calcium content	-	(↓) Ca (710 ,3,000 + 9, 15 °C)	Walther et al., 2011

Sl. No	Species	pH/ pCO_2	Other stress	Concentration	Parameter	Exposure duration	Observation	Reference
9	Mud crab *S. serrata*	7.7	Temperature + Oil (Diesel)	34°C + 5 mg L^{-1}	Rates of Ingestion, absorption, respiration, excretion, thermal performance, thermal critical maxima CTmax).	30 days	(↓) I, A (pH 7.7+34 °C + 5 mg L^{-1}), (↑) RR, E (7.7+34 °C + 5 mg L^{-1}), (↓) CTmax,	Baag and Mandal, 2022
10	Blue crabs, *C. sapidus*	7.16–7.33	Dissolved oxygen (Hypoxia)	3.74–4.06 mg L^{-1}	Survival	14 days	(↓) SR 60% (3.74 mg L^{-1}), 49 % (pH 7.16), and 87 % (pH 7.16+ 3.74 mg L^{-1})	Tomasetti et al., 2018
11	Red king crab *P. camtschaticus*	7.8	Temperature	Ambient +2 and +4 °C	Survival, growth, and moult	184 days	(↓) SR (7.8+ 4°C), (↓) IMP (7.8+ 4°C), (↓) GR (4°C),	Swiney et al., 2017
12	Estuarine fiddler crab *L. thayeri*	6.2	Temperature	30 °C	Survival, embryonic development	10 days	(↓) SR (30 °C), HEV (pH 6.2 + 30 °C)	Pardo and Costa, 2021
13	Fiddler crab *L. thayeri*	7.0, 6.3	Temperature and Salinity	Temperature (20, 25, 30, 35, 40 °C) Salinity (10, 20, 30, 40, and 50 psu)	Survival, osmoregulation, oxygen consumption	-	CD (40 °C). SHR (pH 6.3). (↓) OC (pH combined with Temperature and salinity).	Andrade et al., 2022

Absorption (A), Arcomere length (AL), Calcification rate (CR), Calcium (Ca), Carbohydrate (CHO), Catalyse (CAT), Claw pinching strength (CPS), Complete death (CD), Excretion (E), Feed breaking Time (FBT), Feed identifying time (FIT), Feed intake (FI), Food consuming time (FCT), Food handling time (FHT), Growth (GR), Heat shock protein (HSP), Heat tolerance (HT), Higher egg volume (HEV), Ingestion (I), Inter moult periods (IMP), Molting (M), Oxygen consumption (OC), Pray predation Time (PPT), Prey profitability (PP), Respiration rate (RR), Strong hyperosmoregulators (SHR), Superoxide dismutase (SOD), Survival (SR), Thermal critical maxima (CTmax.

Aim and Objectives

3. AIM AND OBJECTIVES

Marine crabs are diverse crustaceans that play a key role in ecological and economic perspectives. Nonetheless, like other aquatic invertebrates, marine crabs are being affected by various environmental stresses. Ocean acidification (OA) is one of the serious environmental issues that cause detrimental effects on marine crustaceans, including crabs. Earlier studies revealed the adverse effect of OA on marine crabs. However, studies on the effect of OA on the economically important marine mud crab *Scylla serrata* have not been investigated so far. Therefore, the present study was focused on the following objectives.

- To evaluate the effect of CO_2 driven OA on seawater properties, survival, growth, moulting rate, and nutritional index of marine mud crab *S. serrata.*
- To determine the impact of OA on digestive enzymes, biochemical constituents, mineral, and chitin levels of *S. serrata.*
- To estimate the amino acids and fatty acid profile of *S. serrata* exposed to OA.
- To study the influence of OA on antioxidants, metabolic enzymes, and alkaline phosphatase of *S. serrata.*
- To investigate the interactive effect of OA and the heavy metal cadmium on *S. serrata.*

Chapter I

4. CHAPTER I: Effect of CO_2 driven ocean acidification on seawater properties, survival, growth, moulting rate, and nutritional index of marine mud crab *Scylla serrata*

Introduction

Seawater, the vast expanse of saline water covering approximately seventy per cent of the Earth's surface, is a complex and dynamic environment with unique properties. Seawater plays a crucial role in shaping our planet's climate, sustaining marine life, and influencing various geological processes. Seawater's average pH and salinity are 8.2 and 35 ppt, respectively (USEPA, 2022). Seawater is essential to marine animals' survival, growth, reproduction, and overall well-being in terms of habitat, oxygen and carbon dioxide exchange, nutrient source, osmoregulation, thermoregulation, and reproduction. Seawater quality is of utmost importance for marine animals and the health of our entire planet. Seawater composition and its characteristics are vital in shaping marine ecosystems and supporting the incredible diversity of life in the world's oceans. However, in recent years, there has been a growing concern regarding various issues affecting seawater quality due to anthropogenic activities (Jiao et al., 2015). The major issues faced by the ocean can be described as pollution from various sources, including industrial discharge, agricultural runoff, and improper waste disposal, which poses a significant threat to seawater quality. Contaminants such as heavy metals, chemicals, plastics, and oil spills harm marine life, leading to toxicity, habitat degradation, and reduced biodiversity (Rosa, 2022). Moreover, increased atmospheric CO_2 leads to rising global temperatures, and climate change has profound implications for seawater quality, like elevated ocean temperature, which can cause coral bleaching, sea level rise, and disrupt delicate ecosystems. OA is the excessive absorption of carbon dioxide by seawater caused to the lacking of carbonate ions in the calcareous animals for making their outer shells. The primary cause of atmospheric CO_2 increase is human activities, burning fossil fuels such as coal, oil, and natural gas. Deforestation is another major source of CO_2 increment, reducing the Earth's capacity to absorb CO_2 through photosynthesis. Additionally, various industrial processes, such as cement and chemical manufacturing, release CO_2 as a by-product. Report to the global carbon budget, the top five greenhouse gas emitting countries are China (28.3%), the United States (14.5%),

India (6.6%), Russia (4.9%), and Japan (3.6%) (GCP, 2021). Based on the earlier reports the crabs, shrimps, brine shrimps, and other fishes are struggling to survive due to the decreasing of the oceanic pH by the OA process (Miller et al., 2016; Long et al., 2021; Muralisankar et al., 2021). Invertebrates like corals, brine shrimp, sea urchins, shrimps, crabs, etc., showed negative impacts on survival, growth, foraging and reproduction exposed to OA (Baumann et al., 2012; Dodd et al., 2015; Campbell et al., 2016; Tasoff and Johnson, 2019; Thangal et al., 2022). Crustaceans have significant economic value through the seafood industry, job creation and trade. They also play important ecological roles in nutrient cycling, trophic interactions, biodiversity maintenance and environmental monitoring. Crabs play a vital role in the economy and ecology of crustaceans. Economically, they hold substantial value as highly exclusive seafood. They have a vital role in ecosystems through their feeding habits and interactions with other organisms. Certain species of crabs are utilized as bio-indicators due to their sensitivity to environmental changes and ability to reflect their ecosystems' health. The Marine crabs such as fiddler crabs *Genus uca*, blue crab *C. sapidus*, and mud crab *S. serrata* have been used as bioindicators for various environmental issues, including heavy metal pollution, pesticide pollution, OA, etc. (Giblock and Crain, 2011; Salvat-Leal et al., 2020; Flint et al., 2021). The crabs *C. bairdi*, *P. camtschaticus*, *C. magister*, *H. araneus*, *P. herbstii*, *L. aequispinus* and *A. hassleri* exposed to the acidified seawater had shown a negative impact on the survival, growth, moulting, feeding rate and behavior (Long et al., 2013; Dodd et al., 2015; Miller et al., 2016; Wang et al., 2018; Reinhardt, 2020; Manríquez et al., 2021; Long et al., 2021). The mud crab *S. serrata* is an Indo-Pacific species with an outstanding nutritional index, high economic value, and feasible seafood in the international market (Bhuiyan et al., 2022). According to the report of FAO (2020), *S. serrata* contributes about 248.8 thousand tons of crabs to the world fisheries sector. Also, they are used as a bioindicator due to their higher sensitivity to biotic and abiotic stress (El-Kahawy et al., 2021; Flint et al., 2021). Earlier studies reported the effect of OA on some marine crab species. Nonetheless, the impact of OA on the economically important crab *S. serrata* is not studied so far. Hence, the present chapter focused on investigating the effect of OA on the survival, growth, moulting, and nutritional index of the commercially important mud crab *S. serrata*.

4.2 Materials and Methods

4.2.1 Procurement of mud crab

The mud crab instars (*S. serrata*, first instar) were purchased from Rajiv Gandhi Center for Aquaculture (RGCA-MPEDA) Sirgali, Tamilnadu, India. Four hundred crab instars were transported in a five-litter transparent polythene cover filled with oxygenated natural seawater and hideouts to prevent cannibalism. Instars were accommodated in an ambient laboratory environment for one week in FRP tank (500 L) with seawater [pH (8.2 ± 0.07), salinity (35 ± 0.8 ppt), temperature (26 ± 1°C), dissolved oxygen (7 ± 0.1 mg L^{-1}), total alkalinity (1861±38 µmol kg^{-1}), and ammonia (0.1 ± 0.05 mg L^{-1})] with continuing aeration and hideouts. During the acclimatized period, instars were fed with 20g of (100% /body mass) frozen Artemia biomass with 235 ± 2.3 mg g^{-1}, 199 ± 5mg g^{-1} and 97 ± 2 mg g^{-1} of protein, carbohydrate, and amino acid, respectively twice per day (40% morning and 60% night). About 20% of seawater from the accommodated tank was renewed, and the unfed feed and faeces were removed during the renewing process.

4.2.2 Manipulation of seawater

The pH manipulation setup was planned as per the earlier method of Riebesell et al. (2007). In short, the acidification set-up consists of fifteen parts, which include a CO_2 cylinder, cylinder valve, pressure meter, flow meter, CO_2 gas tube, CO_2 valve, CO_2 diffuser, pH meter, pH probe, aerator, air controller, air vent, air stone, aquaria (50 L) and PVC pipes (Fig.. 4). The experimental system consisted of six aquaria with pH 8.2 (control), 7.8 (IPCC expected ocean pH), 7.6, 7.4, 7.2, and 7.0 were the pCO_2 level of 267.81, 588.03, 970.67, 1521.71, 2480.39, and 3731.44 µatm respectively. Seawater pH was manipulated by manually releasing pure CO_2 (99.9%) using a CO_2 valve through a rubber insulation tube that ends at a CO_2 diffuser to diffuse the CO_2 in the seawater of each aquarium until the desired pH was reached. The pH of each aquarium was monitored continuously using a pH meter (individual probes were always dipped in each aquarium with a separate pH meter throughout the experiment), and adjusted the desired pH manually by releasing the CO_2. Water quality factors, such as salinity, dissolved oxygen, ammonia, and total alkalinity, were evaluated according to the standard methods of APHA (2005). The carbonate ions (CO_3^{2-}), bicarbonate ions (HCO_3^-), partial pressure

of CO_2, a saturation of calcium carbonate for calcite (ΩCa) and aragonite (ΩAr), and total CO_2 (TCO_2) were determined using CO_2 calculator prescribed by Robbins et al. (2010) during the experimental period

4.2.3 Experimental setup on S. serrata

After seven days of acclimatization, a total number of 120 crabs of *S. serrata* (0.6 ± 0.05 cm length and 0.05±0.01 g weight) (Fig.. 5) were transferred into six aquaria (50 L capacity with 58 cm diameter and 26 cm height) for six different desired pH exposures (pH 8.2, 7.8, 7.6, 7.4, 7.2, and 7.0). Each aquarium was partitioned with 20 pre-holed (3.2 ± 0.23 mm × 32) PVC pipe cages (9 cm diameter and 20 cm height). Individual crabs were placed in each PVC pipe cage to prevent cannibalism (Fig. 4). The properties of seawater (salinity, temperature, dissolved oxygen, total alkalinity, and ammonia) were monitored daily. The desired pH of each experimental aquarium was employed and sustained using CO_2 gas (as detailed in the seawater acidification section) during the 60-day experimental duration. The crab instars were fed with frozen brine shrimp (*Artemia franciscana*) biomass (100% / body mass) for 15 days, and remaining 45 days, chopped white leg shrimp (*Litopenaeus vannamei*) muscle meat (30%/ body mass) with 244 ± 5 mg g^{-1}, 180 ± 8 mg g^{-1}, 188 ± 2mg g^{-1} of protein, carbohydrate and amino acid respectively twice per day (8 hr and 19 hr). The photoperiod was maintained as each 12hr for light and dark. Nearly 25% of aquarium water was renewed daily to maintain water quality, and unfed feed, moults, and crab wastes were collected daily from each aquarium. The same experimental setup was repeated twice to get triplicate results (Total crabs 120×3 = 360).

4.2.4. Survivorship, growth, moulting, and food indices

At the end of 60 days of the experiment, the survivorship, growth (length gain, weight gain and specific growth rate), moulting rate and food directories (feed intake, feed conversion ratio were calculated by following the equations (Tekinay and Davies, 2001).

$$Survivorship\ (\%) = \left(\frac{Number\ of\ live\ crab}{Number\ of\ introduced}\right) \times 100$$

$$Weight\ gain\ (g) = Final\ weight\ (g) - Initial\ weight\ (g)$$

$$length\ gain\ (cm) = Final\ length\ (cm) - Initial\ length\ (cm)$$

$$Specific\ growth\ rate\ (\%day^{-1}) = \left(\frac{\ln(final\ weight) - \ln(initial\ weight)}{total\ days}\right) \times 100$$

$$Molting\ rate\ (no.of\ molt\ day^{-1}) = \frac{Total\ number\ of\ molt}{Total\ days}$$

$$Feed\ intake\ (g\ crab^{-1}d^{-1}) = Feed\ consumption/Crab\ number/days$$

$$Feed\ conversion\ ratio = \frac{Feed\ intake}{Weight\ gain}$$

4.2.5. Statistical analysis

The significant variations of obtained data among pH treatments were compared by Duncan's multiple range test (DMRT) using one-way analysis of variance (ANOVA) in SPSS (16.0) software, and the significant variation was denoted when $p < 0.05$. All the data were communicated as mean ± SD. The survival data, specific growth rate, moister, and ash were arcsine transformed before being subject to one-way ANOVA.

4.3 Results

4.3.1. Physiochemical properties of CO_2 driven seawater

The physicochemical characteristics of seawater, including pH, dissolved oxygen, CO_3, calcite, and aragonite, exhibited a significant decrease ($P<0.05$) in all pH-manipulated seawater treatments (pH 7.8 to 7.0) compared to the control treatment (pH 8.2). However, no significant variation ($P > 0.05$) was observed in salinity, temperature, and alkalinity among the pH treatments (pH 7.8 to 7.0) compared to the control treatment at pH 8.2. Additionally, TCO_2, PCO_2, and HCO_3 levels were significantly higher ($P<0.05$) in the pH 7.8 to 7.0 treatments compared to the control treatment at pH 8.2 (Table 3).

4.3.2. Survivorship and growth

The survival rate of *S. serrata* exhibited a significant decrease ($p < 0.05$) in acidified seawater, particularly at pH 7.6 to 7.0, compared to the control pH of 8.2. The crabs exposed to pH 7.6, 7.4, 7.2, and 7.0 attained reductions in survival by 7%, 12%, 25%, and 30%, respectively, when compared to the control group. However, no significant

difference was observed between the crab groups reared at pH 7.8 and 8.2 ($p > 0.05$) (Table 4 and Fig. 8). The length, length gain, weight, and weight gain of *S. serrata* exposed to all pH treatments showed significant decreases ($p < 0.05$) compared to the ambient pH of 8.2 (Table, 4 and Fig. 6, 7, 8). While the control crabs increased their initial length by five times, those in the pH treatments exhibited growth rates of 4.3, 4, 3.5, 3.3, and 3 times, respectively. Furthermore, the crabs subjected to pH 7.8, 7.6, 7.4, 7.2, and 7.0 showed reductions in weight gain by 33%, 45%, 58%, 70%, and 75%, respectively, compared to the control. The specific growth rate of the crabs demonstrated a significant difference ($p < 0.05$) when reared at pH 7.6 to 7.0 compared to the control, with reductions of 0.92, 0.88, 0.81, and 0.77 times in pH 7.6, 7.4, 7.2, and 7.0, respectively, relative to the control (Table 4 and Fig. 8). However, crabs treated at pH 7.8 and 7.6 showed insignificant variations ($p > 0.05$) in these measurements. However, no significant variance was observed in the specific growth rate of crabs at pH 7.8 compared to pH 8.2 (Table 4 and Fig. 8).

4.3.3 Food directories and moulting rate

The feed intake of crabs exhibited a 50% reduction in all acidification experiments compared to the control, while crabs treated at pH 7.8 to 7.2 showed negligible variation ($p > 0.05$). A significant increase ($p < 0.05$) in the feed conversion ratio was observed in crabs treated with pH 7.0 (75% increase compared to the control) compared to the other pH treatments (8.2, 7.8, 7.6, 7.4 and 7.2) (Table 4 and Fig. 8). Nevertheless, the feed conversion ratio was insignificantly affected at pH 7.8 compared to the control. On the other hand, there was a significant decline ($p < 0.05$) in the moulting rate of crabs exposed to all acidified seawater treatments compared to the control. Crabs reared at pH 7.0 exhibited the highest decrease (22%) in moulting rate compared to the control. Nonetheless, there was no significant difference ($p > 0.05$) in moulting between crabs exposed to pH 7.8 and 7.6, as well as pH 7.2 and 7.0 (Table 4 and Fig. 8).

Table 3: Physicochemical properties of experimental seawater

Parameters	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
pH	8.2 ± 0.05^{a}	7.8 ± 0.05^{b}	7.6 ± 0.05^{c}	7.4 ± 0.02^{d}	7.2 ± 0.03^{e}	7.1 ± 0.06^{f}
Salinity (ppt)	34 ± 0.8^{a}	34 ± 0.7^{a}	34 ± 0.8^{a}	34 ± 0.9^{a}	34 ± 0.9^{a}	34 ± 0.9^{a}
Temperature (ºC)	26 ± 1.1^{a}	26 ± 1.1^{a}	26 ± 1.1^{a}	26 ± 1.1^{a}	26 ± 1.1^{a}	26 ± 1.1^{a}
Alkalinity (µmol kg^{-1})	1861 ± 38^{a}	1789 ± 52^{b}	1756 ± 50^{c}	1756 ± 50^{c}	1756 ± 50^{c}	1756 ± 50^{d}
DO (mg l^{-1})	6.5 ± 0.1^{a}	6 ± 0.4^{b}	5.5 ± 0.2^{c}	5.4 ± 0.15^{d}	5.2 ± 0.1^{e}	4.9 ± 0.1^{f}
TCO_2 (µmol kg^{-1})	1503 ± 14^{e}	1632 ± 20^{d}	1670 ± 28^{d}	1720 ± 33^{c}	1774 ± 32^{b}	1825 ± 28^{a}
$_{P}CO_2$ (µatm)	197 ± 29^{e}	588 ± 64^{e}	971 ± 97^{d}	1522 ± 108^{c}	2480 ± 227^{b}	3731 ± 421^{a}
HCO_3^-(µmol kg^{-1})	1266 ± 45^{e}	1507 ± 4^{d}	$1571. \pm 18^{c}$	1630 ± 29^{bc}	1674 ± 34^{ab}	1699 ± 38^{a}
CO_3 (µmol kg^{-1})	231 ± 32^{a}	110 ± 21^{b}	73 ± 13^{c}	49 ± 8^{cd}	32 ± 6^{d}	22 ± 5^{d}
Ω Ca	5.6 ± 0.7^{a}	2.6 ± 0.5^{b}	1.7 ± 0.3^{c}	1 ± 0.2^{cd}	0.8 ± 0.1^{d}	0.5 ± 0.1^{d}
Ω Ar	3.7 ± 0.5^{a}	1.7 ± 0.3^{b}	1 ± 0.2^{c}	0.8 ± 0.1^{cd}	0.5 ± 0.1^{d}	0.34 ± 0.8^{d}

n = 6; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT).

Table 4: Survival, growth, and food indices of *S. serrata* exposed to acidified seawater at different pH

Parameters	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Survival %	100 ± 0.0^{a}	100 ± 0.0^{a}	93 ± 2.9^{b}	88 ± 2.9^{c}	75 ± 5.0^{d}	70 ± 5.0^{e}
Initial length (cm)	0.6 ± 0.05^{a}	0.6 ± 0.1^{a}	0.6 ± 0.02^{a}	0.63 ± 0.05^{a}	0.63 ± 0.11^{a}	0.64 ± 0.06^{a}
Final length (cm)	3 ± 0.3^{a}	2.6 ± 0.3^{b}	2.4 ± 0.1^{b}	2.1 ± 0.1^{c}	2 ± 0.1^{cd}	1.8 ± 0.2^{d}
Length gain (cm)	2.4 ± 0.4^{a}	2 ± 0.3^{b}	1.8 ± 0.1^{b}	1.5 ± 0.1^{c}	1.3 ± 0.2^{c}	1.2 ± 0.3^{c}
Initial weight (g)	0.1 ± 0.01^{a}	0.1 ± 0.00^{a}	0.1 ± 0.01^{a}	0.1 ± 0.01^{a}	0.1 ± 0.01^{a}	0.1 ± 0.01^{a}
Final weight (g)	4 ± 1.3^{a}	2.7 ± 0.9^{b}	2 ± 0.2^{bc}	1.8 ± 0.4^{cd}	1.2 ± 0.3^{d}	1 ± 0.3^{d}
Weight gain (g)	4 ± 1.3^{a}	2.7 ± 0.9^{b}	2.2 ± 0.2^{b}	1.7 ± 0.4^{bc}	1.2 ± 0.3^{c}	1 ± 0.3^{c}
Molting (No. of molt day^{-1})	1.5 ± 1.4^{a}	1.4 ± 2^{b}	1.3 ± 1.5^{bc}	1.3 ± 1.5^{c}	1.2 ± 1.5^{d}	1.2 ± 1.4^{d}
Feed intake g day^{-1}	0.2 ± 0.1^{a}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.1 ± 0.04^{c}
SGR (% day^{-1})	2.7 ± 0.1^{a}	2.6 ± 0.1^{ab}	2.5 ± 0.04^{b}	2.4 ± 0.1^{bc}	2.2 ± 0.1^{cd}	2.1 ± 0.2^{d}
FCR	0.04 ± 0.01^{e}	0.04 ± 0.01^{e}	0.04 ± 0.02^{d}	0.05 ± 0.02^{c}	0.06 ± 0.03^{b}	0.07 ± 0.03^{a}

n= 60 for survival, moulting, FI, and FCR; n=15 for LG, WG and SGR; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT).

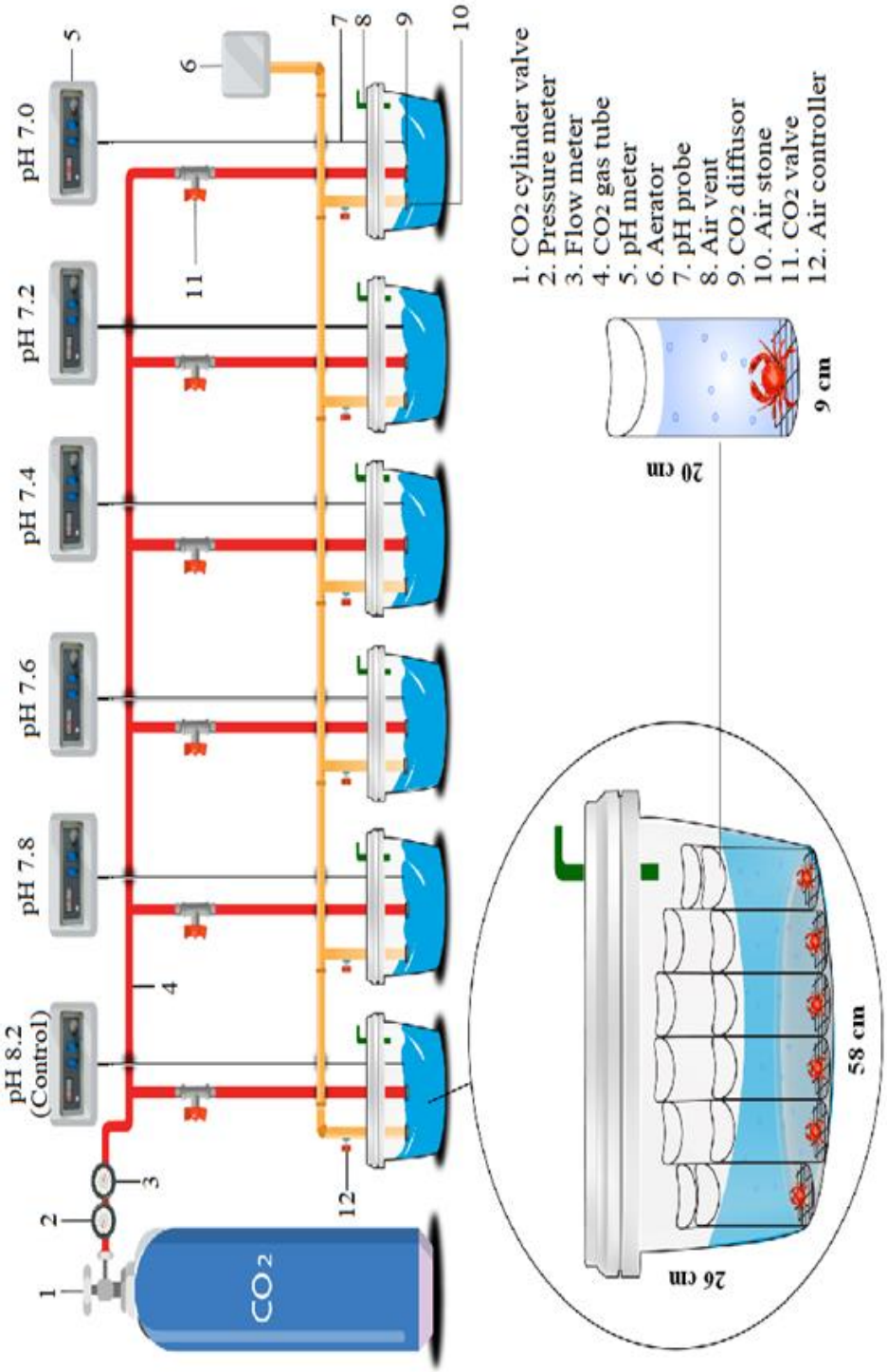


Figure 4: Design of CO_2 experimental setup with multi-cell cage for *S. serrata* instars

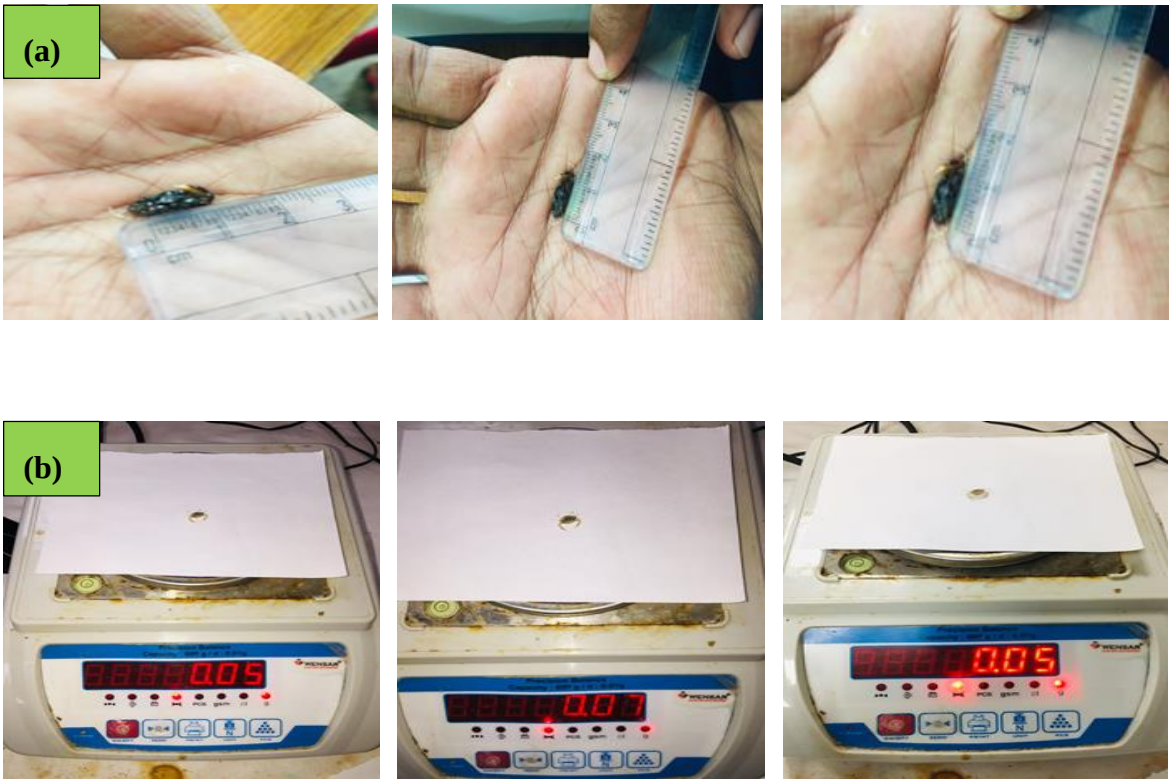


Figure 5. (a) length and (b) weight of *S. serrata* at initial stage


(a)


(b)

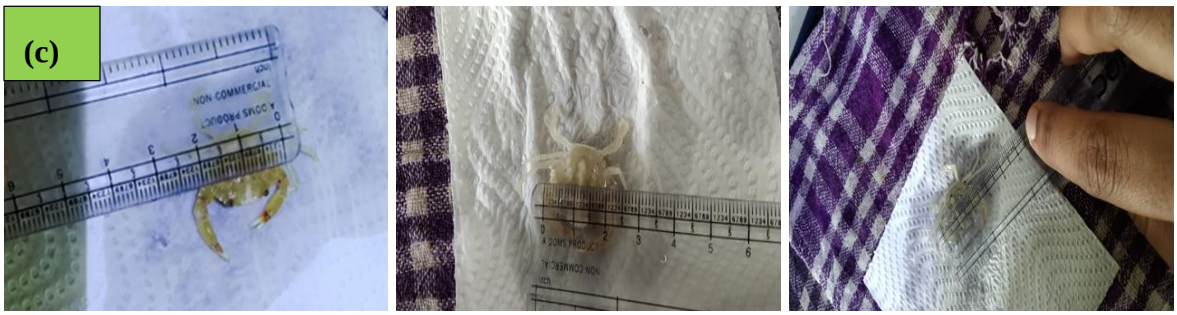
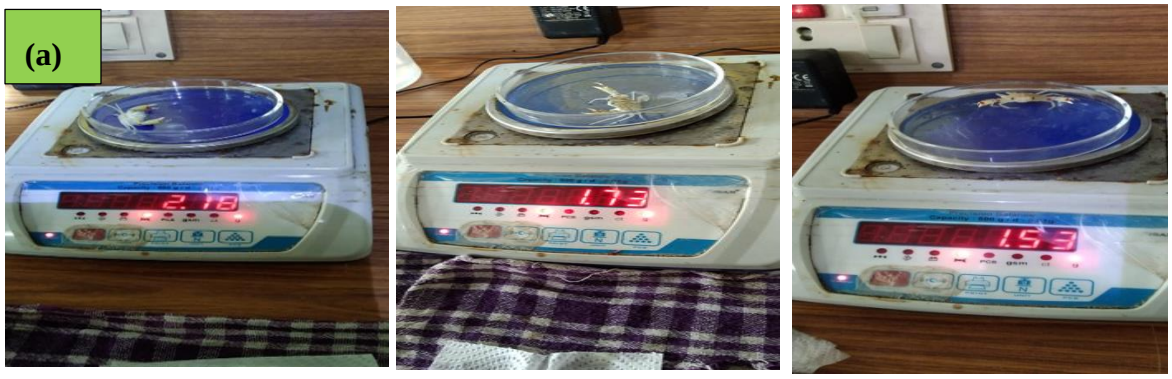

(c)

Figure 6. Length of *S. serrata* after 60 days experiment (a) pH 8.2, (b) pH 7.8, (c) pH 7.6, (d) pH 7.4, (e) pH 7.2, and (f) pH 7.0.


(a)


(b)


(c)

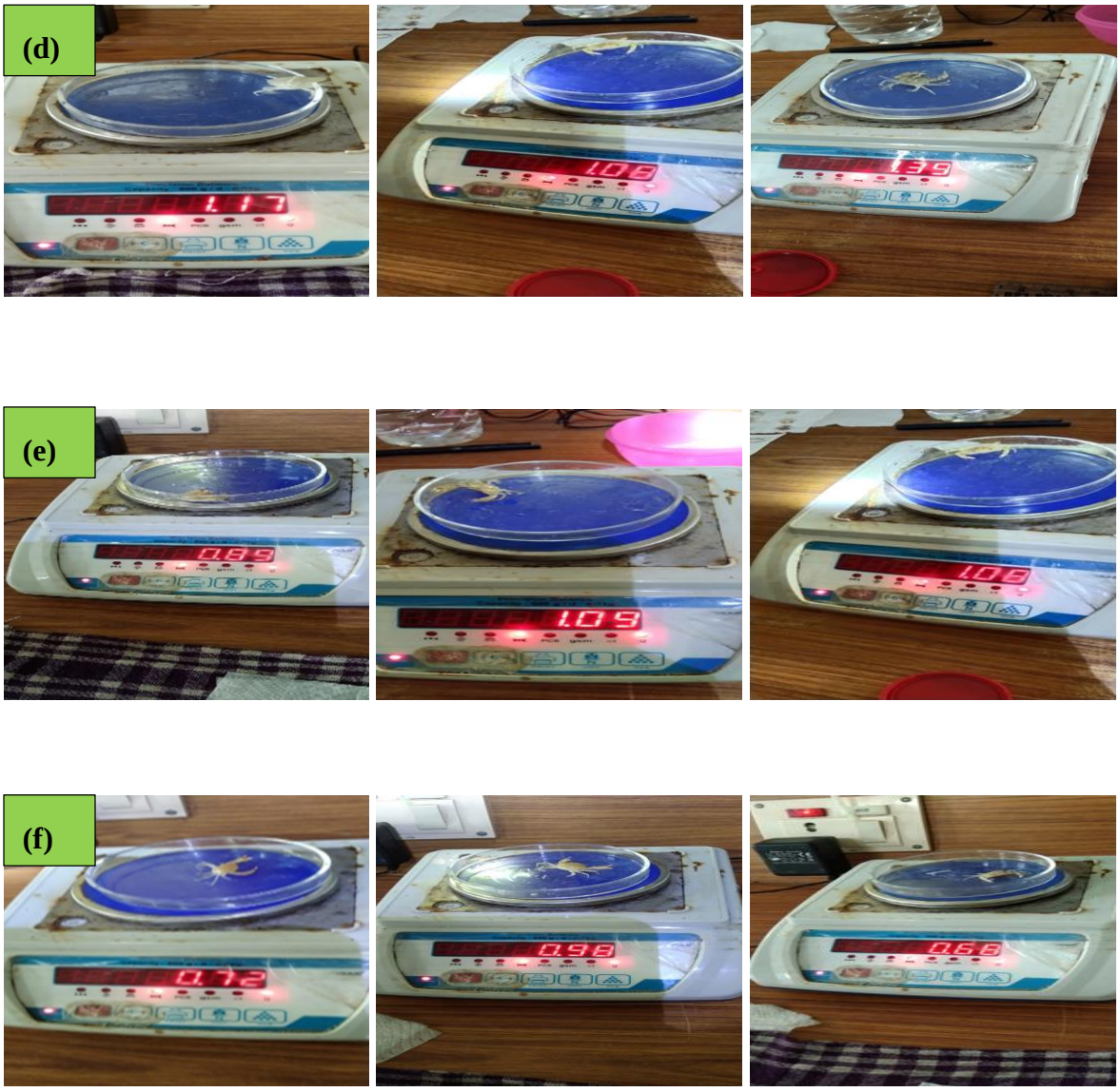


Figure 7. Weight of *S. serrata* after 60 days experiment (a) pH 8.2, (b) pH 7.8, (c) pH 7.6, (d) pH 7.4, (e) pH 7.2, and (f) pH 7.0.

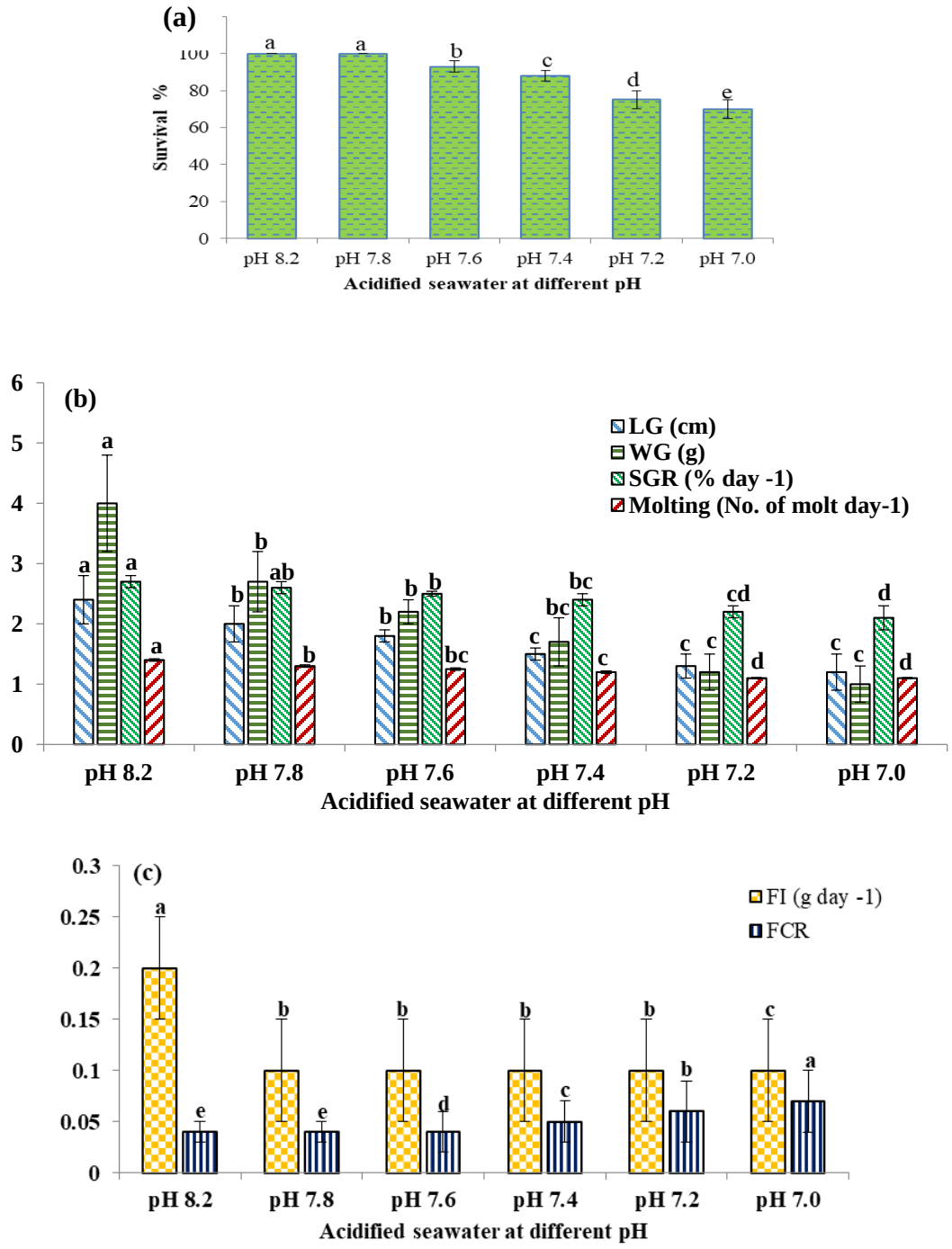


Figure 8: (a) Survival, (b) LG, WG, SGR and moulting and (c) FI and FCR of *S. serrata* exposed to CO_2 driven acidified seawater.n= 60 for survival, moulting, FI, and FCR; n=15 for LG, WG and SGR; mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups. LG: Length gain, WG: Weight Gain, SGR: specific growth rate, FI: feed intake, FCR: Feed Conversion Ratio.

4.4 Discussion

The oceans absorb approximately 30% of atmospheric CO_2 (NOAA, 2020). Increased levels of CO_2 in the atmosphere by human activities, such as burning fossil fuels, deforestation, industrial waste, and vehicle emissions, contribute to elevated pCO_2 and subsequent decreases in pH and carbonate ions within the marine environment. These reductions in bicarbonate levels harm the formation of calcium carbonate in the shells and skeletons of calcareous species. Additionally, the decreasing ocean pH adversely affects the feeding behaviour, reproduction, and physiological regulation of both invertebrates and vertebrates (Dixson et al., 2014; Pansch et al., 2018; Zlatkin and Heuer, 2019). Projections suggest that the ongoing release of CO_2 into the atmosphere will lead to a potential decrease of 0.3 pH units or more in the ocean by 2100 (IPCC, 2007; Penman et al., 2014). The future decline in ocean pH is expected to have severe consequences for the marine ecosystem, directly impacting organisms' behaviour, morphology, and physiology and indirectly affecting higher trophic-level organisms through the reduction of prey availability. Furthermore, changes in seawater chemistry can negatively impact the production of edible marine organisms, including fish and shellfish, thereby affecting seafood safety (Birchenough et al., 2017).

Marine organisms' normal biological and physiological activities heavily rely on seawater's properties. The acidification process, which alters the chemistry of seawater, can have detrimental effects on the health of marine organisms. In the present study, salinity, temperature, and alkalinity were insignificantly affected in all pH experiments, indicating that the induced seawater acidification by CO_2 did not result in significant fluctuations in these factors over the 60 days experiment. However, a noteworthy reduction in dissolved oxygen levels was observed in the acidified seawater, likely attributed to the altered metabolism of the crabs under acidic stress. This increased oxygen consumption from the surrounding environment as a mechanism to tolerate the stress. The elevation of pCO_2 levels increased TCO_2 and HCO_3^- levels in the experimental seawater while reducing saturated calcite and aragonite levels. Consequently, the scarcity of calcium and the formation of bicarbonate ions created challenges for calcareous species in shell formation. Previous studies on OA have also reported significant changes

in pCO_2, bicarbonate, calcite, and aragonite levels (Pedersen et al., 2014; Anand et al., 2021). Furthermore, alterations in the physicochemical characteristics of seawater due to high pCO_2 have been documented by Egilsdottir et al. (2009).

Growth, survival, and food consumption are crucial factors influencing crustacean production. In this study, significant reductions in survival, weight gain, length gain, moulting rate, feed intake, and specific growth rate of *S. serrata* exposed to acidified seawater indicate the detrimental effects on this species. Among the pH treatments, pH 7.4 to 7.0 negatively impacted these parameters in *S. serrata*. Changes in seawater chemistry can affect various physiological processes, such as shell formation, moulting, foraging, key enzyme activity (digestive and metabolic enzymes), hemolymph pH, reactive oxygen species (ROS) production, and cell membrane integrity. These factors appear to induce more stress on *S. serrata*, resulting in reduced survival and growth. Similar harmful effects have been observed in the growth and survival of juvenile Tanner crab (*C. bairdi*) and red crab (*Pleuroncodes planipes*) exposed to elevated pCO_2 (pH 7.5) (Long et al., 2013). Previous studies have also reported reduced survival, growth rate, and hatching in crab species, such as *C. magister* (pH 7.5 and 7.1), *Hyas araneus* (pH 7.2), and *L. aequispinus* (pH 7.5) when cultured in CO_2 enriched seawater (Miller et al., 2016; Long et al., 2021). In the present investigation, a significant decline in the moulting of *S. serrata* under acidified seawater indicates the adverse effects of OA on crab development. OA can reduce the activity of chitinolytic enzymes, such as chitinase and N-acetyl β-D-glucosidase (β-NAGase), which negatively affect moulting in crustaceans (Spindler Barth et al., 1990; Luo et al., 2015; Chen et al., 2019). Liu et al. (2022) observed a significant decrease in chitinase and β-NAGase activity, adversely impacting moulting in the horseshoe crab *T. tridentatus* under an acidified environment (pH 7.3). Similarly, exposure to pH 7.2 negatively affected moulting in the Dungeness crab *C. magister* (Reinhardt, 2020). Also, a significant reduction in daily moulting of the shrimp *L. Vannamei* exposed to OA has been reported by Muralisankar et al. (2021).

4.5 Conclusion

The present investigation demonstrated that the CO_2-driven OA led to the elevation of pCO_2, thereby reducing the levels of pH, carbonates ions, saturated calcite, and aragonite in the seawater, creating challenges to shell formation in the experimental crab *S. serrata*. Besides, the acidic stress can be responsible for poor feed intake, which causes poor growth, moulting and survival of *S. serrata.*

Chapter II

5 CHAPTER II: Impact of ocean acidification on digestive enzymes, biochemical constituents, minerals and chitin level of *Scylla serrata*

5.1 Introduction

Digestive enzymes are specialized proteins produced by the digestive system of animals. These enzymes break complex food molecules into smaller, more easily absorbable nutrients. Marine animals have diverse diets, ranging from plankton to larger prey. Therefore, digestive enzymes are essential for marine animals, enabling efficient digestion, nutrient absorption, and energy acquisition from various food sources. Digestive enzymes aid in the breakdown of proteins, carbohydrates, and lipids in food sources. Some marine animals have specialized diets that require specific enzymes for digestion. Imbalance in the secretion of digestive enzymes (trypsin, maltase, amylase, and lipase) by aquatic animals under stressful environments like temperature and ammonia has been studied earlier (Bakke et al., 2010; Amin et al., 2016; Cao et al., 2021). Studies on ocean acidification (OA) on digestive enzyme activities show that OA negatively impacts marine animals' digestive enzyme activities. For instance, the animals like fish (*Dicentrarchus labrax*), shrimp (*Ancylocaris brevicarpalis*), molluscs (*Mytilus coruscus*) and crabs (*Portunus trituberculatus*) exposed to the acidified seawater showed notable decreases in lipase, amylase, and alkaline phosphatase activities (Lin et al., 2020; Cominassi et al., 2020; Wang et al., 2020; Prakash et al., 2022).

Biochemical constituents refer to the various organic and inorganic compounds present in the bodies of marine animals. These constituents are essential in marine organisms' physiological processes, metabolism and overall functioning. Proteins are crucial biochemical constituents in marine animals serving as building blocks of tissues, enzymes and hormones. Proteins are vital in structural support, muscle contraction, immune function, and biochemical reactions necessary for growth, reproduction, and maintaining homeostasis (LaPelusa and Kaushik, 2023). Carbohydrates serve as an important source of energy for marine animals. They are broken down into simple sugars, which are utilized in cellular respiration to generate ATP, the primary energy currency of cells. Carbohydrates also play a role in energy storage, structural support, and cell signalling (Albert et al., 2002). Lipids, including fats and oils, serve multiple functions in

marine animals. They are an efficient energy storage form, providing insulation and buoyancy. Lipids are also integral components of cell membranes, playing a role in maintaining membrane integrity and facilitating various cellular processes. Additionally, lipids are precursors for synthesising hormones and serve as important signalling molecules (Horn and Jaiswal, 2019). Amino acids are the building blocks for protein synthesis and low molecular weight elements (Wu et al., 2014). The presence and balance of these biochemical constituents are vital for marine animals' survival, growth, reproduction, and overall health. Any disruptions or imbalances in these constituents can harm marine organisms' physiological processes and ecological interactions. The CO_2-driven OA seriously impacts the biochemical profile of marine species, which causes drops in nutritional availability in marine animals. The animals such as fish (*Chiloscyllium plagiosum*), clam (*Ruditapes philippinarum*), shrimp (*Litopeneaus vannamei*), brine shrimp (*Artemia franciscana*), and crabs (*C. maenas, P. criniticornis* and *Acanthocyclus hassleri*) exposed to the acidified seawater shown a significant decreases in muscle protein, carbohydrate, amino acid and lipid contents (Hammer et al., 2012; Lopes et al., 2018; Turra et al., 2019; Manríquez et al., 2020; Muralisankar et al., 2021; Thangal et al., 2021).

Minerals are inorganic substances crucial in various physiological processes, structural support, and overall health (Lall, 2003). Minerals such as calcium, magnesium, and phosphorus are fundamental for forming and maintaining skeletal structures in marine animals. Calcium carbonate is a key component of the shells and exoskeletons, providing structural support and protection. Minerals like sodium, potassium, chloride, and magnesium are involved in osmoregulation, which helps regulate fluid balance, ion concentrations, and overall cellular homeostasis (Molnar and Gair, 2015). Some minerals serve as cofactors for enzymes essential for various metabolic reactions in marine animals. Iron is a haemoglobin component that transports oxygen in the blood, while zinc is involved in enzyme activity and immune function. Carbonate and bicarbonate ions, derived from dissolved minerals in seawater, help buffer and regulate pH levels, ensuring proper acid-base balance and maintaining the physiological function of aquatic animals. The availability and balance of minerals are essential for the survival and well-being of marine animals. Any deficiencies or imbalances in mineral intake can adversely affect growth, reproduction, immune function, and overall health. The decreases in

seawater pH showed a decrease in muscle minerals levels of crustaceans like shrimps (Muralisankar et al., 2021) and crabs (*P. camtschaticus, C. bairdi, P. herbstii,* and *Pelia tumida*) due to acidified seawater can prevent the intake of minerals from water and food (Long et al., 2013; Swiney et al., 2016; Rankin et al., 2019; Dodd et al., 2021).

Chitin is a polysaccharide composed of linear chains of amino sugar and is found in the extracellular matrix of invertebrates, including crustaceans (Moussian, 2019). It serves crucial roles in providing maximum support to the exoskeleton, maintaining the organism's shape, and defending against predators (Hendriks et al., 2015). The study observed that exposure to acidified seawater with a pH of 7.0 significantly decreased chitin content in crabs. This indicates that the acidified environment had a severe impact on chitin biosynthesis. Chitin synthesizes, and chitinolytic enzymes involved in chitin synthesis in arthropods play a vital role in this process (Merzendorfer and Zimoch, 2003). The decrease in chitin levels and interference with moulting observed in the crabs suggest that OA can disrupt the activity of the enzymes responsible for chitin biosynthesis, leading to impaired shell formation and moulting. The loss of exoskeleton production in marine organisms can result in reduced survival, vulnerability to predators, and decreased claw hardness, among other consequences like capturing and feeding prey. Earlier studies have also reported reductions in chitin levels in acidified seawater treatments for tanner crabs (*C. bairdi*) and white leg shrimp (*L. vannamei*) (Mustafa et al., 2015; Muralisankar et al., 2021; Dickinson et al., 2021) since earlier reports stated that the detrimental effect of OA on the digestive enzymes, biochemical constituents, minerals and chitin of marine crabs, the impact of OA in these parameters on the economically important edible crab *S. serrata* is not yet addressed. Hence, the current chapter aimed to estimate the possible effect of the OA on the digestive enzymes (protease, amylase, and amylase), biochemical constituents (protein, carbohydrate, lipid and amino acids), minerals (sodium, potassium and calcium) and chitin level of marine mud crab *S. serrata*.

5.2 Materials and Methods

5.2.1 Procurement of crabs, sweater manipulation, and experimental setup

Collection and acclimatization of mu crab instars, seawater manipulation, experimental setup, and statistical analysis were done as stated in Chapter-I in 2.1, 2.2, 2.3 and 2.5 sections.

5.2.2 Digestive enzyme activity determination

The whole crab from each pH treatment was homogenized in ice-cold double distilled water. The resulting mixture was centrifuged at 7840 ×g under 4 ◦C for 15 minutes. The supernatant obtained after centrifugation served as a crude enzyme source for digestive enzyme analysis. Furne et al. (2005) described the casein hydrolysis method to measure protease activity, with tyrosine as a standard. Amylase activity was determined using the starch hydrolysis method outlined by Bernfeld et al. (1955), with maltose as the standard. Lipase activity was assessed according to the previous method prescribed by Furne et al. (2005), which involved the hydrolysis of triacylglycerol into free fatty acids using virgin olive oil as the substrate. The measurement of lipase activity defined one unit as the hydrolysis of one micro equivalent of fatty acids from triacylglycerols for 1 hour, under pH 8.0 at 37 ◦C. The specific activity of these enzymes was expressed as units per milligram of protein, calculated as the total activity of the enzyme divided by the amount of soluble protein (in milligrams) present in the crude enzyme source. The concentration of soluble protein in the crude enzyme extracts was determined using the standard method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard. The detailed protocol for the digestive enzyme is provided in the annexure section.

5.2.3 Biochemical constituents of the S. serrata

Determining the total soluble protein in the crab muscle was performed as per the standard procedures of Lowry et al. (1951). Briefly, 0.1 g of crab muscle tissue was homogenized in 80% ethanol and centrifuged at 1960 ×g for 15 minutes at 4 ◦C. The resulting precipitate was dissolved in 1 N NaOH to a final volume of 1 mL. Subsequently, 5 mL of alkaline copper solution was added, and the mixture was incubated at room temperature for 20 minutes. After the incubation period, 0.5 mL of Folin–Ciocalteu phenol reagent

was added and incubated for 20 minutes. The intensity of the developed colour was measured at 650 nm using a UV–visible spectrophotometer against a blank solution. Bovine serum albumin was used as a freshly prepared standard.

Estimating total amino acid levels were employed using the ninhydrin reaction method described by Moore and Stein (1948). In this procedure, 0.5 g of crab muscle tissue was homogenized with a 1:1 ratio of sodium tungstate and 0.66 N H_2SO_4 (2 mL total volume). The homogenate was then centrifuged at 1960 ×g for 10 minutes at 4 ◦C. 0.5 mL of ninhydrin reagent (4%) and 4.5 mL of distilled water were added to 0.5 mL of the supernatant. The test tubes were sealed and placed in a water bath at 80 ◦C until a purple colour developed. After cooling the tubes to room temperature, the colour intensity was measured at 540 nm using a UV–visible spectrophotometer against a blank solution. Leucine was used as a freshly prepared standard.

Determining total carbohydrates involved the hydrolysis of polysaccharides into simple sugars, following the method described by Roe (1955). Briefly, 0.5 g of crab muscle tissue was homogenized in 2 mL of 80% ethanol and centrifuged at 1960 ×g for 15 minutes at 4 ◦C. The supernatant was transferred to a test tube, and 4 mL of fresh anthrone reagent (0.2 g anthrone in 100 mL of ice-cold concentrated H_2SO_4) was added. The mixture was then placed in a boiling water bath for 10 minutes, followed by cooling in a dark room for 10 minutes. The intensity of the developed colour was measured at 620 nm against a blank using a UV–visible spectrophotometer. Glucose was used as the standard.

The Folch et al. (1957) method was used to extract the lipids using a chloroform-methanol mixture to estimate total lipid content. The estimation of lipid content was performed following the sulphophosphovanillin method described by Barnes and Black Stock (1973). For this analysis, 0.5 g of crab muscle tissue was homogenized in 4 mL of the chloroform-methanol mixture (2:1 v/v). Then, 0.2 mL of NaCl (0.9%) was added to the extraction, and the mixture was stored overnight. The lower lipid layer was carefully separated and dried in a vacuum desiccator. The dried product was dissolved in 0.5 mL of concentrated H_2SO_4, placed in a boiling water bath for 10 minutes, and then cooled to room temperature. Subsequently, 5 mL of sulphophosphovanillin reagent was added to

the solution, mixed well, and undisturbed for 30 minutes. The colour intensity was measured at 520 nm using a UV–visible spectrophotometer against a blank solution. Virgin olive oil was used as the standard. The detailed protocol for the biochemical constituents is provided in the annexure section.

5.2.4 Determination of ash and moisture contents in S. serrata

The standard procedures of AOAC, 1997 determined the tissue ash and moister contents of crabs exposed to acidified seawater. For ash determination, precisely 1 g of the sample was carefully weighed and placed in a pre-weighed crucible, ensuring it was moisture-free. The crucible containing the sample was placed in a muffle furnace and maintained at 600°C for 6 hours. Subsequently, the crucibles were transferred to a desiccator using metal tongs and allowed to cool to room temperature. The crucibles were weighed promptly to prevent moisture absorption. The percentage of ash present in the crab sample was determined using the following equation (AOAC, 2005); the detailed protocol for the ash is provided in the annexure section

$$Ash\ (\%) = \left(\frac{Weight\ of\ ash}{Weight\ of\ sample}\right) \times 100$$

The moisture content of the sample is eliminated through volatilization induced by heat. The remaining material, free from moisture, is called dry matter. Precisely 1g of the crab sample from each experimental group was carefully transferred to a pre-weighed dish and placed in an oven at 105ºC overnight. Subsequently, the dishes were removed from the oven and placed in a desiccator to cool. Once cooled, the samples were weighed to determine their weight without moisture. The following equations were calculated using the dry matter and moisture quantities (APHA, 2005). The detailed protocol for the moisture is provided in the annexure section.

$$Moisture\ (\%) = \left(\frac{Initial\ weight\ of\ sample\ (g) - Final\ weight\ of\ sample\ (g)}{Initial\ weight\ of\ sample\ (g)}\right) \times 100$$

5.2.5 Minerals and chitin levels in S. serrata

The mineral contents, specifically potassium (K), sodium (Na), and calcium (Ca), in the crab tissue from each pH treatment, were determined using a Flame Photometer (Labtronics LT-671) following the method described by Jeffery (1989). In brief, 1 g of

crab tissue from each pH experiment was digested in 10 ml of triacid (a mixture of HNO_3, H_2SO_4, and $HClO_4$ in a ratio of 9:2:1) at 80 ºC for 3 hours until a clear solution was obtained. The digested sample was then cooled to room temperature, filtered through a nylon filter (0.8µm), and mixed with 50 ml of double-distilled water for analysis. The analysis involved injecting the digested clear solution into the flame to measure the concentrations of Na, K, and Ca. After analysis, the mineral concentrations were converted to mg/kg based on the weight of the sample used for digestion.

The chitin content of the crab shells was determined following the standard method of Pandharipande and Bhagath (2016). The carapace of crabs from each pH treatment was collected and thoroughly washed with double-distilled water to remove any foreign materials. The washed shells were dried completely in a hot air oven and finely ground to obtain a powder. The dehydrated shells were treated with 7% HCl and heated at 60ºC for 2 hours to remove the phosphate content. The acid-hydrolyzed samples were subsequently treated with 6% w/v sodium hydroxide to reduce the nitrogen content derived from proteins. The samples were carefully washed to remove any residual sodium hydroxide. Each sample was filtered through a nylon filter (0.8µm) and washed multiple times with distilled water to remove trace chemicals and soluble impurities. The filtered samples were dried in a hot oven at 70ºC for 3 hours. The resulting dry product was ground into a powder, and the chitin yield was measured. Approximately 0.50 mg of crab chitin was analyzed using a Fourier transform infrared (FT-IR) spectrometer (JASCO- FT/IR 4100) within an absorption range of 4000 to 400 cm^{-1} to identify specific IR bands.

5.3 Results

5.3.1 Digestive enzymes activity of S. serrata

In this study, the protease activity of *S. serrata* was found to be significantly ($p < 0.05$) decreased in pH 7.8 to 7.0 compared with 8.2 control. At the same time, an insignificant ($p > 0.05$) variation was observed between 7.8 and 7.6 and 7.4 and 7.2. The protease level decreased to 27.45, 49.01, 64.70, 69.60, and 80.39 % in pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, over control. Amylase enzyme activity was notably ($p > 0.05$) decreased in crabs exposed to pH 7.8 to 7.0 compared with control pH (8.2). An insignificant difference was observed between the pH 7.6 and 7.4. Also, the reduction in amylase

activity was decreased to 22.85, 25.71, 26, 28.85, and 37.14 % in pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to the control crabs. The lipase activity was significantly decreased in pH 7.8 to 7.0 compared to control pH 8.2. At the same time, an insignificant variation was observed in pH 7.4 and 7.2. Also, the reduction in lipase activity revealed 10.05, 17.35, 17.80, 18.26, and 26.94 % decreases in pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to the control pH 8.2 (Table 5 and Fig. 9).

5.3.2. Biochemical constituents of S. serrata

The levels of biochemical components such as protein, carbohydrate, amino acids, and lipids were significantly reduced ($p<0.05$) in *S. serrata* reared in acidified seawater compared to the control group. The protein level decreased 25%, 31%, 37%, 50%, and 55% in crabs exposed to pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to the control. Similarly, the carbohydrate level decreased by 36%, 49%, 55%, 59%, and 61% in crabs exposed to pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to the ambient pH 8.2. The levels of amino acids declined by 12%, 18%, 20%, 27%, and 36% in crabs exposed to pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to the control. In terms of lipid content, there was a significant decrease observed in crabs reared at pH 7.4, 7.2, and 7.0, with reductions of 4%, 8%, and 13%, respectively, compared to the control crabs. However, the lipid content in *S. serrata* treated at pH 7.8 and 7.6 showed negligible variations ($p > 0.05$) when compared to the control pH (Table. 6 and Fig.. 10). The moisture content is significantly increased in experimental crabs (pH 7.8 to 7.0) when compared to control pH 8.2. In contrast, the ash content decreased in the crabs subjected to all acidification experiments compared to the control group. The crabs exhibited the maximum decrease (74%) in ash content and the maximum increase (15%) in moisture content when exposed to pH 7.0 compared to the control (Table 6 and Fig. 11).

5.3.3. Minerals and chitin levels in S. serrata

The levels of sodium, potassium, and calcium minerals in crabs subjected to acidified seawater experiments were significantly reduced ($p<0.05$) compared to those in crabs exposed to control pH (8.2). However, there was no significant ($p > 0.05$) change in potassium was observed in pH 7.4 to 7.0 (Table. 6 and Fig. 11). The results showed that crabs exposed to pH levels of 7.8, 7.6, 7.4, 7.2, and 7.0 experienced reductions of 9%,

14%, 47%, 55%, and 58% in sodium levels, 11%, 18%, 20%, 21%, and 39% in potassium levels, and 39%, 47%, 59%, 60%, and 61% in calcium levels, respectively, compared to the control group at pH 8.2.

The crab *S. serrata* was exposed to acidified seawater with pH levels ranging from 7.4 to 7.0, and showed a significant ($p<0.05$) decrease in chitin levels compared to the ambient pH conditions. The crabs attained a nearly 50% reduction in their chitin content when exposed to pH 7.4 to 7.0. However, there were no significant variations ($p > 0.05$) in chitin levels for crabs treated at pH 7.8 and 7.6 compared to the control group (Table 6). The analysis of chitin FT-IR spectra from crabs reared under various OA conditions (pH 8.2 to 7.0) revealed certain characteristic features. The spectra exhibited the presence of O–H and NH groups, which appeared in the range of 3491–3235 cm^{-1} and 3265-3116 cm^{-1}, respectively. A peak at 2888 cm^{-1} indicated the stretching vibrations of C–H, while another peak at 1074 cm^{-1} suggested the occurrence of C–O–C bands. Notably, the prominent features in the chitin spectrum were the peaks at 1500 and 1319 cm^{-1}, representing the stretching vibrations of amide II and III bands, respectively. Additionally, two amide I peak were observed at 1627 and 1649 cm^{-1}, indicating that the chitin structure derived from the crab shells exhibited the α-form (Fig. 12).

Table 5: Digestive enzymes activity of *S. serrata* exposed to acidified seawater at different pH

Parameter	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Protease (U mg protein^{-1})	1.02 ± 0.18^{a}	0.74 ± 0.08^{b}	0.52 ± 0.14^{b}	0.36 ± 0.16^{c}	0.31 ± 0.11^{c}	0.20 ± 0.04^{d}
Amylase (U mg protein^{-1})	3.50 ± 0.16^{a}	2.70 ± 0.19^{b}	2.60 ± 0.15^{c}	2.59 ± 0.18^{c}	2.49 ± 0.13^{d}	2.20 ± 0.15^{e}
Lipase (U mg protein^{-1})	2.19 ± 0.11^{a}	1.99 ± 0.16^{b}	1.81 ± 0.14^{c}	1.80 ± 0.12^{d}	1.79 ± 0.11^{d}	1.60 ± 0.15^{e}

mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT).

Table 6: Biochemical constituents, chitin, and minerals contents of *S. serrata* exposed to acidified seawater at different pH

Parameter	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Protein (mg g-1)	228.01±6.81^{a}	171.38±9.32^{b}	156.22±14.80^{c}	144.47±15.96^{d}	114.47±8.10^{e}	101.88±4.09^{f}
Carbohydrate (mg g^{-1})	85±10^{a}	54±6.3^{b}	43±8^{c}	38.4±2.6^{d}	35±1.1^{d}	33±2.6^{d}
Amino acid (mg g^{-1})	204±14^{a}	180±13^{b}	167±10^{bc}	164±7^{cd}	149±17^{d}	130±27^{e}
Lipid (mg g^{-1})	24±0.6^{a}	24±0.6^{a}	24±0.5^{a}	23±1.2^{b}	22±0.6^{c}	21±0.4^{d}
Moisture (%)	65±1^{e}	67±1.5^{d}	69±0.5^{c}	71±0.5^{c}	73±0.5^{b}	75±1^{a}
Ash (%)	8.7±1.2^{a}	3.7±1.2^{b}	3.7±0.6^{b}	3±0.6^{b}	3±0.6^{b}	2.3±0.6^{b}
Chitin (mg g^{-1})	0.9±0.01^{a}	0.9±0.01^{a}	0.93±0.01^{ab}	0.9±0.01^{bc}	0.9±0.01^{c}	0.9±0.01^{d}
Na (mg kg^{-1})	185±4.3^{a}	168±0.2^{b}	159±0.7^{c}	98±0.5^{d}	84±0.2^{e}	78±0.5^{f}
K (mg kg^{-1})	28±0.2^{a}	25±0.2^{b}	23±0.4^{c}	22.5±0.4^{c}	22±1^{c}	17±0.4^{d}
Ca (mg kg^{-1})	51±0.3^{a}	31±0.3^{b}	27±0.2^{c}	21±0.5^{d}	20.5±0.3^{e}	20±0.6^{f}

mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT). Na, Sodium; K, Potassium; Ca, Calcium.

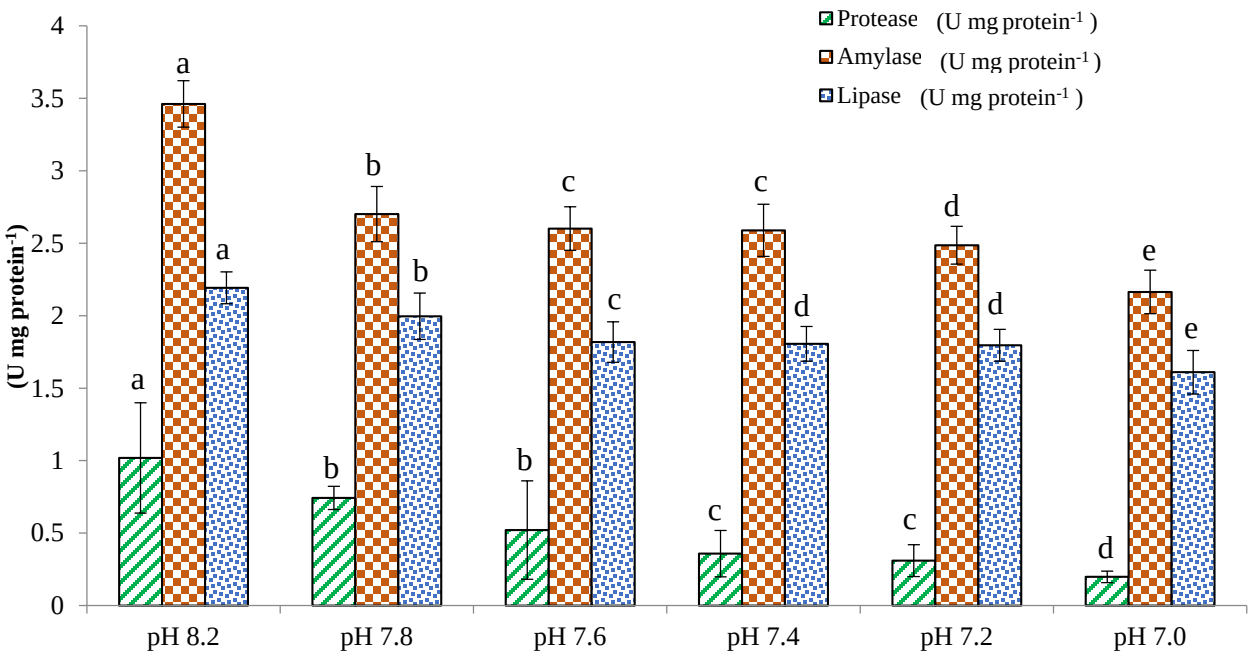


Figure 9: Protease, amylase, and lipase of *S. serrata* exposed to CO_2 driven acidified seawater. n= 3; mean ± SD; bars sharing different letters in each parameter are considered significant at $p<0.05$ compared to control (pH 8.2) and other pH-treated groups.

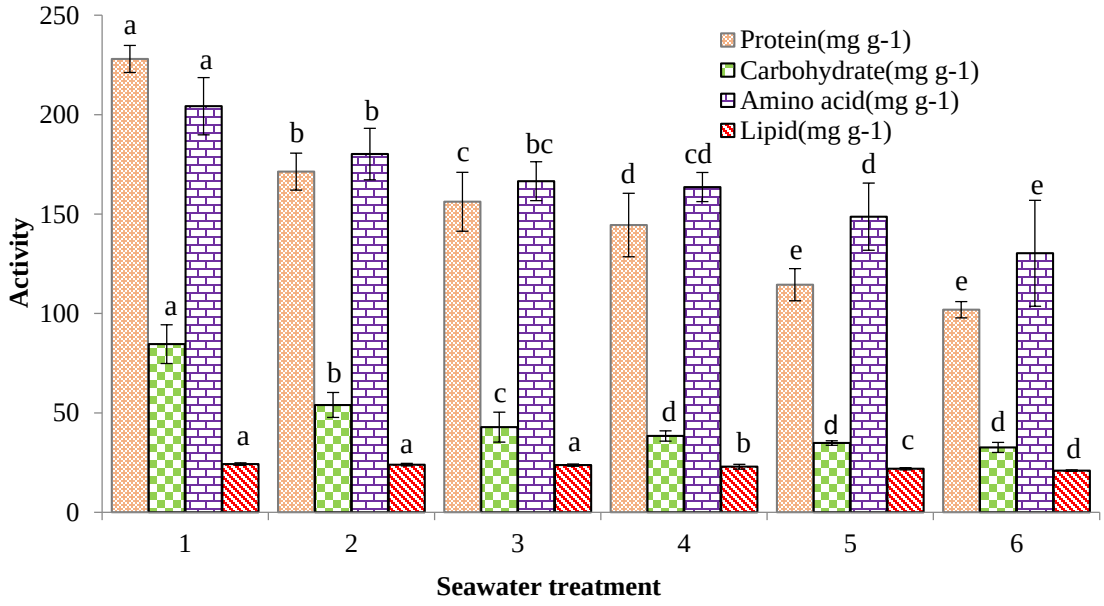


Figure 10: Protein, carbohydrate, amino acid, and lipid of *S. serrata* exposed to CO_2 driven acidified seawater. n= 3; mean ± SD; bars sharing different letters in each parameter are considered significant at $p<0.05$ compared to control (pH 8.2) and other pH-treated groups.

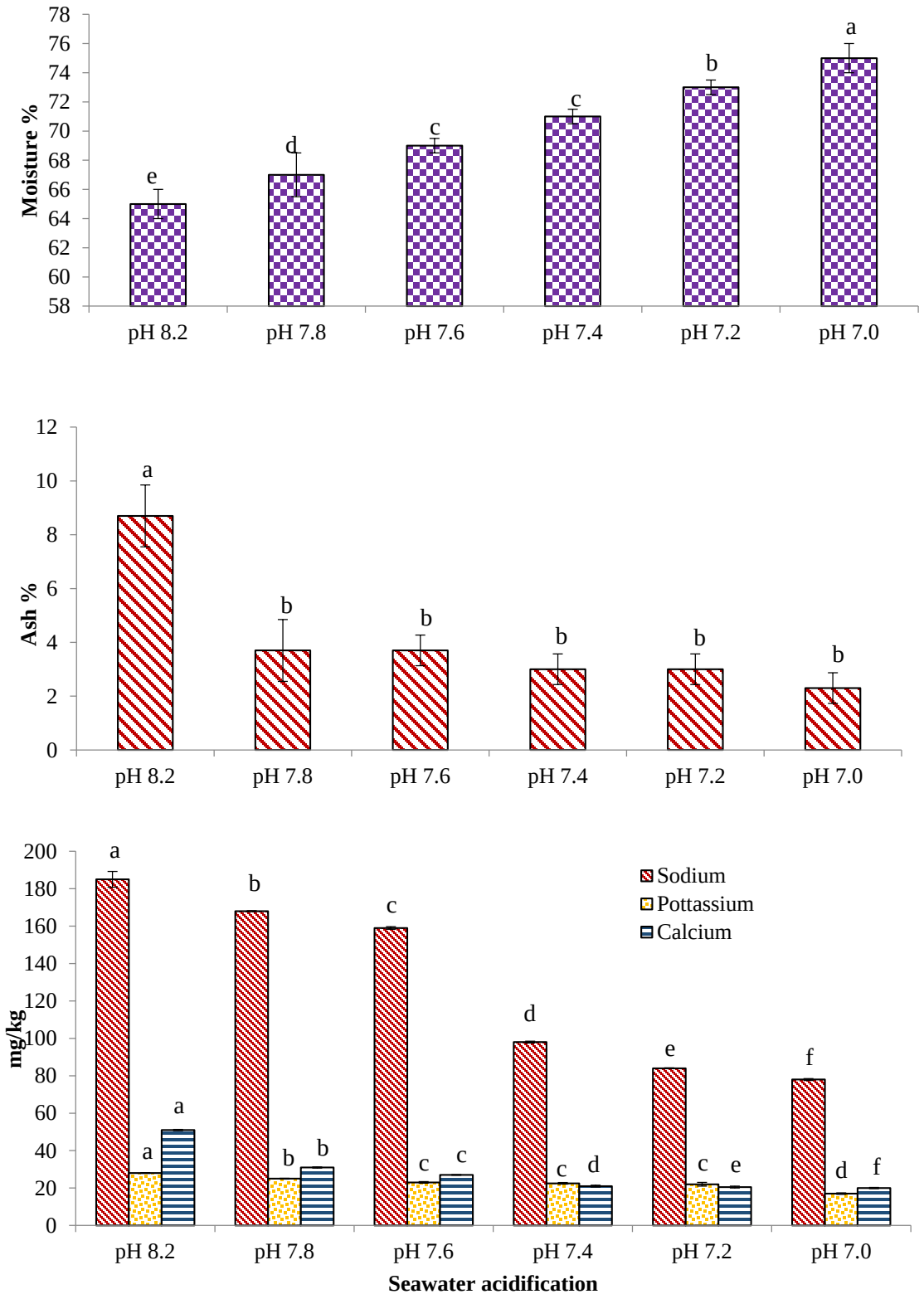


Figure 11: Ash, moisture, and minerals of *S. serrata* exposed to CO_2 driven acidified seawater. n= 3; mean ± SD; bars sharing different letters in each parameter are considered significant at $p<0.05$ compared to control (pH 8.2) and other pH-treated groups.

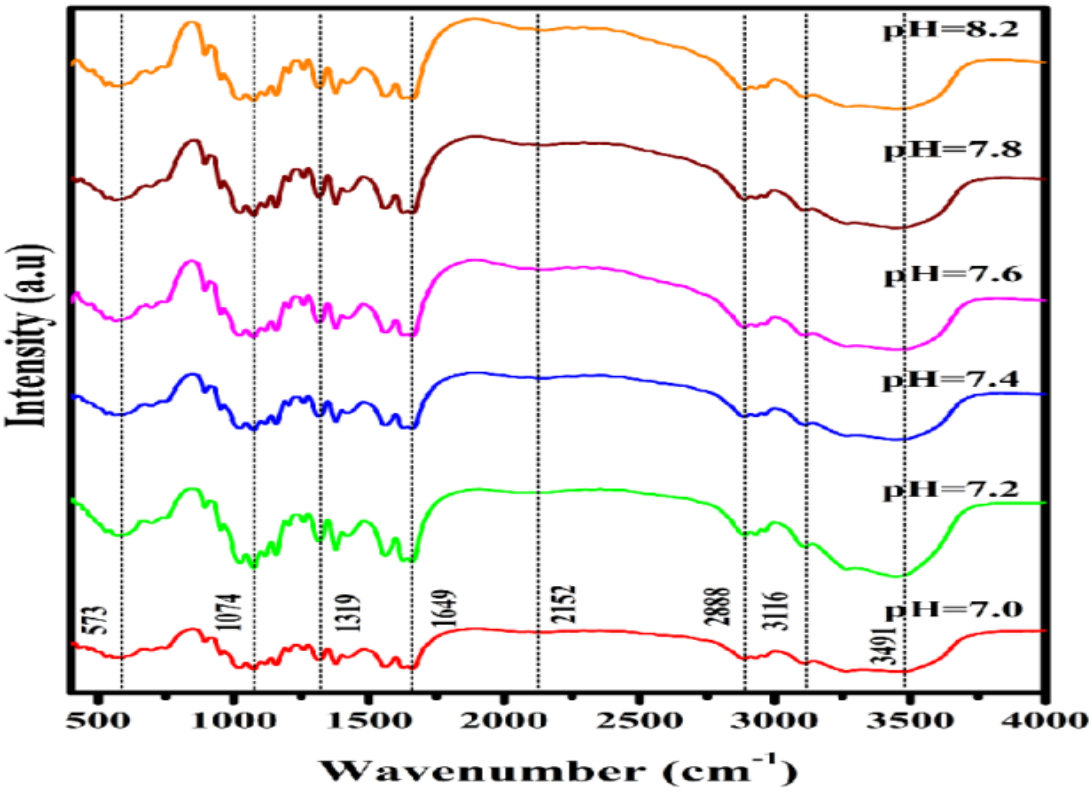


Figure 12: FTIR analysis of chitin extracted from *S. serrata* shells.

5.4 Discussion

Digestive enzymes in crustaceans play a crucial role in ensuring their survival and well-being and have a key role in nutritional physiology and regulating the growth and moult cycle (Lovett and Felder, 1990). Proteases are essential for various physiological processes in crustaceans, including the breakdown of proteins, digestion, nutrient absorption, moulting, tissue remodelling, and immune defence. Amylase in crustaceans is responsible for digesting and breaking complex carbohydrates into simpler sugars. Lipase in are responsible for lipids' digestion, absorption, and metabolism. They play a crucial role in energy acquisition, growth, and the regulation of lipid homeostasis in these organisms. They facilitate nutrient absorption, contribute to energy metabolism, and allow crustaceans to adapt to different dietary sources. In the present study, CO_2-driven OA influenced the poor secretion of digestive enzymes like protease, amylase and lipase in *S. serrata*, indicating OA's negative impact on crabs. The reductions in the lipase activity of the experimental crab might be due to the switch from the lipid and protein metabolism in the hepatopancreas (Carter et al., 2013). Also, the reductions in the digestive enzyme such as protease, amylase and lipase may be due to the reductions in feed intakes (Charron et al., 2015) of the crabs in acidified environments. In a study conducted by Lin et al. (2020) on the Chinese crab species *P. trituberculatus* they exhibited decreased activities of protease, amylase, and trypsin enzymes after being exposed to elevated pCO_2 levels (750 and 1500 µatm) for four weeks. The high pCO_2 (~1600 µatm) negatively affect the trypsin and amylase activity of *Solea senegalensis* larvae under acidified condition (Pimental et al., 2015). Lipase and trypsin activity reduction in high pCO_2 (1750 µatm) was observed in sea bass *Dicentrarchus labrax* (Cominassi et al., 2020).

Protein, lipids, amino acids, and carbohydrates are vital biochemical elements crucial in organisms' physiological and biological processes. This study observed significant decreases in these biochemical components in *S. serrata* crabs exposed to acidified seawater induced by elevated CO_2 levels in seawater. These findings indicate that the lowered pH adversely affected the biochemical constituents of *S. serrata*, highlighting the detrimental impact of OA on the physiological activities of the experimental crabs. Food utilisation decreased, such as reduced feed intake and increased energy demand under acidic stress. Thereby catabolism of tissue protein, carbohydrates,

and lipids for high energy production to tolerate the stress by *S. serrata,* which resulted in substantial reductions in the production of proteins, carbohydrates, lipids, and amino acids. Previous research by Turra et al. (2019) reported a more pronounced decrease in lipid content in hermit crabs (*P. criniticornis*) reared at a pH of 7.7 for 120 days. Similar reductions in biochemical elements, including total protein, amino acids, lipids, and carbohydrates, have been observed in shrimp species such as *L. vannamei* and brine shrimp *A. franciscana* when subjected to various acidified environments ranging from pH 7.8 and 3.5 respectively reported by Muralisankar et al. (2021) and Thangal et al. (2021). Previously a reduction in amino acid content in tiger shrimp (*Penaeus monodon*) exposed to a pH of 7.5 for four weeks was recorded (Hsieh et al., 2021).

Minerals are vital in numerous biological and physiological functions within living organisms (Lall, 2003). Calcium, potassium, and sodium are important minerals for nutrient absorption, muscle contraction, nerve signal transmission, and fluid balance in animals, including crustaceans. The present study's findings indicate that reducing essential minerals in *S. serrata* crabs reared in various acidified pH environments suggests that the acidification process may hinder the uptake of mineral salts from water and diets (Liu et al., 2020). Moreover, the decrease in calcium, particularly in the form of calcium carbonate, within the surrounding environment can lead to inadequate calcification levels in crabs inhabiting an acidified environment. The unavailability of essential minerals negatively affects organisms, including hyponatremia, weakness, fatigue, and compromised skeletal strength (Karppanen et al., 2005). Earlier studies have reported significant reductions in calcification in various crab species, such as red king crab *P. camtschaaticus* (pH 7.7), snow crab *C. bairdi* (pH 7.8 and 7.5), and Atlantic crab *Panopeus herbstii* (pH 7.86, 6.98, and 6.97) when exposed to seawater with low pH levels (Long et al., 2013; Swiney et al., 2016; Dodd et al., 2021). Similarly, Rankin et al. (2019) observed a reduction in calcium and magnesium levels in decorator crabs *Pelia tumida* (pH 7.74) exposed to acidified water during five weeks experiment. The alteration of pH due to elevated CO_2 has also been found to affect the characteristics of sodium, potassium, and calcium in the mineralized cuticle of crab species such as *P. camtschaticus* and blue crab *P. platypus* (pH 7.8 and 7.5), as studied previously by Coffey et al. (2017).

Chitin, an amino sugar-based linear chain polysaccharide in the extracellular matrix of invertebrates, including crustaceans, serves critical functions. It supports the exoskeleton, maintains the organism's shape, and offers defence against predators (Hendriks et al., 2015; Moussian, 2019). The present study reveals a significant decrease in chitin levels in crabs exposed to acidified seawater with a pH of 7.0, indicating the severe impact of the acidified environment on chitin biosynthesis. Chitin synthetases and chitinolytic enzymes play key roles in chitin synthesis in arthropods (Merzendorfer and Zimoch, 2003). The observed reductions in chitin levels and moulting disruption in crabs suggest that ocean acidification could disrupt the enzymes responsible for chitin biosynthesis, leading to impaired shell formation and moulting. The loss of exoskeleton production in marine organisms results in reduced survival, diminished ability to escape predators, and decreased claw hardness. Previous studies have also reported a decrease in chitin levels in tanner crab *C. bairdi* (at pH 7.8 and 7.5) and white leg shrimp *L. vannamei* (at pH 7.4, 7.5, 7.2, and 7.0) exposed to acidified seawater (Mustafa et al., 2015; Muralisankar et al., 2021; Dickinson et al., 2021). These findings further support the understanding that acidification negatively affects chitin production in various crustacean species, which can harm their survival and physical characteristics.

The chitin FTIR spectrum obtained in this study exhibits similarities to previous reports on crustaceans, including crabs reported by Fernando et al. (2016) and Mohan et al. (2021). Furthermore, the FT-IR analysis in this study reveals significant structural changes in the chitin composition of *S. serrata* crabs reared in acidified environments compared to those raised in the control pH of 8.2. Although the spectra of chitin from both control and experimental groups are similar, notable broadening was observed in spectral bands for the CH ring at 855 cm^{-1} and amide I at 1649 cm^{-1} in crabs reared at pH 7.4 to 7.0 when compared to the control group. Additionally, the spectra of C-O-C at 1074 cm^{-1}, amide III at 1319 cm^{-1}, amide II at 1500 cm^{-1}, N-H stretching at 3116 cm^{-1}, and O-H stretching at 3419 cm^{-1} exhibit noticeable broadening in *S. serrata* chitin at pH 7.0 compared to both the control group and other pH treatments. These alterations indicate the impact of the acidified environment on the quality of chitin in crabs. Similar findings have been observed in the cuticle of the carapace of the crab *C. bairdi* exposed to pH 7.5 (Dickinson et al., 2021).

Additionally, changes in the FT-IR spectrum of shell powders from sea urchin *Salmacis virgulata* exposed to pH 7.8 and 7.6 have been noticed compared to the control pH of 8.2 (Anand et al., 2021).

5.5 Conclusion

The present chapter revealed that crabs under OA had decreased digestive enzymes, leading to poor nutrient utilization, including minerals. Besides, under acidic environments, biochemical elements are utilized for energy production to tolerate the toxic stress, which might be the reason for decreased biochemical constituents in crabs. The qualitative and quantitative changes of chitin in crabs under OA suggest the negative impact of OA on crabs under changing seawater chemistry, such as low pH, decreased carbonates, calcite and aragonite.

Chapter III

6 CHAPTER III: Amino acids and fatty acids profile of *Scylla serrata* exposed to ocean acidification

6.1 Introduction

Amino acids are the building blocks of proteins, essential macromolecules in living organisms. Amino acids are organic compounds composed of an amino group ($-NH_2$), a carboxyl group (-COOH), and a side chain (R group). Amino acids are crucial for synthesising proteins, which play diverse roles in animals; they provide the necessary building blocks for forming new tissues, including muscles, organs, and skin. The demand for amino acids increases during the rapid growth of organisms, like larval stages or reproductive phases. Amino acids are vital for synthesising and activating enzymes, enabling metabolic processes such as digestion, respiration, and energy production. Enzymes facilitate chemical reactions, ensuring efficient nutrient utilization and metabolism. In animals, amino acids such as glycine and proline are involved in osmoregulation and ion balance. They help maintain cellular integrity and fluid balance, ensuring the proper functioning of cells and organs in varying salinity conditions. Some amino acids, such as cysteine and glutathione, act as antioxidants and detoxifying agents in animals. They help neutralize harmful reactive oxygen species (ROS) and detoxify toxins and pollutants, protecting cells and tissues from oxidative damage. Amino acids are essential for the proper functioning of the immune system in marine animals. They contribute to synthesising antibodies, cytokines, and immune cell receptors, supporting the body's defence against pathogens and maintaining immune homeostasis. Adequate intake of essential amino acids is crucial for animals' growth, development, health, and overall physiological functioning. Their importance extends to various biological processes, supporting marine organisms' complex ecology and survival. Each amino acid possesses its unique biological function and metabolic role. For instance, leucine transforms acetyl-CoA and acetyl-acetic acid as a ketone-producing amino acid. These intermediates hold significance in carbohydrate and lipid metabolisms. Lysine, found in muscle tissue, is involved in various functions, such as the absorption of calcium from the intestinal tract, promoting skeletal growth, and forming collagen. Histidine, an essential amino acid, participates in metabolic processes like histamine production related to allergic and inflammatory reactions. It also plays a vital role in osmoregulation and energy production

during emergencies or harsh conditions (Abe and Ohmama, 1987). Cystine, required for proper vitamin B6 utilization, aids in healing burns and wounds and breaking down mucus deposits in conditions like bronchitis and cystic fibrosis. Additionally, cysteine may contribute to communication between immune system cells. Arginine is crucial for energy metabolism, particularly in maintaining glycolysis under hypoxic conditions (Gade and Grieshaber, 1986). It also plays roles in cell division, wound healing, ammonia removal, immune function, and hormone release (Tapiero et al., 2002; Stechmiller et al., 2005; Witte and Barbul, 2003). Methionine, a sulfur-containing essential amino acid, is pivotal in metabolism. It is a potent antioxidant, neutralizing free radicals generated during various metabolic processes. Methionine also plays a significant role in lipid metabolism and the proper functioning of the immune system. Isoleucine, leucine, and tryptophan serve major bodily functions, including protein synthesis, providing direct energy to muscle tissue, and various essential metabolic processes. Tryptophan also affects the immune system (Waithe et al., 1975; Moffett and Namboodiri, 2003). Phenylalanine is involved in the synthesis of tyrosine and regulates the production of neurotransmitters that influence neuronal function and cell metabolism (Li et al., 2007). Proline acts as a pathogen-killing agent, a signalling molecule, and an immune stimulator. Glycine is directly involved in calcium influx through glycine-gated channels in the cell membrane and participates in producing hemoproteins as heme. Glutamine acts as a neurotransmitter, contributes to the malate shuttle, and is involved in cellular metabolism. Alanine is directly involved in inhibiting apoptosis, stimulating lymphocyte proliferation, and enhancing antibody production, possibly through cellular signalling mechanisms (Li et al., 2007). Glutamic acid is a key component in cellular metabolism, plays a role in neurotransmission, and has significant involvement in cellular metabolism (Sapolsky, 2005). Crabs and other aquatic animals rely on dietary sources to acquire essential amino acids. Variations can influence the amino acid composition in marine animals in seawater properties like ammonia, salinity, and pH. These changes in environmental conditions have been shown to impact the amino acid profiles of aquatic organisms (Pinto et al., 2007; Hsieh et al., 2021; Rahi et al., 2021). The changes in amino acids level in aquatic animals such as crabs (*Eriocheir sinensis*), shrimp (*P. monodon* and *L. vannamei*) and brine shrimp (*A. franciscana*) under stress environments (salinity and low pH) have been studied earlier (Long et al., 2019;

Thangal et al., 2020; Hsieh et al., 2021; Muralisankar et al., 2021). The excess emission of anthropogenic CO_2 cause decreasing in seawater pH known as OA. These drops in oceanic pH negatively affect the amino acid level in marine animals like *C. maenas* exposed to acidified seawater have notable decreases in amino acids (Hammer et al., 2012). Likewise, the tiger shrimp *P. monodon* showed a notable decrease in essential amino acids contents like valine, threonine, methionine, isoleucine, histidine, phenylalanine, lysine, and arginine exposed to the CO_2-driven acidified seawater (Hsieh et al., 2021). Moreover, decreases in total amino acids in the muscle of *L. vannamei* and *A. franciscana* exposed to acidified seawater have been observed earlier (Thangal et al., 2020; Muralisankar et al., 2021).

Fatty acids are a type of lipid composed of a hydrocarbon chain with a carboxyl group at one end. Fatty acids serve as a concentrated energy source for animals. When metabolized, fatty acids yield more than twice the amount of energy compared to carbohydrates and proteins. Marine animals can store excess energy in fat, sustaining metabolic needs during food scarcity or energy-demanding activities. Fatty acids like omega-3 and omega-6 fatty acids are important for maintaining cell membrane health and flexibility. Fatty acids are involved in synthesising hormones and signalling molecules in marine animals. They are precursors for producing prostaglandins, which play roles in inflammation, reproduction, and immune response regulation. Marine animals cannot synthesize some fatty acids. They must be obtained from their diet, which are called essential fatty acids, such as omega-3 (alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) and omega-6 (linoleic acid and arachidonic acid) fatty acids. They are crucial for proper growth, reproduction, immune function, and overall health. Marine animals obtain essential fatty acids by consuming algae, phytoplankton, and other food sources rich in these fatty acids. A proper balance of fatty acids, including essential acids, is crucial for marine animals' health, growth, reproduction, and overall well-being. Their diverse roles extend to energy metabolism, cellular structure, hormone synthesis, defence mechanisms, and reproductive processes, allowing marine animals to adapt and thrive in their marine environments (Song et al., 2019; Rahi et al., 2021). Studies that reveal the changes in the fatty acids composition of marine animals, such as shrimps (*L. vannamei*, *Fenneropenaeus chinensis*, and *Marsupenaeus*

japonicas) and crabs (*S. serrata*, *C. pagurus*, and *C. maenas*) under stress environment (Cuculescu et al., 1995; Wang et al., 2007; Fan et al., 2019; Meng et al., 2019; Ren et al., 2020), however, the effect of OA on the amino acids and fatty acids composition of marine animals including crustaceans is very limited. Hence, the present chapter investigated the possible effect of amino and fatty acids composition in the marine crab *serrata* subjected to CO_2-driven ocean acidification.

6.2 Materials and Methods

6.2.1 Procurement of crabs, sweater manipulation, and experimental setup

Collection and acclimatization of mu crab instars, seawater manipulation, experimental setup, and statistical analysis were done in chapter-I's 2.1, 2.2, 2.3 and 2.5 sections.

6.2.2 Analysis of amino acids composition of crabs

The crabs' muscle tissue was analysed using a modified high-performance liquid chromatography (HPLC) method, as described by Umayaparvathi et al. (2014). In short, 100 mg of powdered crab tissue was dried at 120 °C and then digested with 1 ml of 6N HCl at 110 °C for 24 hours. The resulting mixture was subsequently dried using a vacuum evaporator. A reverse phase HPLC system (Hitachi LaChrom L-7000) with an Octadecylsilyl silica gel column was utilized for amino acid separation, operating at 34 °C. The mobile phase consisted of 850 ml of a 1.52% triethylamine solution adjusted to pH 3 with phosphoric acid and 150 ml of a propanol mixture. In brief, 20 µL of the hydrolyzed sample was injected into the instrument and processed accordingly. A UV detector at 220 nm was employed for detection purposes. The amino acids present in the sample were quantified by comparing their peaks to those of standard amino acids. The results were reported in grams per kilogram of dry weight. The detailed protocol for the amino acid profile is provided in the annexure section

6.2.3 Analysis of fatty acids composition of Crabs

The fatty acid content in the muscle tissue of crabs was assessed using gas chromatography (GC) through the direct FAME (Fatty Acid Methyl Ester) method, as described by O'Fallon et al. (2007). Briefly, 0.5 g of dried crab powder was combined

with 0.7 ml of 10N KOH and 5.3 ml of MeOH. The mixture was then incubated at 55 ºC and cooled to room temperature. Next, 0.58 ml of 24N sulfuric acid was added to the reaction and incubated at 55 ºC before being cooled. Following this, 3 ml of hexane was introduced to the reaction, and centrifugation at 1500 rpm was performed to separate the clear hexane layer (FAME). The FAME was collected in a GC vial and utilized to analyse fatty acids. The fatty acid composition was determined using a GC system with a flame ionization detector (GC-FID) (Shimadzu GC-2014). For analysis, an auto-injector injected one microliter of the FAME sample into the capillary column phase (SHRT 2560: Size 100 mm× 25 mm × 0.25 µm). The oven temperature was initially set at 100 ºC and then increased at a rate of 4 ºC per minute until reaching 240 ºC, which was maintained for one hour. Nitrogen was the carrier gas with a 0.5 ml/min flow rate. The sample's retention time and peak area were compared to standard fatty acids to quantify each fatty acid in the samples. The results were expressed as a percentage relative to 1µ1 of the methylated fatty acid sample. The detailed protocol for the fatty acid profile is provided in the annexure section

6.3 Results

6.3.1. Amino acid composition of crabs

The composition of amino acids in the muscle of *S. serrata* revealed ten essential amino acids (EAA) and ten nonessential amino acids (NEAA). The EAA such as threonine, arginine, histidine, valine, methionine, isoleucine, phenylalanine, leucine, and tryptophan were notably decreased ($p < 0.05$) in the muscle of crabs reared in OA trials compared to that in control. While an insignificant ($p > 0.05$) variations were observed in amino acids like threonine at pH 7.6 to 7.2, arginine at pH 7.4 to 7.0, histidine at pH 7.4 to 7.0, valine at pH 7.6 to 7.2, methionine at pH 7.6 to 7.2, isoleucine at pH 8.2 and 7.8, and 7.4 to 7.0, phenylalanine at pH 7.6 to 7.0, leucine at pH 7.6 to 7.2, and tryptophan at pH 7.4 to 7.0 of crabs reared at acidified seawater. The lysine level of the crab subjected to the acidified seawater showed a significant elevation in pH 7.6 compared to the other pH (pH 7.8, 7.4, 7.2 and 7.0), including control (pH 8.2). The NEAA such as aspartic acid, glutamic acid, asparagine, serine, glycine, alanine, cysteine, tyrosine and proline were significantly ($p < 0.05$) decreased in crabs subjected to pH 7.8 to 7.0 when compared

to that in control pH 8.2. While an insignificant ($p > 0.05$) difference was observed in the aspartic acid (at pH 7.6 to 7.2), glutamic acid (at pH 7.6 to 7.2), asparagine (at pH 7.4 to 7.0), serine (at pH 7.6 to 7.0), glycine (at pH 7.4 to 7.0), alanine (7.4 to 7.0), cysteine (7.6 to 7.2), tyrosine (7.6 to 6.2), and proline (at pH 7.4 to 7.0) of crabs exposed to the acidic environment. At the same time, the amino acid glutamine showed a notable increase in crabs at pH 7.6 and significantly decreased at pH 7.4 to 70 compared to the ambient pH (8.2). Furthermore, compared to the control group, the total sum of EAA and NEAA gradually decreased in crabs grown in all acidified seawater trials. In this context, insignificant changes in the ratio of EAA/NEAA were noticed in crabs subject to OA treatments compared to control crabs (Table 7 and Figure 13).

6.3.2. Fatty acids composition of crabs

In the present investigation, a total of 26 fatty acids was detected, comprising 13 saturated fatty acids (SFA), eight monounsaturated fatty acids (MUFA), and five polyunsaturated fatty acids (PUFA) (Table. 8). A notable decrease ($p > 0.05$) in SFA like myristic acid, pentadecanoic acid, palmitic acid, and heneicosanoic acid were observed in pH 7.6 to 7.0, 7.8 to 7.0, 7.8 to 7.0, and 7.8 respectively. In comparison, an insignificant difference ($p < 0.05$) was noted in the crabs' pentadecanoic acid and palmitic acid between the pH 7.6 and 7.4. At the same time, the heneicosanoic acid was noticed as below the detectable level in crabs exposed to pH 7.6 to 7.0. Nonetheless, butyric acid, margaric acid, stearic acid, arachidic acid, docosanoic acid, and lignoceric acid showed a gradual increase in pH 7.6, 7.8, 7.8, 7.8, 7.8, and 7.6 respectively when compared to the crabs in ambient pH 8.2. At the same time, insignificant variations were observed between the pH 8.2 and 7.8, 7.4 and 7.2, 7.4 and 7.2, and 7.6 to 7.2 in butyric acid, margaric acid, stearic acid, and arachidic acid, respectively. Moreover, the docosanoic acid and lignoceric acid were below detectable levels in crabs at pH 7.4 to 7.0 and 7.0, respectively. The lauric acid and methyl ester were only detected in the pH 7.6 to 7.0 and 7.0, respectively. Furthermore, the lauric acid of crabs exposed to acidified seawater gradually increased from pH 7.6 to 7.0. In MUFA, palmitoleic acid, eicosenoic acid, and linolenic acid were observed to be significant decreases ($p < 0.05$) in pH 7.8 to 7.0 when compared to the ambient pH 8.2. In comparison, an insignificant variation was observed in the palmitoleic acid of crabs exposed to pH 7. 8 and 7.6. Gradual increases in oleic

acid, linoleic acid, and eicosadienoic acid were observed in pH 7.8, 7.8, and 7.6, respectively. In contrast, an insignificant variation was observed between the pH 8.2 and 7.2, 7.2 and 7.0, 8.2 and 7.8 in oleic acid, linoleic acid, and eicosadienoic acid, respectively. The nervonic acid and α-linoleate were only detected in crabs under ambient pH 8.2. In the case of PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) showed a gradual increase in crabs treated with pH 7.8 to 7.0 compared to control pH 8.2. Also, trans-linoleate, alpha linolenate and arachidonic acid were only detected in the 7.8 and 7.6 compared to crabs exposed to control and another pH. Furthermore, it was observed that the sum of SFA and MUFA was decreased in crabs exposed to pH 7.4 to 70. In this context, the sum of PUFA and PUFA/SFA was notably raised in crabs treated with OA (pH 7.8 to 7.0). At the same time, MUFA/SFA was decreased in crabs exposed to OA treatment (pH 7.8 and 7.4 to 7.0) (Table 8 and Fig. 14).

Table 7: Amino acids composition (g/ kg dry weight) of crabs exposed to different OA treatments

	Amino acid	pH 8.2 (Control)	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
EAA	Threonine	0.430 ± 0.01^{a}	0.423 ± 0.04^{b}	0.301 ± 0.03^{c}	0.302 ± 0.01^{c}	0.302 ± 0.02^{c}	0.126 ± 0.01^{d}
	Arginine	0.325 ± 0.03^{a}	0.319 ± 0.02^{b}	0.302 ± 0.02^{c}	0.107 ± 0.02^{d}	0.108 ± 0.01^{d}	0.109 ± 0.01^{d}
	Histidine	0.393 ± 0.01^{a}	0.183 ± 0.01^{b}	0.092 ± 0.01^{c}	0.093 ± 0.02^{d}	0.093 ± 0.01^{d}	0.112 ± 0.01^{d}
	Valine	0.299 ± 0.02^{a}	0.293 ± 0.03^{b}	0.214 ± 0.02^{c}	0.215 ± 0.03^{c}	0.215 ± 0.02^{c}	0.093 ± 0.02^{d}
	Methionine	0.308 ± 0.01^{a}	0.203 ± 0.01^{b}	0.197 ± 0.01^{c}	0.198 ± 0.01^{c}	0.198 ± 0.01^{c}	0.125 ± 0.02^{d}
	Iso-leucine	0.301 ± 0.02^{a}	0.302 ± 0.02^{a}	0.295 ± 0.03^{b}	0.093 ± 0.01^{c}	0.093 ± 0.01^{c}	0.092 ± 0.02^{c}
	Phenylalanine	0.458 ± 0.04^{a}	0.398 ± 0.01^{b}	0.112 ± 0.04^{c}	0.112 ± 0.04^{c}	0.112 ± 0.04^{c}	0.119 ± 0.01^{cd}
	Leucine	0.421 ± 0.03^{a}	0.403 ± 0.05^{b}	0.292 ± 0.02^{c}	0.292 ± 0.02^{c}	0.293 ± 0.02^{c}	0.213 ± 0.02^{d}
	Lysine	0.230 ± 0.02^{b}	0.212 ± 0.01^{c}	0.302 ± 0.01^{a}	0.141 ± 0.01^{d}	0.142 ± 0.01^{d}	0.142 ± 0.01^{d}
	Tryptophan	0.419 ± 0.01^{a}	0.203 ± 0.01^{b}	0.119 ± 0.01^{c}	0.037 ± 0.02^{d}	0.038 ± 0.02^{d}	0.037 ± 0.01^{d}
∑EAA		**3.584**	**2.939**	**2.226**	**1.59**	**1.59**	**1.168**
NEAA	Aspartic acid	0.423 ± 0.03^{a}	0.314 ± 0.02^{b}	0.203 ± 0.01^{c}	0.204 ± 0.01^{c}	0.204 ± 0.01^{c}	0.213 ± 0.02^{d}
	Glutamic acid	0.299 ± 0.02^{a}	0.293 ± 0.01^{b}	0.117 ± 0.02^{c}	0.118 ± 0.01^{c}	0.119 ± 0.01^{c}	0.109 ± 0.02^{d}
	Asparagine	0.398 ± 0.01^{a}	0.339 ± 0.01^{b}	0.325 ± 0.01^{c}	0.301 ± 0.02^{d}	0.301 ± 0.02^{d}	0.302 ± 0.01^{d}
	Serine	0.209 ± 0.03^{a}	0.203 ± 0.02^{b}	0.192 ± 0.03^{c}	0.193 ± 0.03^{c}	0.193 ± 0.03^{c}	0.196 ± 0.01^{c}
	Glutamine	0.116 ± 0.01^{b}	0.113 ± 0.01^{b}	0.442 ± 0.04^{a}	0.052 ± 0.01^{c}	0.053 ± 0.01^{c}	0.053 ± 0.01^{c}
	Glycine	0.403 ± 0.04^{a}	0.209 ± 0.03^{c}	0.309 ± 0.01^{b}	0.125 ± 0.01^{d}	0.125 ± 0.02^{d}	0.126 ± 0.01^{d}
	Alanine	0.492 ± 0.02^{a}	0.192 ± 0.01^{b}	0.103 ± 0.01^{c}	0.092 ± 0.01^{d}	0.092 ± 0.01^{d}	0.092 ± 0.01^{d}
	Cystine	0.360 ± 0.02^{a}	0.356 ± 0.04^{b}	0.292 ± 0.03^{c}	0.292 ± 0.01^{c}	0.293 ± 0.02^{c}	0.093 ± 0.02^{d}
	Tyrosine	0.449 ± 0.01^{a}	0.319 ± 0.01^{b}	0.112 ± 0.01^{c}	0.113 ± 0.01^{c}	0.113 ± 0.02^{c}	0.032 ± 0.02^{d}
	Proline	0.210 ± 0.01^{a}	0.203 ± 0.01^{b}	0.093 ± 0.02^{c}	0.029 ± 0.02^{d}	0.029 ± 0.01^{d}	0.028 ± 0.02^{d}
∑NEAA		**3.359**	**2.541**	**2.188**	**1.519**	**1.522**	**1.244**
∑AA		**6.943**	**5.48**	**4.414**	**3.109**	**3.112**	**2.412**
EAA/NEAA		**1.066**	**1.156**	**1.017**	**1.046**	**1.044**	**0.938**

AA, Amino acids; EAA, Essential amino acids; NEAA, Nonessential amino acids; mean ± standard deviation (n=3); values within the same row sharing the same superscript are insignificant ($p < 0.05$) (One-way ANOVA and subsequent post hoc multiple comparisons using DMRT).

Table 8: Fatty acids composition (%/ 1 µl methylated fatty acids fatty acid) of in crabs exposed to different OA treatments

	Fatty acid	pH 8.2	pH 7.8	7.6	pH 7.4	pH 7.2	pH 7.0
	Butyric acid (C4:0)	0.095 ± 0.01^{c}	0.099 ± 0.01^{c}	0.362 ± 0.01^{a}	0.226 ± 0.01^{b}	0.057 ± 0.01^{d}	0.027 ± 0.01^{e}
	Decanoic Acid (C10: 0)	0.114 ± 0.01^{a}	BDL	BDL	BDL	BDL	BDL
	Myristic acid (C14:0)	1.195 ± 0.05^{a}	1.195 ± 0.02^{a}	1.650 ± 0.37^{b}	0.827 ± 0.25^{c}	0.786 ± 0.28^{d}	0.708 ± 0.07^{d}
	Pentadecanoic acid (C15:0)	0.940 ± 0.04^{a}	0.939 ± 0.01^{b}	0.767 ± 0.02^{c}	0.780 ± 0.05^{c}	0.713 ± 0.25^{d}	BDL
	Palmitic acid (C16:0)	9.412 ± 1.01^{a}	8.305 ± 0.51^{b}	7.201 ± 0.84^{c}	7.254 ± 0.52^{c}	6.190 ± 0.69^{e}	6.321 ± 0.04^{d}
	Margaric acid (C17:0)	1.381 ± 0.01^{e}	3.681 ± 0.32^{a}	2.212 ± 0.01^{b}	1.933 ± 0.13^{c}	1.963 ± 0.01^{c}	1.522 ± 0.01^{d}
	Stearic acid (C18:0)	4.742 ± 0.21^{e}	9.347 ± 0.91^{a}	8.999 ± 0.93^{b}	7.106 ± 0.18^{d}	7.149 ± 0.53^{d}	7.311 ± 0.83^{c}
	Arachidic acid (C20:0)	0.265 ± 0.01^{b}	0.374 ± 0.01^{a}	0.212 ± 0.03^{c}	0.205 ± 0.03^{c}	0.238 ± 0.01^{c}	0.189 ± 0.01^{d}
	Heneicosanoic acid (C21:0)	1.622 ± 0.50^{a}	0.076 ± 0.01^{b}	BDL	BDL	BDL	BDL
	Docosanoic acid (C22:0)	0.136 ± 0.02^{b}	0.393 ± 0.02^{a}	0.105 ± 0.01^{c}	BDL	BDL	BDL
	Lignoceric acid (C24:0)	0.077 ± 0.01^{e}	1.197 ± 0.05^{b}	1.619 ± 0.23^{a}	0.213 ± 0.02^{c}	0.089 ± 0.01^{d}	BDL
	Lauric acid (C12:0)	BDL	BDL	0.108 ± 0.01^{d}	0.140 ± 0.01^{c}	0.244 ± 0.01^{b}	0.289 ± 0.01^{a}
	Methyl ester (C8:0)	BDL	BDL	BDL	BDL	BDL	0.012^{a}
∑SFA		**19.97**	**25.6**	**23.23**	**18.68**	**17.42**	**16.37**
MUFA	Palmitoleic acid (C16:1)	7.964 ± 0.81^{a}	4.479 ± 0.21^{c}	4.475 ± 0.09^{c}	4.284 ± 0.13^{e}	4.697 ± 0.80^{b}	4.420 ± 0.05^{d}
	Oleic acid (C18:1)	5.621 ± 0.91^{c}	6.873 ± 0.57^{a}	6.581 ± 0.90^{b}	5.172 ± 21^{d}	5.546 ± 0.71^{c}	4.696 ± 0.08^{e}
	Eicosenoic acid (C20:1)	1.623 ± 0.11^{a}	1.153 ± 0.08^{b}	0.948 ± 0.01^{c}	0.853 ± 0.01^{d}	0.848 ± 0.01^{e}	0.794 ± 0.01^{f}
	Nervonic acid (C24:1)	0.193 ± 0.02^{a}	BDL	BDL	BDL	BDL	BDL
	Linoleic acid (C18:2)	1.977 ± 0.21^{e}	4.107 ± 0.09^{a}	2.716 ± 0.27^{d}	3.168 ± 0.04^{b}	3.036 ± 0.13^{c}	3.047 ± 0.09^{c}

	Linolenic acid (C18:3)	1.912 ± 0.25^{a}	1.287 ± 0.01^{b}	0.947 ± 0.01^{c}	0.903 ± 0.01^{d}	0.901 ± 0.01^{d}	0.839 ± 0.01^{e}
	Eicosadienoic acid (C20:2)	2.137 ± 0.11^{b}	2.205 ± 0.05^{b}	11.08 ± 0.01^{a}	1.110 ± 0.98^{c}	0.831 ± 0.05^{d}	0.689 ± 0.01^{e}
	α-linolenate (C20:3)	0.269 ± 0.01^{b}	BDL	BDL	BDL	BDL	BDL
∑ MUFA		**21.69**	**20.1**	**26.74**	**15.49**	**15.85**	**14.48**
PUFA	Eicosapentaenoic acid (EPA)	17.18 ± 0.90^{f}	23.02 ± 1.01^{e}	25.71 ± 1.10^{d}	30.12 ± 2.03^{a}	30.03 ± 2.18^{b}	29.51 ± 1.81^{c}
	Docosahexaenoic acid (DHA)	22.58 ± 0.18^{f}	25.76 ± 1.05^{e}	27.43 ± 1.08^{d}	29.92 ± 2.06^{c}	30.99 ± 2.01^{b}	40.51 ± 2.91^{a}
	Trans-linoleate. (C18:2)	BDL	2.58 ± 0.09^{a}	1.02 ± 0.01^{b}	BDL	BDL	BDL
	α-linolenate (C18:3)	BDL	9.01 ± 0.90^{a}	1.44 ± 0.03^{b}	BDL	BDL	BDL
	Methyl arachidonic acid (C20:4)	BDL	9.64 ± 0.83^{a}	8.56 ± 0.08^{b}	BDL	BDL	BDL
∑ PUFA		**39.76**	**70.01**	**64.16**	**60.04**	**61.02**	**70.02**
MUFA/SFA		**1.08**	**0.78**	**1.15**	**0.82**	**0.9**	**0.88**
PUFA/SFA		**1.99**	**2.73**	**2.76**	**3.21**	**3.5**	**4.27**

SFA: saturated fatty; USFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; BDL: Below detectable limit; Nonessential amino acids; mean ± standard deviation (n=3); values within the same row sharing the same superscript are insignificant ($p < 0.0$). (One-way ANOVA and subsequent post hoc multiple comparisons using DMRT)

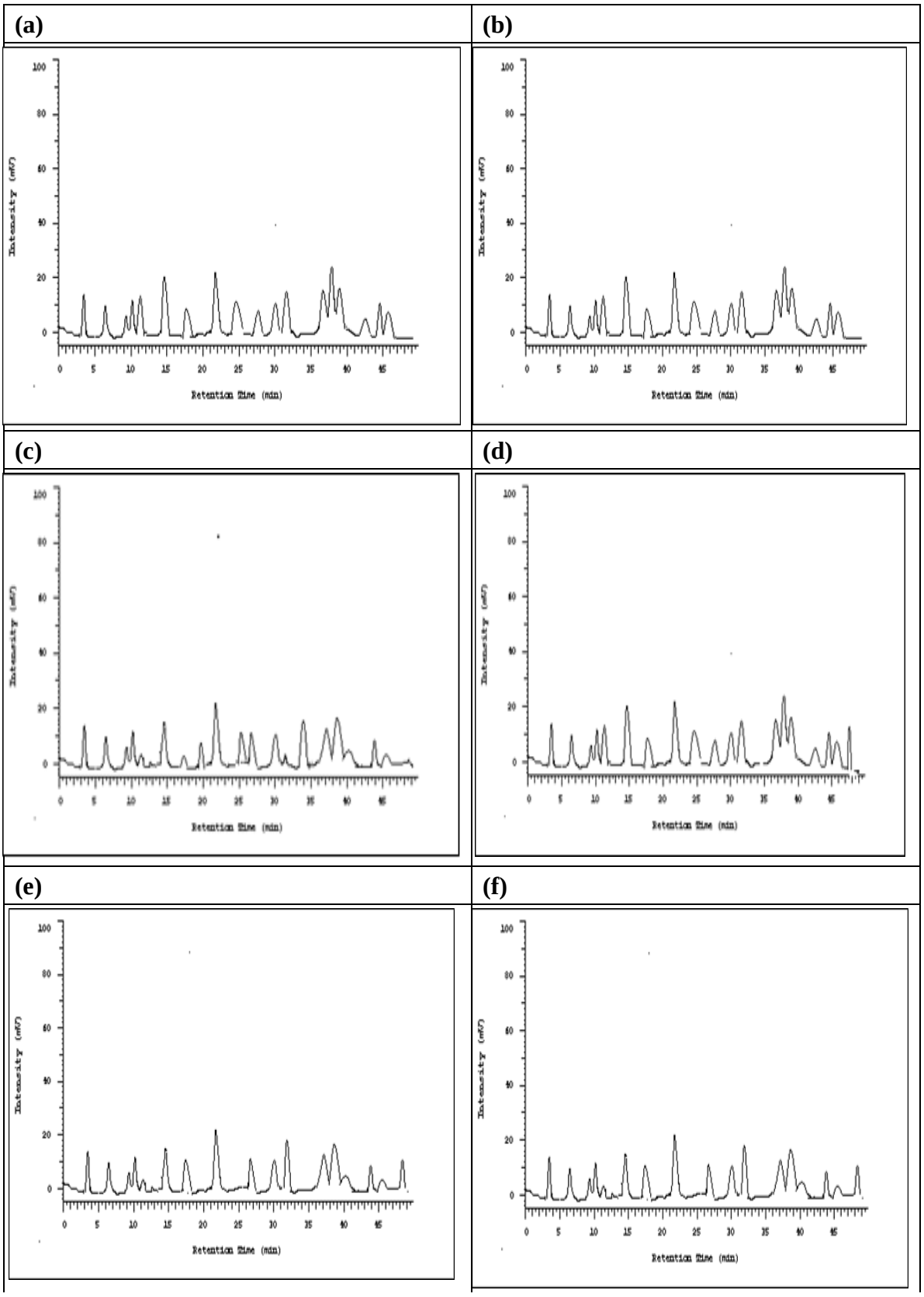
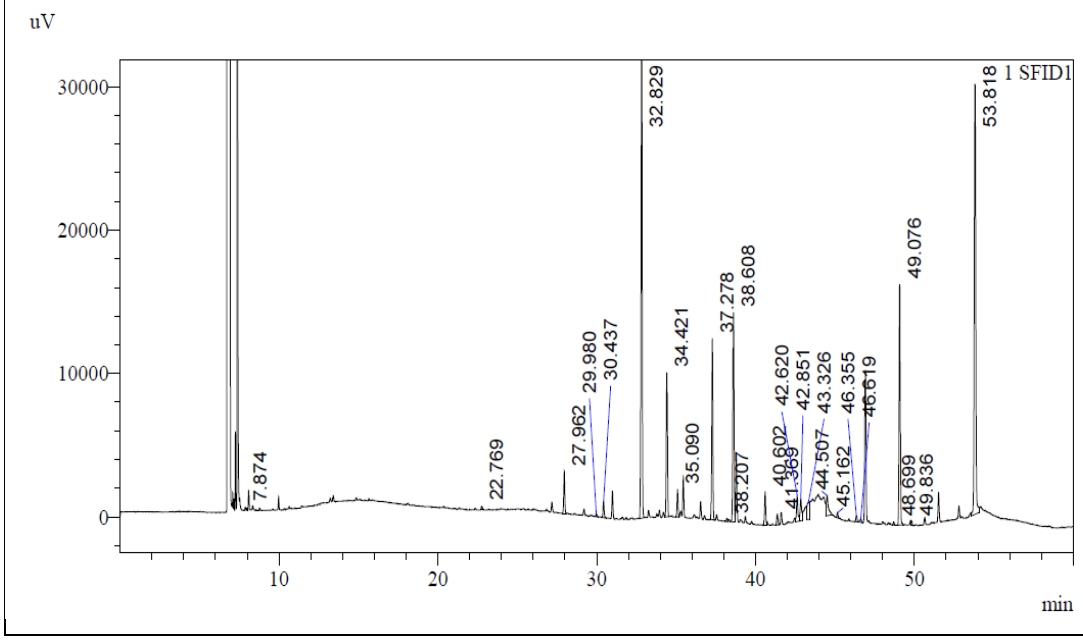


Figure 13. Chromatogram of the amino acid profiles of the experimental crabs exposed to a) pH 8.2, b) pH 7.8, c) pH 7.6, d) pH 7.4, e) pH 7.2, f) pH 7.0

(a)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38.608
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29.980
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42.620
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46.619
7.874
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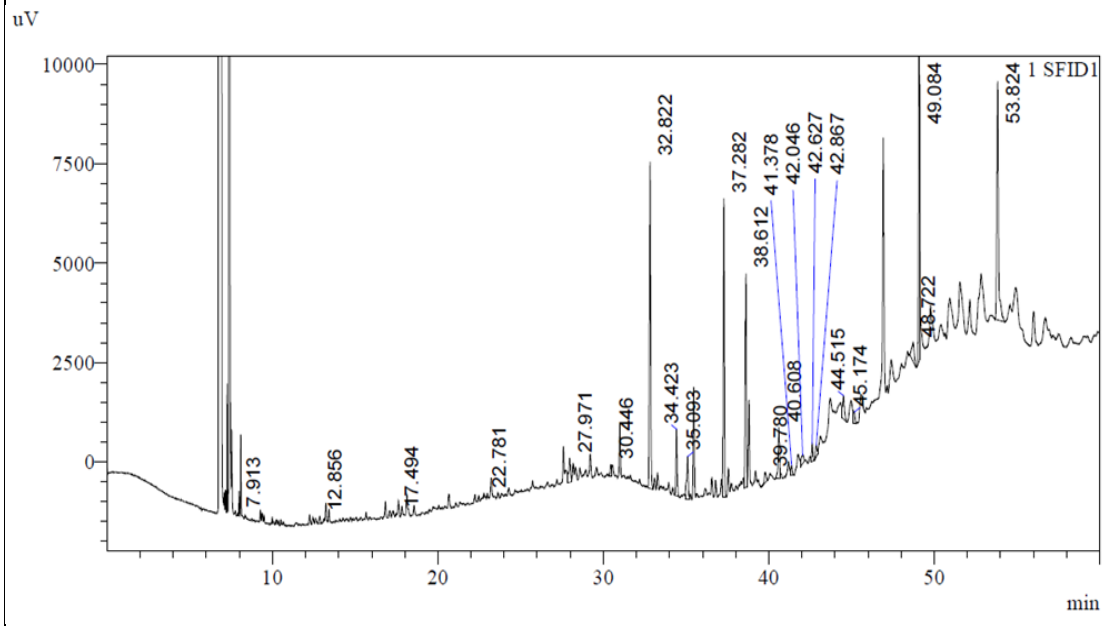
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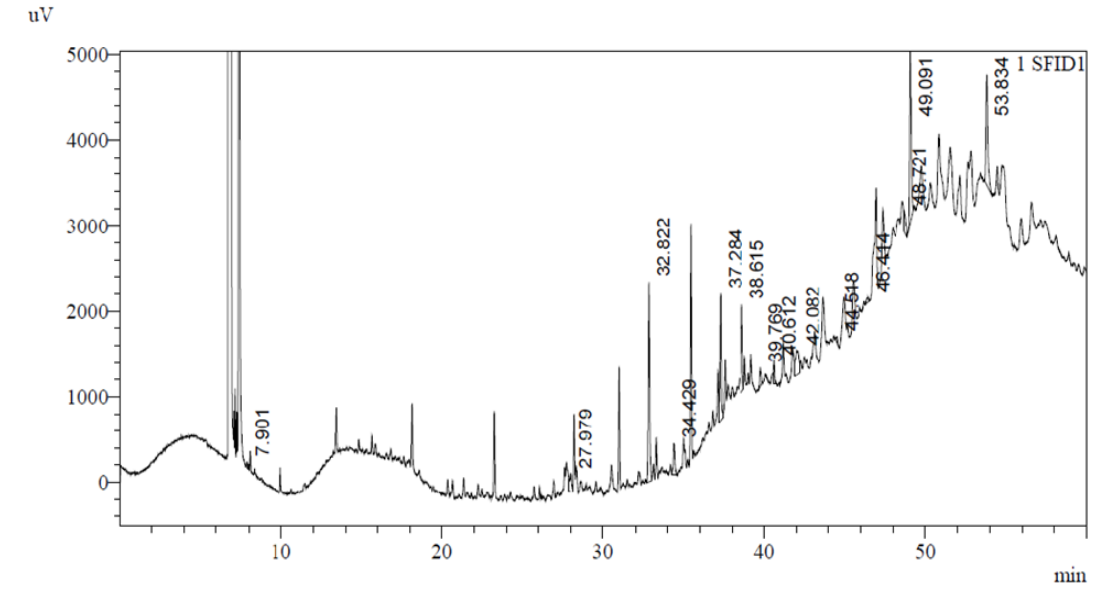
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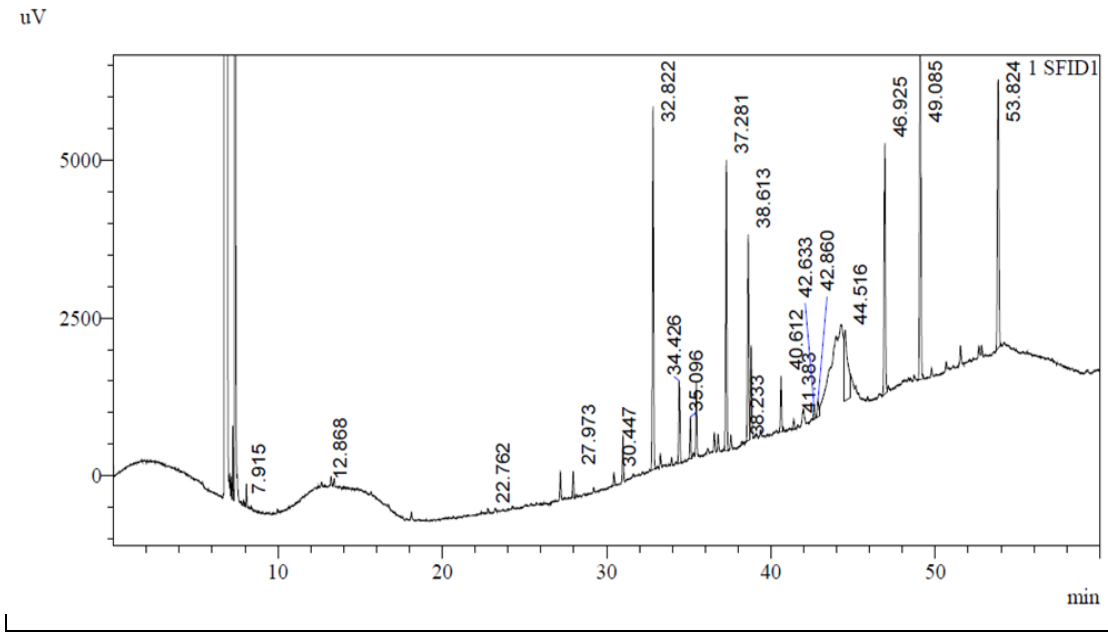
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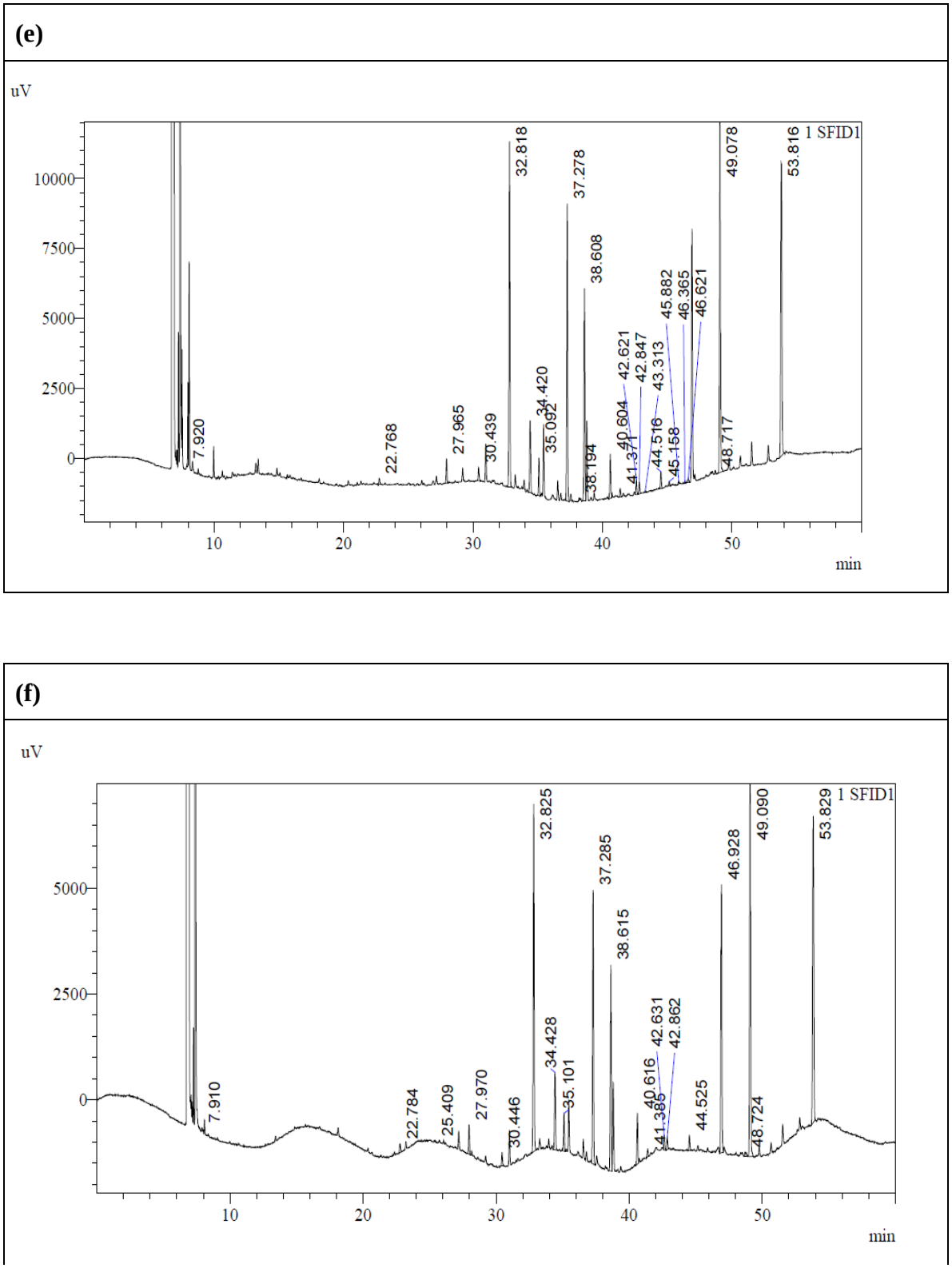


Figure 14. Chromatogram of the fatty acid profiles of the experimental crabs exposed to a) pH 8.2, b) pH 7.8, c) pH 7.6, d) pH 7.4, e) pH 7.2, f) pH 7.0

6.4. Discussion

Proteins play a crucial role in enhancing growth and regulating the physiological processes of an organism. Proteins are composed of amino acids, which have essential functions in cellular metabolism and carbohydrate and lipid metabolism in animals. Certain amino acids, known as essential amino acids (EEA), are indispensable for the metabolism and growth of animals, including crustaceans. Aquatic animals like crustaceans must acquire these amino acids from their diet. Changes in seawater properties such as ammonia, salinity, and pH can influence the amino acid composition in crustaceans, including crabs (Pinto et al., 2007; Hsieh et al., 2021; Rahi et al., 2021). In the current study, it was observed that there was a significant decrease in both EAA (threonine, arginine, histidine, valine, methionine, isoleucine, phenylalanine, leucine, and tryptophan) and NEAA (aspartic acid, glutamic acid, asparagine, serine, glycine, alanine, cysteine, tyrosine, and proline) in *S. serrata* exposed to acidified seawater environments. This decline suggests that the fluctuations in the chemical properties of seawater caused by increased CO_2 levels lead to physiological stress, followed by a substantial reduction in these amino acids in crabs. Additionally, significant reductions in both EAA and NEAA were observed in crabs reared under acidified seawater conditions with a pH range of 7.8 to 7.0, indicating the limited production of these amino acids under pH stress, leading to poor physiological activities. Furthermore, EAA lysine and NEAA glutamine are essential for metabolising macronutrients (protein, carbohydrate, and lipid), providing energy to cells and protein and DNA synthesis. In the present study, the insignificant changes in lysine and glutamine in crabs exposed between pH 8.2 (control) and 7.8 and significant elevation of these amino acids in pH 7.6 compared to control and other pH exposure indicates these amino acids did not affect up to pH 7.8 and crabs could produce these amino acids under pH 7.6 to tolerant acidic the stress. Besides, a significant reduction of lysine and glutamine in crabs exposed to pH 7.4 to 7.0 suggests that the availability of these amino acids was considerably decreased in crabs under this acidic stress, leading to poor physiological activities, followed by survival and growth. Moreover, the decrease in the total sum of EAA, total amino acids, and the ratio of EAA/NEAA in crabs exposed to all acidified seawater treatments compared to the control group revealed the detrimental impact of acidified environments on the utilization,

synthesis, and balance of amino acids in *S. serrata*. Similar findings were observed in tiger shrimp (*P. monodon*) exposed to acidified seawater derived from increased CO_2 levels (pH 7.5), where there was a significant reduction in EAA (threonine, isoleucine, valine, methionine, phenylalanine, lysine, histidine, and arginine) and NEAA (aspartic acid, serine, glutamic acid, alanine, cysteine, leucine, and tyrosine) contents (Hsieh et al., 2021). Previous studies have also reported significant reductions in the total amino acids of shrimp *L. vannamei* and brine shrimp *A. franciscana* reared in acidified environments (Thangal et al., 2020; Muralisankar et al., 2021).

Fatty acids are fundamental components of various lipids and play crucial roles in cellular membranes, energy production, and structural integrity. Unsaturated fatty acids, such as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), have diverse functions in biochemical and physiological processes, including digestion, metabolism, hormonal regulation, neural development, immune response, and reproduction in aquatic animals. While organisms can synthesize fatty acids internally, many animals, including crustaceans, cannot produce essential *n*-3 and *n*-6 fatty acids. Consequently, these organisms rely on dietary intake or precursors like linoleic and linolenic acids (Hermsen et al., 2021). Moreover, changes in abiotic factors such as salinity and temperature can also impact the utilization and synthesis of fatty acids in fish and crustaceans (Song et al., 2019; Rahi et al., 2021). In the present study, the significant decrease in saturated fatty acids (SFA) such as myristic acid, pentadecanoic acid, palmitic acid, and heneicosanoic acid in crabs exposed to acidified seawater environments indicates that OA can disrupt the synthesis of these SFAs in crabs, leading to reduced energy transfer to the cells. Moreover, the below-detectable level of the heneicosanoic acid in the pH 7.6 to 7.0 indicates that the crabs struggled to produce this fatty acid under acidic stress. The increases in butyric acid, margaric acid, stearic acid, arachidic acid, docosanoic acid, and lignoceric acid at pH 7.6, 7.8, 7.8, 7.8, 7.8, and 7.6, respectively in the muscle of the *S. serrata* suggest that these particular pH might be favoured the production of these fatty acids in crabs under acidified environment to tolerant the stress. Moreover, the decline of these fatty acids in crabs under succeeding pH environments indicates that the crabs might be struggling to produce these fatty acids. The detection of the lauric acid at pH 7.6 to 7.0 and methyl ester at pH 7.0 in the muscle of the crabs suggest that these specific pH

might be produced under these acidic conditions to tolerate the extreme acidic stress. However, the overall low amount of total SFAs recorded in crabs reared under acidified environments indicate the negative impact of OA on the synthesis of SFAs in *S. serrata*.

In this study, *S. serrata* exposed to OA exhibited significant decreases in monounsaturated fatty acids (MUFA) such as palmitoleic acid and eicosenoic acid at pH 7.8 to 7.0. These findings suggest that the acidic environment might influence the synthesis of these MUFA, which regulate metabolic processes. The gradual increases of oleic acid, linoleic acid, and eicosadienoic acid at pH 7.8, 7.8, and 7.4, respectively, indicate that the specific pH stress might be favoured for the production of these fatty acids in crabs under acidified environment (Carrillo et al., 2012). Furthermore, the notable decrease of these fatty acids in crabs under subsequent pH treatments shows that the crabs might be Fig.hting to produce these MUFA. Also, the absence of nervonic acid and α-linolenate in crabs exposed to all pH treatments indicates a potential impact on nerve-related functions and the reduction of another omega- 3 fatty acid synthesis. A commendable enhancement in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the crabs exposed to the pH 7.8 to 7.0 when compared to the control pH indicates that the high requirement of de novo synthesis of EPA and DHA in crabs to maintain physiological activities like moulting, development, neurological function and immune function. It is known that crustaceans can convert linoleic acid and linolenic acid into EPA and DHA (Kabeya et al., 2018). Similar findings were observed in copepods (*Parastenhelia* sp.) reared in acidified seawater regarding improvement of EPA, DHA, and arachidonic acid (Jayalakshmi et al., 2016). Besides, the elevations in the total levels of PUFA, and the ratios of PUFA/SFA in *S. serrata* reared in acidified environments suggest a delicate requirement for these fatty acids to tolerate acidic stress and maintain physiological activities. Fatty acids in crustaceans play a crucial role in lipid synthesis, long-term energy sources, maintenance of cell membranes, cell signalling and so on; based on the present results, it suggests that the acidified environment can affect these physiological regulations in crabs by altering the unsaturated fatty acids production.

6.5 Conclusion

The present findings reveal that most essential amino acids and fatty acids were considerably decreased in crabs exposed to OA, which denotes the adverse effect of CO_2 driven OA on amino acids and fatty acids production, leading to poor physiological activities, survival and growth in crabs. Moreover, the increment in amino and fatty acids in crabs suggests high demand for these components to tolerate acidic stress.

Chapter IV

7 CHAPTER IV: Influence of ocean acidification on antioxidants, metabolic enzymes, and alkaline phosphatase activities of *Scylla serrata*

7.1 Introduction

Antioxidants protect cells and tissues from oxidative damage caused by harmful molecules called reactive oxygen species (ROS). Antioxidants like superoxide dismutase (SOD) and catalyze (CAT) are the chief defence enzymes essential for the defence from superoxide anions formed by the oxidative process, phagocytosis, and cell damage (Rathore et al., 1998; Fang et al., 2002; Ighodaro and Akinloy, 2018). Lipid peroxidation (LPO) is a free radicle that can make oxidative degradation of polyunsaturated fats and cell membranes (Hampel et al., 2016). The environmental stressors, such as pollution, UV radiation, and fluctuations in oxygen, temperature, salinity, and pH levels lead to the generation of reactive oxygen species (ROS) in marine animals such as crab (*Neohelice granulata*), shrimp (*L. vannamei*) and brine shrimp (*A. franciscana*), which can cause oxidative damage to cells and tissues (Vargas et al., 2009; Estrada-Cárdenas et al., 2021; Long et al., 2021, Thangal et al., 2021). Antioxidants neutralize ROS and prevent their harmful effects, reducing oxidative stress and maintaining cellular health. Also, antioxidants help to maintain the integrity of cellular components, such as lipids, proteins, and DNA. ROS can cause LPO, protein oxidation, and DNA damage, leading to cellular dysfunction and potential long-term consequences. Also, antioxidants play a role in the immune response of animals. They help modulate the immune system by regulating the production of immune cells and cytokines. Previous reports observed that the OA-mediated stress in marine animals like crabs (*T. tridentatus* and *P. trituberculatus*), shrimp (*L. vannamei*), and brine shrimps (*A. sinica* and *A. franciscana*) (Zheng et al., 2015; Lin et al., 2020; Muralisankar et al., 2020; Thangal et al., 2021; Liu et al., 2022) that lead to producing considerable changes in antioxidants defence enzymes (SOD, CAT, glutathione S-transferase (GST), and glutathione peroxidase (GPx)) and LPO had been observed (Furtado et al., 2015; Zheng et al., 2015; Lin et al., 2020; Muralisankar et al., 2021; Thangal et al., 2021; Liu et al., 2022).

Metabolic enzymes are proteins that facilitate and regulate biochemical reactions involved in various animal metabolic processes. They are crucial in converting nutrients into energy, synthesizing essential molecules, and maintaining overall metabolic homeostasis. Metabolic enzymes are involved in energy production and utilization in marine animals. They facilitate the breakdown of carbohydrates, proteins, and fats through glycolysis, the citric acid cycle, and oxidative phosphorylation. These enzymes help extract energy from nutrients and convert it into adenosine triphosphate (ATP), the primary energy currency of cells. Metabolic enzymes break complex nutrients into smaller, more absorbable forms. Metabolic enzymes are essential for the efficient functioning and survival of marine animals. They enable energy production, nutrient assimilation, biosynthesis of vital molecules, detoxification processes, metabolic regulation, and adaptation to changing environmental conditions. The glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) play crucial roles in various biological processes within crustaceans' bodies and indicate impaired or compromised liver function. The GOT, also known as aspartate aminotransferase (AST), is an enzyme involved in the interconversion of aspartate and α-ketoglutarate and has a chief role in the synthesis and breakdown of amino acids (Vroon and Israili, 1990). It helps the transfer of amino groups between aspartate and α-ketoglutarate, which is essential for protein synthesis and the generation of energy (Bhagavan and Ha, 2011). Also, they play an essential role in detoxification and help to identify tissue damage. The GPT, also known as alanine aminotransferase (ALT), is another enzyme in amino acid metabolism. It catalyzes the interconversion of alanine and α-ketoglutarate. GPT participates in the metabolism of amino acids, particularly alanine. It facilitates the transfer of amino groups between alanine and α-ketoglutarate, contributing to protein synthesis and energy production (Bhagavan and Ha, 2011). Animals under the stress caused by pollution, temperature, heavy metal, hypoxia, salinity, etc., can exhibit the fluctuations in metabolic enzymes like GOT and GPT (Yang et al., 2020; Estrada-Cárdenas et al., 2021; Wang et al.., 2022) Moreover, the low seawater pH condition due to the excess emission of anthropological carbon dioxide making the marine animals are under stressful environment that leads to changes in metabolic enzymes such as GOT, GPT, and ALP in marine animals such as hermit crab *P. tanneri* (Kim et al., 2016), copepods

Calanus acuspes and *Ruditapes philippinarum* (Carter et al., 2013), shrimp *L. vamnnamei* (Muralisankar et al., 2021), and brine shrimp *A. franciscana* (Thangal et al., 2021) have been noticed earlier.

Alkaline phosphatase (ALP) is a membrane-bound metalloenzyme found in various organisms that play a key role in processing metabolites and the biomineralization process (Szabo and Ferrier, 2014). ALP is an appropriate indicator for examining the physiological state of an animal under acidic stress. (Zambonino-Infante et al., 2008). Norris and Rao (1935) reported that alkaline phosphatase activity declined by nearly 50% due to the low pH in marine invertebrates. Since ALP is involved in the regulation of mineralization processes, aiding in the deposition of calcium carbonate or calcium phosphate that forms the structural components of their skeletons. ALP plays a role in detoxifying harmful substances, including certain organic compounds and pollutants, in marine animals. The decrease in seawater pH negatively affects the ALP activities in many marine animals. For instance, the decreased activity of ALP was observed in shrimp *Ancylocaris brevicarpalis* and sea anemone *Stichodactyla haddoni* at low pH conditions (Prakash et al., 2022). The crab *T. tridentatus* subjected to the acidified environment showed a decreased ALP activity (Liu et al., 2022). Nevertheless, the effect of OA on the antioxidants, lipid peroxidation, and metabolic enzymes of marine animals, including crabs, is limited. Moreover, to our knowledge, no one report is available on the impact of OA in these parameters of edible marine mud crab *S. serrata*. Therefore, the present chapter aimed to estimate the possible effect of the OA on antioxidants (SOD and CAT), LPO, metabolic enzymes (GOT and GPT), and ALP levels in *S. serrata*.

7.2 Materials and Methods

7.2.1 Procurement of crabs, sweater manipulation, and experimental setup

Collection and acclimatization of mu crab instars, seawater manipulation, experimental setup, and statistical analysis were done as stated in Chapter-I's 2.1, 2.2, 2.3 and 2.5 sections.

7.2.2 Estimation of antioxidants and lipid peroxidation

After 60 days, each crab from the respective pH treatments was subjected to ice anaesthesia. The tissue was then extracted by homogenizing it with ice-cold tris buffer (10% w/v) at pH 7.4 and centrifugation at 2415×g for 20 minutes at 4°C. The resulting supernatant was collected and utilized as an enzyme source for evaluating the levels of antioxidants such as superoxide dismutase (SOD) and catalase (CAT), as well as lipid peroxidation (LPO). The total soluble protein content in each crab's tissue was determined using the standard method described by Lowry et al. (1951).

SOD activity in the crab was assessed by measuring the autoxidation of pyrogallol (10 mM) in tris buffer at pH 7.0, as Marklund and Marklund (1974) prescribed. The activity of SOD was reported as U/mg protein. In brief, the reaction mixture for autoxidation consisted of 2 ml of the buffer containing DETAPAC, 0.5 ml of 2 rnM pyrogallol and 1.5 ml water. Initially, the rate of autoxidation of pyrogallol was noted at an interval of one minute to three minutes. The assay mixture for the enzyme contained 2 ml of 0.05 M Tris-HCI buffer, 0.5 ml pyrogallol, aliquots of the homogenate and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol autoxidation after adding the enzyme was noted. Iron accelerates pyrogallol autoxidation even in trace amounts. DETAPAC acts as a chelator and thus prevents interference from Fe^{2+} as well as from Cu^{2+} and Mn^{2+}.

CAT activity in the crab was measured using the method of Sinha (1972). Hydrogen peroxide (H_2O_2) was utilized as the substrate in phosphate buffer, and the CAT activity was expressed as µmol of H_2O_2/min/mg protein. In short, the reaction was initiated by adding 1.0 ml of phosphate buffer (0.01M, pH 7.1), 0.5 ml of H_2O_2 (0.2 M) and 0.4 ml of distilled water successively to 0.5 ml of tissue homogenate. After 60 seconds, the reaction was stopped by adding 2.0 ml of dichromate-acetic acid reagent. Further, the tubes were kept in a boiling water bath for 10 min and cooled at room temperature. The absorbance of the chromophore was read at 620 nm. A system devoid of enzymes served as the control.

LPO in the crab was evaluated according to Ohkawa et al. (1979) by measuring the production of thiobarbituric acid reactive substances (TBARS) and reported as nanomoles of malondialdehyde (MDA). Briefly, the tubes containing 1 ml of tissue

homogenate (10% w/v in 50 mM phosphate buffer, pH 7.4) were subsequently mixed with 1 ml of tris buffer (0.02 M, pH 7.5), 1 ml of 10% trichloro acetic acid and 1.5 ml of thiobarbituric acid (1.5%). The reaction mixture was boiled for 15 min in the boiling water bath and cooled at room temperature. The content was centrifuged at 100 g for 20 min, and the supernatant was collected. The absorbance of the supernatant was measured at 535 nm against the reagent blank. The detailed protocol for the antioxidant estimation is provided in the annexure section.

7.2.3 Estimation of metabolic enzymes (GOT and GPT)

The activity of metabolic enzymes, including glutamic oxaloacetate transaminase (GOT) and glutamic pyruvate transaminase (GPT), in the crabs exposed to acidified seawater was evaluated using established methods (Reitman and Frankel, 1957) and a testing kit (HiMedia Laboratories Pvt. Ltd. Mumbai, Maharashtra, India). In summary, 100 mg of crab tissue was homogenized in a 0.25 M sucrose solution. The homogenate was then centrifuged at 2415×g for 25 minutes at 4ºC, and the resulting supernatant was used to analyse GOT and GPT activity for GOT analysis. The substrate solution, L-Aspartic acid (500 µL; pH 7.4), was added with a 100 µl sample and incubated at 37 ºC for 1 hr. Then, 500 µl of 2, 4-dinitrophenyl hydrazine was added and allowed to stand for 20 min at room temperature. Then 3 ml of freshly prepared 4 N sodium hydroxide solution was added to the above solution. The colour development was read at 505 nm using a spectrophotometer within 15 min. Sodium pyruvate (160 U/L) was used as a calibrator. For GPT analysis, buffered L-Alanine, 2- Oxoglutarate substrate (500 µl; pH 7.4) was added with 100 µl sample and incubated at 37 ºC for 20 min. With this, 500 µl of 2, 4-dinitrophenyl hydrazine was added and allowed to stand at room temperature for 30 min. Then, 3 ml of freshly prepared 4 N sodium hydroxide solution was added. The colour development was read at 505 nm using a spectrophotometer within 15 min. Sodium pyruvate (170 U/L) was used as a calibrator. The activity of both GOT and GPT was expressed as U/ml. The detailed protocol for the metabolic enzyme activity is provided in the annexure section.

7.2.4 Estimation of alkaline phosphatase

The alkaline phosphatase (ALP) activity was determined using the method described by Kind and King (1954). In brief, the entire crab, excluding the carapace, eyestalk, and legs, was homogenized in 1 ml of ice-cold citrate buffer (50 mM) and then centrifuged at 9961×g at 4 °C for 10 minutes. Four test tubes were prepared: blank (B), standard (S), control (C), and test (T). 1.05 ml of double distilled water was added to the B tube, while the remaining three tubes (S, C, and T) received 1 ml each. Next, 1000 µl of buffer reagent and 100 µl of (disodium phenyl phosphate) substrate reagent was added to all tubes. The contents were mixed thoroughly and incubated at 37 °C for 3 minutes. For the T tube, 50 µl of the extracted sample was added, while 50 µl of phenol solution was added as a standard for the S tube. These tubes were mixed well and incubated for 15 minutes at 37 °C. Finally, 1000 µl of the colour reagent (Phenol + 4-amino antipyrine) was added to all tubes, and only the C tube received an additional 50 µl of the sample. The absorbance of each tube (B, S, C, and T) was measured at 510 nm using a UV-visible spectrophotometer, with distilled water serving as the reference. The detailed protocol for the ALP is provided in the annexure section.

7.3 Results

7.3.1 Activity of SOD, CAT and Lipid peroxidation

In the present study, the SOD, CAT, and LPO levels in the crab *S. serrata* reared in all acidified seawater were significantly higher ($p < 0.05$) compared to the crabs in the control pH of 8.2. The elevation of SOD, CAT, and LPO levels in the crabs showed a gradual increase from pH 7.8 to 7.0. Specifically, the crabs exhibited a 1.42, 1.58, 1.75, 2.24, and 2.68 fold increase in SOD, 1.35, 1.53, 1.70, 2.18, and 2.68 fold increase in CAT, and 1.62, 1.92, 2.10, 2.71, and 3.10 fold increase in LPO at pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to crabs exposed the control pH (8.2) (Table 9 and Fig. 15).

7.3.2 Activity of GOT and GPT enzymes

The metabolic enzyme GOT and GPT levels were significantly enhanced ($p < 0.05$) in *S. serrata* exposed to acidified seawater compared to the control pH of 8.2. Among the different pH treatments, *S. serrata* exposed to pH 7.0 exhibited the highest levels of

metabolic enzymes, followed by pH 7.2 to 7.8. The levels of GOT in the crabs increased by 1.06, 1.22, 1.29, 1.30, and 1.44 times, while the levels of GPT in the crabs increased by 1.25, 1.26, 1.29, 1.32, and 1.51 times at pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, compared to the control crabs exposed to pH 8.2 (Table 10 and Fig. 16).

7.3.3 Activity of alkaline phosphatase

The activity of alkaline phosphatase (ALP) was significantly reduced ($p < 0.05$) in *S. serrata* exposed to pH 7.2 and 7.0 compared to crabs reared at pH 8.2. The levels of ALP decreased by 0.58, 0.58, 0.41, 0.16, and 0.09 times at pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, relative to the control pH. However, crabs treated at pH 7.8 to 7.4 showed no significant difference in ALP levels compared to the control (Table 10 and Fig. 16).

Table 9: Antioxidants and lopid peroxidation status of *S. serrata* exposed to acidified seawater at different pH

Parameters	pH 8.2 (control)	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
SOD	5.59 ± 0.28^{f}	7.97 ± 0.31^{e}	8.85 ± 0.29^{d}	9.81 ± 0.37^{c}	12.57 ± 0.32^{b}	14.99 ± 0.38^{a}
CAT	0.54 ± 0.01^{f}	0.73 ± 0.02^{e}	0.83 ± 0.01^{d}	0.92 ± 0.03^{c}	1.18 ± 0.03^{b}	1.46 ± 0.02^{a}
LPO	2.48 ± 0.23^{f}	4.03 ± 0.22^{e}	4.77 ± 0.08^{d}	5.23 ± 0.11^{c}	6.74 ± 0.24^{b}	7.69 ± 0.29^{a}

n = 3; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT).

Table 10: Metabolic enzymes activity of *S. serrata* exposed to acidified seawater at different pH

Parameters	pH 8.2 (control)	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
GOT	28.75 ± 0.67^{e}	30.71 ± 0.92^{d}	35.11 ± 0.65^{c}	37.32 ± 0.07^{b}	37.49 ± 0.18^{b}	41.42 ± 2.11^{a}
GPT	21.30 ± 0.75^{c}	26.82 ± 1.59^{b}	27.00 ± 0.39^{b}	27.67 ± 1.14^{b}	28.22 ± 3.77^{b}	32.31 ± 2.93^{a}
ALP	1.95 ± 0.05^{a}	1.22 ± 0.03^{ab}	1.21 ± 0.02^{ab}	0.89 ± 0.21^{ab}	0.31 ± 0.03^{b}	0.22 ± 0.02^{b}

n = 3; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT).

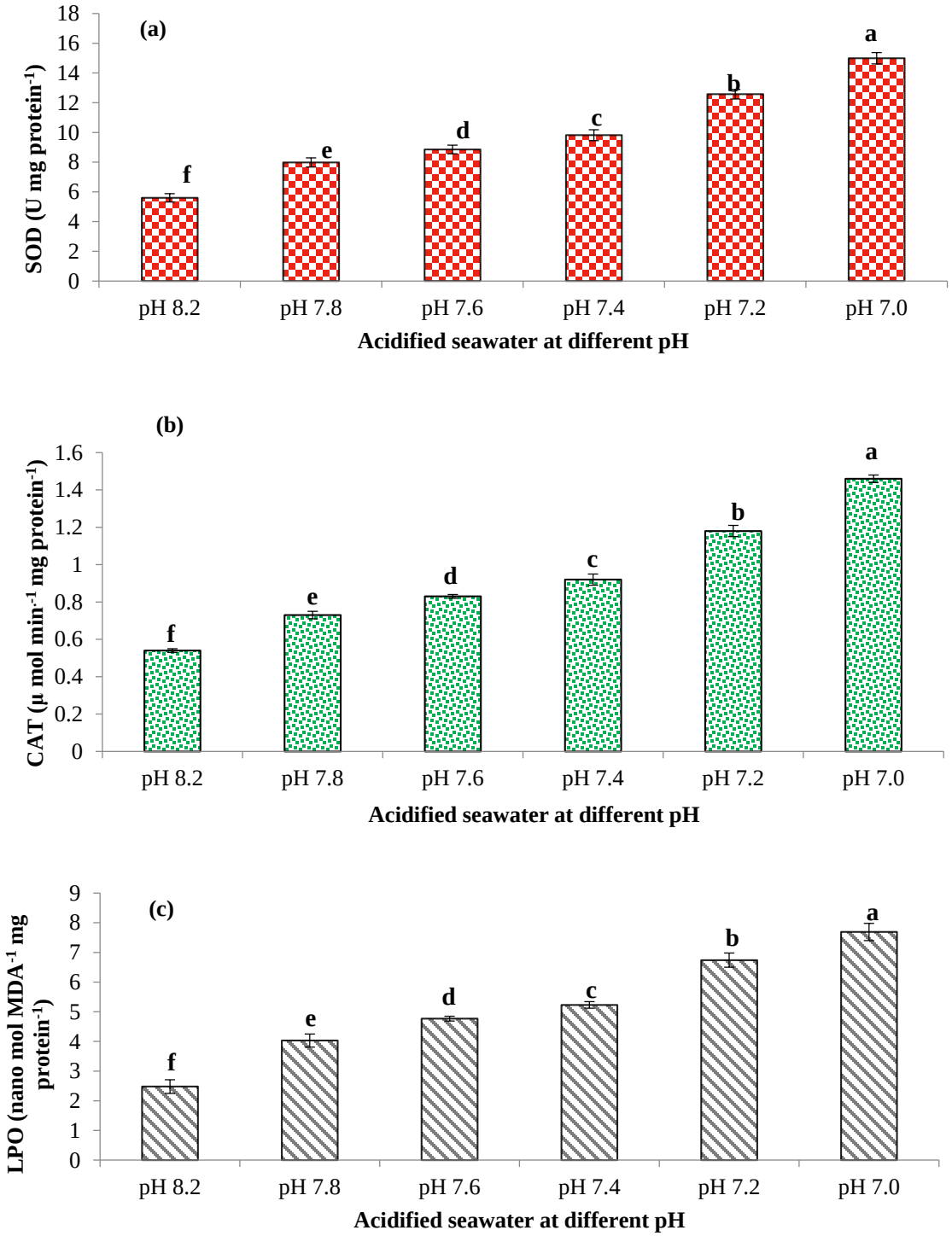


Figure 15: (a) SOD, (b) CAT, and (c) LPO of *S. serrata* exposed to CO_2 driven acidified seawater. n = 3 (three samples from each treatment), mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH-treated groups. SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation.

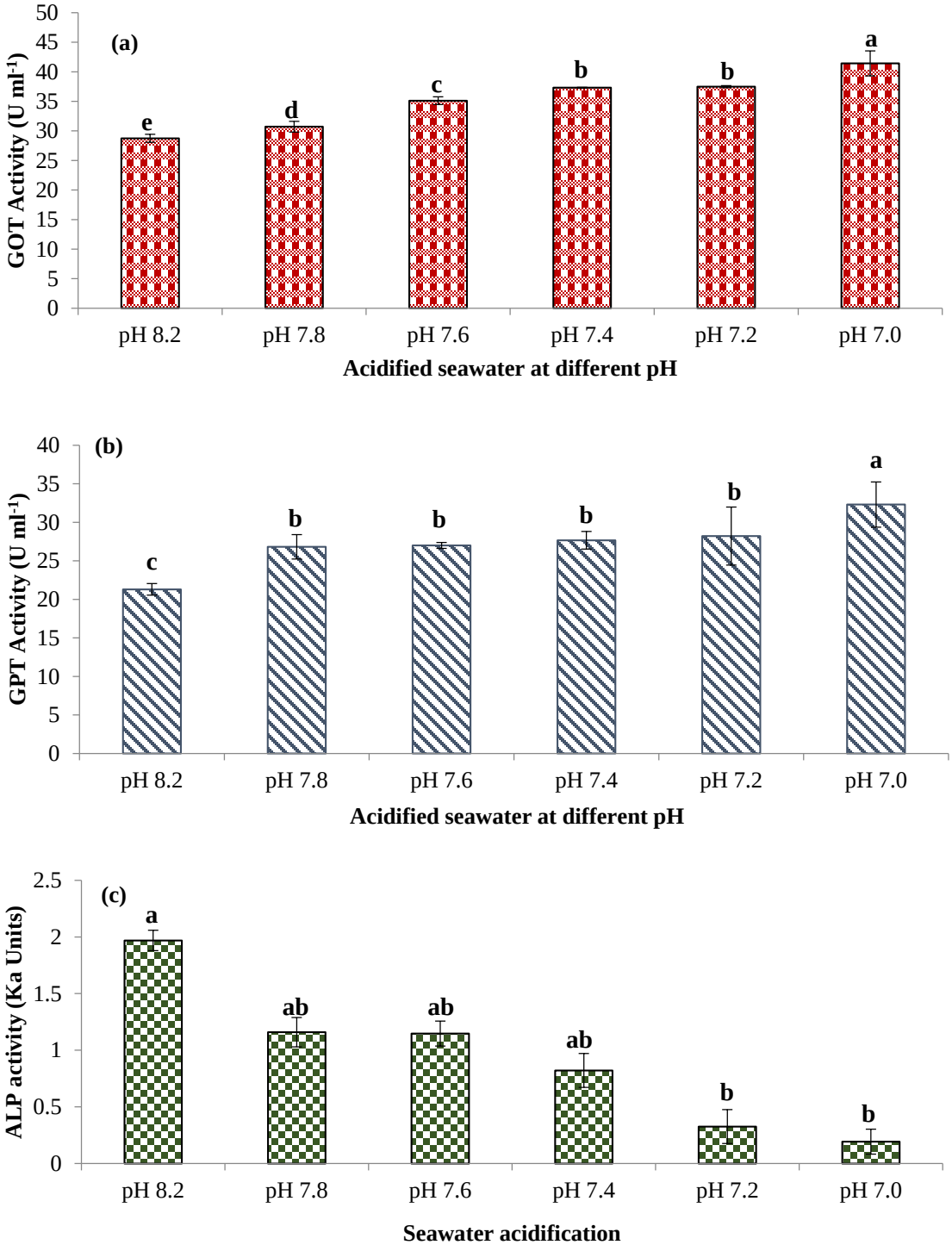


Figure 16: (a) GOT, (b) GPT, and (c) ALP of *S. serrata* exposed to CO_2 driven acidified seawater. n = 3 (three samples from each treatment), mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH-treated groups. GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvate transaminase; ALP, Alkaline phosphatase.

7.4 Discussion

Antioxidants play a crucial role in protecting cells from damage caused by free radicals. SOD and CAT are important enzymes involved in defending cells against reactive oxygen species, such as superoxide anion radicals, which are generated during normal metabolic processes, oxidative stress, and tissue damage (Rathore et al., 1998; Fang et al., 2002; Ighodaro and Akinloye, 2018). LPO is a chain reaction initiated by free radicals (Hampel et al., 2016), which can lead to the oxidative degradation of polyunsaturated fats and cell membranes, the primary targets within a biological system. In the present study, a significant increase in SOD and CAT levels was observed in the mud crab *S. serrata* reared in acidified seawater due to CO_2-induced acidification compared to the control, indicating an enhanced production of these antioxidant enzymes in response to the elevated levels of oxygen-free radicals caused by pH stress. Crabs under acidified seawater environment had more H^+ ions in the hemolymph, which might cause hemolymph acidosis followed by metabolic acidosis. This metabolic acidosis might be responsible for the elevated level of ROS that leads to more antioxidants as a defense mechanism. Furthermore, acidic conditions induced cell membrane damage, evidenced by the elevated levels of LPO in *S. serrata*. Similar findings have been reported in horseshoe crab *T. tridentatus* exposed to acidified seawater (pH 7.3) for 28 days exposure showed significant increases in glutathione peroxidase, SOD, CAT, and malondialdehyde (Liu et al., 2022). Additionally, alterations in SOD, glutathione S-transferase (GST), and LPO have been observed in the Chinese crab *P. trituberculatus* exposed to a high CO_2 for four weeks exposure to seawater environment (Lin et al., 2020). Elevation of GST, CAT, and SOD has also been reported in shrimp species such as *L. vannamei* (at pH 7.6 to 7.0 for 60 days) and brine shrimp species like *A. sinica* (at pH 7.8 and 7.6 for two weeks) and *A. franciscana* (at pH 7.8 and 6.8 for 15 days) exposed to acidic seawater (Furtado et al., 2015; Zheng et al., 2015; Muralisankar et al., 2021; Thangal et al., 2021).

GOT and GPT enzymes have critical functions in diverse physiological processes, including digestion, cellular respiration, transcription, and energy storage. Alterations in the levels of these metabolic enzymes can indicate liver impairment and metabolic stress in organisms. Animals under stress conditions need much more energy. Metabolic enzymes like GOT and GPT play a crucial role in producing more energy by metabolising

nutrients, thereby increasing these enzymatic activities. The observed alterations in GOT and GPT in *S. serrata* reared in acidic seawater suggest that ocean acidification can induce metabolic stress in crabs. These highlight the potential toxicity of pH variations in seawater on crab populations. The modulation of metabolic enzymes can directly impact organisms' growth, survival, and biochemical and mineral compositions. Similar increases in metabolic enzymes have been observed in the deep-sea hermit crab *Pagurus tanneri* under acidic stress conditions (pH 7.6 and 7.1) (Kim et al., 2016). Elevation in GOT and GPT levels under acidified conditions has also been reported in copepods such as Pseudo *Calanus acuspes* and bivalve species like *Ruditapes philippinarum* (Ross et al., 2011). Additionally, the crab *P. cinctipes* and shrimp species like *L. vannamei* have significantly increased GOT and GPT levels under acidic stress (Carter et al., 2013; Muralisankar et al., 2021). These findings further support that changes in seawater pH can induce metabolic alterations in various crustacean species.

Alkaline phosphatase (ALP) is a type of membrane-bound metalloenzyme comprising multiple isoenzymes that play a crucial role in metabolite processing and biomineralization and serves as a reliable indicator for assessing the physiological condition of animals experiencing acidic stress (Zambonino-Infante et al., 2008; Szabo and Ferrier, 2014) in marine invertebrates. The present study demonstrates the adverse effects of extreme acidic stress (pH 7.2 and 7.0) on ALP activity in *S. serrata* crabs, indicating compromised nutrient assimilation and digestion as a means of conserving energy to regulate intracellular acid-base balance (Michaelidis et al., 2005; Rosa et al., 2014) that lead to poor metabolism and growth. Similar reductions in ALP activity have been observed in marine animals, including shrimp (*Ancylocaris brevicarpalis*) and sea anemones (*S. haddoni*), under acidified conditions (Prakash et al., 2022). Additionally, a decrease in ALP activity has been documented in the horseshoe crab *T. tridentatus* exposed to an acidified environment with a pH of 7.3 (Liu et al., 2022).

7.5 Conclusion

Results of the present investigation indicate the oxidative stress, cell membrane damage, and metabolic stress of crabs under CO_2-driven acidified seawater environments (pH 7.8 to 7.0) with evidence of significant elevations in antioxidants, lipid peroxidation and metabolic enzymes. Besides, the significant decreases in the ALP of crabs in acidic stress (pH 7.2 and 7.0) cause poor nutrients and digestion for conserving energy to regulate the intracellular acid base balance, leading to poor physiological activities and growth.

Chapter V

8 CHAPTER V: Interactive effect of ocean acidification and the heavy metal cadmium on *Scylla serrata*

8.1 Introduction

Ocean is a huge saltwater source covering nearly 71% of the earth and acts as a huge heat reservoir, influencing the climate patterns, hydrological and carbon cycles (Caldecott, 2008). Marine has a chief role in regulating greenhouse gases and is dominant in mitigating climate change (Dutta, 2016). The interspace between the atmosphere and the seawater is a vibrant borderline of the earth, which control the interchanges of materials that stimulate the atmosphere's chemistry. At present, the earth system is facing considerable deviations in worldwide biogeochemical (carbon, nitrogen, sulphur, phosphorus cycles, etc.) and physical (raining, weather, islands, rivers, oceans) processes caused by anthropogenic emission of greenhouse gases (Ciais et al., 2014). The specific effects of greenhouse gases are predictable, become more severe and cause massive consequences on the Earth (Collins et al., 2013). Currently, marine environments are being polluted by various pollutants like plastics, industrial chemicals, pesticides, heavy metals, etc. (Bashir et al., 2020; Landrigan et al., 2020).

The source for the surplus release of CO_2 is visibly recognized as anthropogenic activities due to the usage of fossil fuels, land-use discharges, and industrial operations (IPCC, 2014; Quéré et al., 2018), which cause adverse impacts on the ecosystem, including ocean acidification (OA). The global emission of CO_2 has increased from 34.1 GT to 37.9 GT from 2010 to 2019, respectively (Triollet and Martinez, 2020). The oceans can absorb 30% of atmospheric carbon dioxide emissions (NOAA, 2020). The atmospheric CO_2 enters the ocean by air-to-sea equilibration and is circulated by the ocean carbon cycle (Abas and Khan, 2014). Increases in dissolved CO_2 concentration lead to a decrease in the surface seawater pH (Caldeira and Wickett, 2003). OA is the absorption of CO_2 by seawater that initiates the carbonic acid, leading to declining seawater pH, reducing the calcium carbonate and increasing bicarbonate ions (Doney et al., 2009). At the end of the 21^{st} century, the average outward seawater pH will decrease between 0.2 to 0.4 units due to the excess emission of CO_2 to the atmosphere (Hoegh-Guldberg, 2014; Fitzer et al., 2014). It has been predicted that not only the acidity but also the temperature of seawater will

increase by approximately 1.5 to 4.0 °C due to ocean warming at the end of the 21st century (Hoegh-Guldberg et al., 2018). In addition, OA leads to disturbing the marine ecosystem functioning, like the production of small organisms at a low level, which can affect seafood productivity (Doney et al., 2020). Researchers have recently proved that CO_2-driven ocean acidification negatively impacts marine animals, including coral reefs (Mongin et al., 2021). The decline of ocean pH due to the elevated pCO_2 cause the decline of calcification and another negative impact on physiological activities such as carbonate loss of the sea urchin *Salmacis virgulata* (Anand et al., 2021), reduction in survival, growth, and biochemical of the shrimp *L. vannamei* (Muralisankar et al., 2021), elevation in antioxidants such as superoxide dismutase, catalase and lipid peroxidase in crab *S. serrata* (Thangal et al., 2022). It is harder to custom calcium carbonate outer shells and skeletons of marine organisms such as pteropods, corals, and oysters (Lemasson et al., 2017; Hoegh-Guldberg et al., 2017; Meng et al., 2019; Rajan et al., 2023). Invertebrates like shrimps, corals, crabs, sea urchins, brine shrimp, etc., showed adverse effects on survival, growth, foraging and reproduction subjected to OA (Baumann et al., 2011; Dodd et al., 2015; Campbell et al., 2016; Tasoff and Johnson, 2019; Thangal et al., 2022).

Recently, the accumulation of heavy metal in the land and water is huge compared to the past era (Pinzón-Bedoya et al., 2020). The major sources of heavy metals are mining, tailings, industrial wastes, agricultural runoff, battery industries, paint factories, etc. Copper, lead, zinc, cadmium, mercury and arsenic are heavy metals released into the seawater from various sources (Sun et al., 2020). Among them, some are trace, and some are highly toxic to the animals, which creates severe health issues due to the poison, bioaccumulation and long-lasting (Frid and Caswell, 2017; Mitra et al., 2022). Amidst metals, cadmium (Cd) is one of the top metal pollutants in the world (Su et al., 2020), having an atomic number of 48 and a mass number of 112. The toxic properties of Cd are well documented in animals. Bioaccumulation of the Cd increases the ROS, which influences the oxidization of biological macromolecules and results in several physiological damages to tissues and organs (Thevenod, 2009; Revathi et al., 2011). Cadmium leads to genetic alterations (HSP70, ATP6L, Prx3, and TRX) in crustaceans like the Chinese mitten crab *E. sinensis* (Tang et al., 2019) and freshwater crab *Sinopotamon henanense* (Sun et al. 2016). Studies have investigated the interactive effect of declining ocean pH

and heavy metals on marine species (Stewart et al., 2016; Cao et al., 2018; Zhao et al., 2021; Cryer et al., 2022). The reduction in the oceanic pH water leads to lower OH^- and CO_3^{2-} concentrations, which raises the concentration of metal ions (Millero et al. 2009). Studies revealed that the combined effect of Cu and ocean acidification cause a negative impact on biochemical parameters, molecular pathways, health status and physiological function in clams and scallops (Cao et al., 2022) and suppresses calcification and decreasing respiration in corals *Stylophora pistillata* (Cryer et al., 2022). The negative impact on the survival, calcification, reproduction, biochemicals and antioxidants in the animals *Mytulis edulis*, *Stylophora pistillata*, *Cyprinus carpio* and *E. sinensis* exposed to the acidified seawater were reported earlier (Rajeshkumar et al., 2017; Cao et al., 2018; Zhao et al., 2021; Cryer et al., 2022). However, the studies of the impact of Cd in crustacean species are fragmentary. The seawater acidification will lead shellfish to absorb 10 to 13% of cadmium (Chen et al., 2020), increasing this metal's toxicity in organisms.

The crab *S, serrata* is an Indo-Pacific species having high nutrient market values (Keenan et al., 1998; Paterson, 2011). According to the report of FAO (2020), the mud crab *S. serrata* contributes about 248.8 thousand tons to world fisheries. Nevertheless, *S. serrata* is being used as a bioindicator due to their higher sensitivity to biotic and abiotic stress (El-Kahawy et al., 2021; Flint et al., 2021). Furthermore, crabs are susceptible to changes in the ocean carbon chemistry (Whitely, 2011); hence, the modifications in the carbon chemistry in the adjacent environment can create variations in the moulting, immunity, regulation of acid-base balance and calcification which reasoning to lack survival and effortlessly vulnerable to several biotic and abiotic elements (Roleda et al., 2012; Taylor et al., 2015, Rehman et al., 2021). The detrimental effect of OA on marine crabs, including *S. serrata, H. araneus, C. magister,* and *L. aesquispisnus* has been reported earlier (Miller et al., 2016; Long et al., 2021; Thangal et al., 2022). However, the studies on the combined effect of the heavy metal Cd and ocean acidification on marine crabs is limited. Hence, the present study aimed to evaluate the effect of OA on the toxicity of Cd on growth, survival, moulting, feed index, biochemical constituents, antioxidants, metabolic enzymes and haemocyte population on the crab *S. serrata*.

8.2. Materials and Method

8.2.1. Green crab instars maintenance

Totally 800 green crabs (*S. serrata*) instars (carapace length 0.6 ± 0.0 cm) were brought from Rajiv Gandhi Center for Aquaculture, Thirumullaivasal, Tamilnadu, India. Instars (0.6 cm long and 0.09 g weight) were transported in two six-litter transparent plastic covers packed with oxygenated seawater and seagrasses to avoid Fig.hts and cannibalism. Instars were lodged in a 500 L fibre tank at research laboratory condition for ten days, providing continuous aeration. The crabs were fed 100% of their body mass two times (40% during the day and 60% at night) daily with the brine shrimp (Alava et al., 2017). About ¼ of seawater was renewed daily from the stock tank, and unfed feed and wastes were removed without disturbing the crabs.

8.2.2. Experimental setup

After accommodation, exactly 120 crabs instar (carapace length 0.7 ± 0.0 cm) were reared in six aquaria (20×6) which served as acidification with pH 8.2 (control), 7.8 (estimated ocean pH by IPCC in 2100), 7.6, 7.4, 7.2, and 7.0. Further, 120 crabs were reared in six other aquaria (20×6) served as acidification with 0.01 mg l^{-1} of $CdCl_2$ (pH 8.2+0.01 mg l^{-1}, 7.8+0.01 mg l^{-1}, 7.6+0.01 mg l^{-1}, 7.4+0.01 mg l^{-1}, 7.2+0.01 mg l^{-1}, and 7.0+0.01 mg l^{-1}) for sixty days. Crabs were individually housed in a cage system prepared as stated in Chapter-I. Both experiments were done in triplicate. The concentration of Cd was selected as per the earlier studies of Cao et al. (2018). Manipulating seawater pH was done by manually releasing clean CO_2 (99.9 %) using a CO_2 valve through a rubber insulation tube that ends with a CO_2 diffuser to diffuse the CO_2 in the seawater. The pH of each aquarium was constantly observed using individual pH meters, and the pH was adjusted manually to the desired pH by releasing the CO_2. During the experimental period, the crab instars were fed with brine shrimp for the first week and chopped squid (*Uroteuthis edulis*) and shrimp (*Penaeus monodon*) muscle meat for the remaining days twice per day (Ganesh et al., 2015). Nearly half of the experimental water was changed daily to maintain a healthy environment. Throughout this period, water chemistry (salinity, temperature, dissolved oxygen (DO), alkalinity) was assessed by the

standard method (APHA 2005), and total CO_2 (TCO_2), partial carbon dioxide ($_PCO_2$), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), a saturation of calcite (ΩCa), and aragonite (ΩAr) were computed by CO_2 calculator designed by Robbins et al. (2010).

8.2.3. Survival, development, moulting, and food index

On the final day of the trial, the survival, morphometry [weight gained (WG), length gained (LG), and specific growth rate (SGR)], moulting and food indices [feed conversion ratio (FCR) and feed intake (FI) were evaluated by following the calculations (Takiney and Davies, 2001).

$$Survivorship\ (\%) = \left(\frac{Number\ of\ live\ crab}{Number\ of\ introduced}\right) \times 100$$

$$Weight\ gain\ (g) = Final\ weight\ (g) - Initial\ weight\ (g)$$

$$length\ gain\ (cm) = Final\ length\ (cm) - Initial\ length\ (cm)$$

$$Specific\ growth\ rate\ (\%day^{-1}) = \left(\frac{\ln(final\ weight) - \ln(initial\ weight)}{total\ days}\right) \times 100$$

$$Molting\ rate\ (no.of\ molt\ day^{-1}) = \frac{Total\ number\ of\ molt}{Total\ days}$$

$$Feed\ intake\ (g\ crab^{-1}d^{-1}) = Feed\ consumption/Crab\ number/days$$

$$Feed\ conversion\ ratio = \frac{Feed\ intake}{Weight\ gain}$$

8.2.4. Estimation of biochemical components

The biochemical constituents in the crabs' muscles were determined per standard methods, as stated in Chapter II. In brief, the estimation of total tissue proteins in *S. serrata* was performed as per the standard method of Lowry et al. (1951), and the assessment of total amino acids was done by the method of Moore and Stein (1948). The level of carbohydrates in the crab tissue was determined by the method of Roe (1955). The content of lipids in crab tissue was extracted and estimated using a 3:1 ratio of chloroform-methanol mixture (Folch et al., 1957; Barnes and Blackstock, 1973). AOAC (1995) procedure was adopted to determine the moisture and ash contents in the carcass crabs.

8.2.5. Analysis of antioxidants and lipid peroxidation

The level of superoxide dismutase (SOD) and catalase (CAT), and lipid peroxidation (LPO) in the crabs were determined as per earlier methods, as stated in Chapter IV. In short, After the end of 60 days, all experimental crabs were sedated with ice cubes and the flesh was extracted by homogenizing within frozen tris buffer (pH 7.4, 10% w/v), separated by rotating a cooling centrifuge (2415×g for 20 minutes at 4°C) and the supernatant was used as enzyme source to diagnose the level of SOD and CAT, and LPO. The level of total soluble protein in each crab's tissue was estimated using Lowry et al. (1951) method. SOD level was done by the autoxidation of pyrogallol (10 mM) in tris buffer (pH 7.0) procedure described by Marklund and Marklund (1974), and the activity of SOD expressed as U/mg protein. The activity of CAT was done by the method described by Sinha (1972), and the substrate was considered hydrogen peroxide (H_2O_2) in phosphate buffer. CAT activity was expressed as µmol of H_2O_2/min/mg protein. The activity of LPO level was done by the formation of thiobarbituric acid reactive substances prescribed by Ohkawa et al. (1979), and the activity was stated as nanomoles of malondialdehyde (MDA).

8.2.6. Activity of metabolic and alkaline phosphatase enzyme analysis

The activity of metabolic enzymes glutamic oxalate transaminase (GOT) and glutamic pyruvate (GPT) transaminase of the treated crab was performed per earlier methods of Reitman and Frankel (1957), as stated in Chapter IV. In short, 1 gm of crab tissue was crushed in 0.25 M sucrose and centrifuged at 6000 rpm for 20 min at -4ºC and supernatant was utilized for analysis of GOT and GPT activities. For GOT, L- aspartic and GPT, L- alanine was considered subtracts, and pyruvate was used as a calibrator for both activities. Finally, the GOT and GPT activity was expressed as U/ml. The alkaline phosphatase (ALP) level was estimated using Kind and King's (1954) procedure, as stated in Chapter IV, using 0.1 ml of disodium phenyl phosphate as substrate reagent, 0.05 ml of phenol solution as standard and 1 ml of dye reagent (Phenol + 4-amino antipyrine), The intensity of reactions was measured at 510 nm in a UV spectrophotometer.

8.2.7. Enumeration of total haemocytes

Accurately, 100µl haemolymph was withdrawn from the joint of the merus of crab using a 1 ml insulin syringe pre-filled with 900µl of anticoagulant (1:1:1 ratio of 10 mM of sodium citrate with pH 7.5, 10 mM Tris-HCl, and 250 M of sucrose respectively). From the above stock, 50 µl of anti-coagulated haemolymph was added to 10% of 50 µl formalin and incubated for 30 minutes. After incubation, 50 µl of formalin was mixed with anti-coagulated blood and diluted using a 50 µl phosphate buffer solution. From this, 50 µl diluted haemolymph was mixed with 20 µl rose Bengal stain (0.01 gm rose Bengal stain diluted in 50% ethanol) and incubated for 10 minutes. Finally, the THC was counted by using a haemocytometer (Neubauer improved, Germany) and observed under a light microscope at RP10X (Labomed, OPTIcx), and the cell was counted by using the formula,

$$THC\ (\times 10^6\ cells\ ml^{-1} = \frac{(Counted\ cells \times Depth\ of\ chamber \times Dilution\ water)}{Number\ of\ 1\ mm\ square}$$

8.2.8. Analysis of Cd in the seawater and crabs

The level of Cd was analyzed in the experimental seawater and whole crabs using an atomic absorption spectrometer (Shimadzu AA-7000, Japan). The organic phase of seawater was prepared as per the earlier methods (Arumugam et al., 2018; Vinothkannan et al., 2022) using ammonium pyrrolidine dithiocarbamate, isobutyl methyl ketone, and nitric acid followed by subjected to Cd determination. The Cd level in the crabs was determined per the standard procedures of AOAC (1995) using triple acid (9:2:1 ratio of nitric acid, sulfuric acid, and perchloric acid) digested sample.

8.2.9. Statistical analysis

The data were all symbolized as mean ±SD. The significant difference between OA experiments (different pH treatments) and OA+ Cd experiments (different pH treatments with Cd) was determined using one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Whereas the noticeable difference between each parameter of each OA and OA+ Cd was analyzed using a two-tailed paired sample 't-test. Moreover, two-way ANOVA was subjected to transformed data to know the interactions of pH and Cd. However, the parameters like salinity, temperature, DO, SGR, FI, FCR,

carbohydrate, lipid, SOD, CAT, LPO, GOT, GPT, and ALP retained only one-way ANOVA and "t" test due to their significant difference during normal distribution analysis. All the analyses were achieved using IBM SPSS (20.0) software.

8.3. Results

8.3.1 Physicochemical parameters of seawater

In acidification treatment, the pH, carbonate (CO_3), calcite (ΩCa), and aragonite (ΩAr) of the seawater were considerably decreased from pH 7.8 to 7.0 when equated to the ambient pH 8.2. While ΩCa and ΩAr show a negligible variance in pH 7.2 and 7.0. The experimental setup's salinity, temperature and dissolved oxygen (DO) exhibited an insignificant variation in all pH when matched to pH 8.2. The alkalinity of the experimental seawater was significantly reduced in pH 7.2 and 7.0 when equated to ambient pH 8.2. However, an insignificant variation was observed in 7.8 to 7.4 compared to the control pH 8.2. The level of total carbon dioxide (TCO_2), partial carbon dioxide (pCO_2), and bicarbonate (HCO_3^-) showed significant increases in pH 7.8, 7.6, 7.4, 7.2 and 7.0 when matched to the ambient pH 8.2 (Table 11).

In ocean acidification and cadmium treatment, the seawater's pH, CO_3^{2-}, ΩCa, and ΩAr considerably decreased from pH 7.8 to 7.0 when equated to the ambient pH 8.2. The TCO_2, PCO_2, and HCO_3 levels significantly increased from pH 7.8 to 7.0 once equated to pH 8.2+ Cd. The level of DO and alkalinity shows a significant decline in pH 7.4 to 7.0 when matched to pH 8.2+Cd. Nevertheless, an insignificant variation was observed in pH 7.8 and 7.6 when matched to the pH 8.2+Cd. There was no considerable variation in salinity and temperature in OA+ Cd (Table 11) treatments. In the seawater chemistry such as Alkalinity, TCO_2, pCO_2, CO_3^{2-}, ΩCa, and ΩAr, considerable changes were observed in OA+ Cd treatments when compared to the OA treatments alone as per paired sample t-test (Table 11). In addition, the two-way ANOVA indicated that, ocean acidification process influenced the pH of seawater. In this context, pH and its interaction with Cd influenced the alkalinity, TCO_2, pCO_2, CO_3, ΩCa and Ω Ar of experimental seawater (Table 12).

8.3.2. Survival, development, Molting and food indices

In the present findings, in both acidification treatments (OA and OA+ Cd), the crab's survival rate notably dropped in pH 7.2 and 7.0 when matched to the ambient pH 8.2. Nonetheless, a minor change in survival was noted in crabs subjected to pH 7.8 to 7.4 when equated to pH 8.2 (Table 13 and Fig. 17). Meanwhile, the survival rate showed an insignificant variation between the crabs exposed to all OA and OA+ Cd treatments as per paired sample 't-test. In OA treatments, the crab's length and length gain notably decreased in pH 7.6 to 7.0 when matched to the ambient pH 8.2. While insignificant changes were noticed in pH 7.8 when equalled to control pH 8.2. In OA+ Cd treatments, the length and length gain of the crab was notably decreased in pH 7.8 to 7.0 and pH 7.6 to 7.0, respectively when compared to the ambient pH 8.2. While a minor deviation was found in crabs treated at pH 7.8 and 7.6, however, minor variations were detected in length gain at pH 7.8 when compared to the control pH 8.2 (Table 13 and Fig. 17). The length and length gain of crabs were observed to be notably dropped in Cd with pH 8.2 and 7.8 compared to respective OA groups. In both OA and OA+ Cd treatments, the weight and weight gain notably dropped from pH 7.8 to 7.0 when equated to pH 8.2 (control). While an insignificant variation was detected in pH 7.8 and 7.6. Moreover, the weight and weight gain showed a significant reduction in pH 7.8, 7.4, 7.2 and 7.0 of OA+ Cd crabs when compared to the OA group crabs as per paired sample 't-test (Table 13 and Fig. 17). The moulting rate of crabs in OA treatments shows a notable drop in pH 7.6 to 7.0 once equalling the ambient pH of 8.2. While a slight variation was observed in pH 7.8 when matched to the control pH 8.2. In OA+ Cd treatments, the moulting rate showed significant variations in pH 7.0 only when related to the other pHs (pH 8.2 to 7.2), while an insignificant difference was noted in pH 7.8 to 7.2 concerning control pH 8.2 (Table 13 and Fig. 17). Meanwhile, no significant difference was found between the OA and OA+ Cd groups as per the 't-test. The specific growth rate (SGR) and feed intake (FI) of OA treatments showed a considerable reduction in pH 7.8 to 7.0 and pH 7.4 to 7.0, respectively, compared to pH 8.2 (control) (Table 13 and Fig. 17). A notable decline in SGR and FI was noted in pH 7.8 and 7.6, and 7.8 to 7.2 respectively once equated to that in ambient pH 8.2. In the case of the OA+ Cd experiment, the SGR and FI were notably dropped in pH 7.8 to 7.0, and pH 7.0, respectively, when compared to pH 8.2.

However, pH 7.8 and 7.6 showed minor deviations in SGR and 7.8 to 7.2 in FI. Considerable changes in SGR and FI were noted in pH 7.4 to 7.0 and 7.8 to 7.0 in OA and OA+ Cd experiments, respectively, as per the 't-test. In both OA and OA+ Cd treatments, a notable upsurge was found in the feed conversion ratio (FCR) of crab treated at pH 7.2 and 7.0 when matched to that in control pH 8.2. An insignificant difference was observed in pH 7.8 to 7.4 compared to the control pH 8.2. No notable changes were observed in the FCR of the crabs exposed to the OA and OA+ Cd treatments as per the paired sample 't-test (Table 13 and Fig. 17). Further, the two-way ANOVA indicated that the OA process influenced the crab *S. serrata*, while Cd and OA+ Cd did not influence the survival. Whereas OA, Cd and OA+ Cd influenced LG and WG in crabs. Moreover, OA and Cd notably influenced crabs' moulting rate (Table 14).

8.3.3. Biochemical elements

In both OA and OA+ Cd treatments, the protein, carbohydrate, amino acid and lipid levels notably declined in crabs exposed to pH 7.8 to 7.0 compared to the ambient pH 8.2. The carbohydrate level of the crabs subjected to pH 7.8 and 7.6 showed an insignificant difference in OA and OA+ Cd experiments, respectively. Moreover, lipid content was insignificantly altered in crabs reared to Cd with pH 7.8 to 7.4 and 7.2 and 7.0. Meanwhile, the level of protein and amino acids was noted to be decreased in crabs reared in all OA+ Cd treatments compared to that in OA treatments alone. However, a considerable decrease was noticed in carbohydrate and lipid levels of crabs at pH 7.4 + Cd and 7.2, 7.0+ Cd, respectively, as per the paired sampled 't' test (Table 15 and Fig. 18). In this context, individual exposure to OA and Cd and Cd interact with OA were influenced the protein and amino acid levels of the *S. serrata* as per two-way ANOVA (Table 14).

8.3.4. Antioxidants and lipid peroxidation

The level of SOD was remarkably elevated in *S. serrata* exposed to the high pCO_2 seawater (pH 7.8 to 7.0) of the OA experiment compared to the pH 8.2. While CAT and LPO showed a significant elevation in pH 7.6 and 7.0 related to the control pH 8.2 in OA experiments. An insignificant change was observed in the CAT and LPO of crabs exposed between pH 7.8 and 8.2 (control). In OA + Cd treatment, the SOD, CAT and LPO of *S. serrata* showed significant elevation in pH 7.8+Cd to 7.0+Cd when related to

the crab treated to the pH 8.2+ Cd. While in LPO, an insignificant variation was observed in pH 7.8 to 7.2. Moreover, the level of SOD and CAT was noted to be significantly elevated in OA+ Cd exposed groups (pH 8.2 to 7.0+Cd) when compared to the OA groups alone. The level of LPO showed a notable increase in pH 7.8+ Cd and pH 7.0 + Cd when compared to the respective OA-alone groups (Table 16 and Fig. 19).

8.3.5. Metabolic enzyme activity and alkaline phosphate activity

In the OA trial, a notable elevation was recorded in crabs treated to pH 7.8 to 7.0 compared to pH 8.2. While an insignificant was noted in GOT and GPT at pH 7.8 and 7.6, pH 7.6 to 7.0, respectively. In OA + Cd treatments, the GOT and GPT showed a notable elevation in pH 7.8+Cd to 7.0+Cd when matched to pH 8.2+Cd. Nonetheless, an insignificant change was observed in GOT at pH 7.8+Cd to 7.0+Cd. Moreover, In GOT and GPT activity, an insignificant variation was noted in all pH+ Cd treatments. Moreover, the GOT and GPT activities were noted to be a considerable elevation in pH 7.8+Cd to 7.4+Cd, and 8.2 to 7.0, respectively, in OA+ Cd treatments as per the 't-test (Table 17 and Fig. 20). The alkaline phosphatase activity of crabs exposed in both OA and OA+ Cd treatment, showed a noteworthy decrease in 7.8 to 7.0 when compared to the ambient pH. In both OA and OA+ Cd treatment, an insignificant difference was observed in pH 7.8 to 7.0 and 7.8+Cd to 7.4+Cd, respectively. Furthermore, no significant variations were observed between the OA and OA+ Cd experiments as per the paired sampled 't' test (Fig. 20).

8.3.6. Haemocyte counts

The total count of the haemocyte population of *S. serrata* was remarkably decreased in all OA and OA+ Cd experiments when compared to control pH 8.2 and pH 8.2+Cd. Moreover, the population of haemocytes was significantly decreased in all OA+ Cd exposed groups when compared to the respective OA groups as per the "t" test (Table 18 and Fig. 20). In addition, two-way ANOVA calculation shows that pH significantly influenced the haemocyte counts of *S. serrata*. Continues Cd and its interaction with pH failed to influence the haemocyte counts (Table 14). As per the two-way ANOVA, the OA, Cd and OA+ Cd influenced the haemocyte population of the crab *S. serrata* (Table 18 and Fig. 20).

8.3.7. Accumulation of Cd in seawater and crab tissue

The Cd level was insignificantly varied among different pH exposure of OA and OA+ Cd seawater experiments. In this circumstance, a significant accumulation of the Cd in crabs under all OA and OA+ Cd was noticed compared to crabs. The paired sample t-test indicated significant elevations in Cd bioaccumulation of crabs in all OA+ Cd experiments compared to respective OA experiments (Table 19 and Fig. 20).

Table 11: Physicochemical characteristics of the experimental seawater

Parameter		pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Salinity (ppt)	OA	34.2 ± 0.78^{a}	34.2 ± 0.78^{a}	34.2 ± 0.78^{a}	34.2 ± 0.78^{a}	34.2 ± 0.78^{a}	34.2 ± 0.78^{a}
	OA+ Cd	34.0 ± 0.71^{a}	34 ± 0.71^{a}	34 ± 0.71^{a}	34 ± 0.71^{a}	34 ± 0.71^{a}	34 ± 0.71^{a}
Temperature (%)	OA	24.50 ± 0.50^{a}	24.50 ± 0.50^{a}	24.50 ± 0.50^{a}	24.50 ± 0.50^{a}	24.50 ± 0.50^{a}	24.50 ± 0.50^{a}
	OA+ Cd	24.55 ± 0.03^{a}	24.55 ± 0.03^{a}	24.55 ± 0.03^{a}	24.55 ± 0.03^{a}	24.55 ± 0.03^{a}	24.55 ± 0.03^{a}
pH	OA	8.20 ± 0.02^{a}	7.80 ± 0.04^{b}	7.61 ± 0.03^{c}	7.41 ± 0.03^{d}	7.21 ± 0.02^{e}	7.03 ± 0.02^{f*}
	OA+ Cd	8.20 ± 0.01^{a}	7.79 ± 0.04^{b*}	7.60 ± 0.03^{c}	7.40 ± 0.03^{d}	7.20 ± 0.03^{e}	7.01 ± 0.02^{f*}
Dissolved oxygen (mg l^{-1})	OA	7.93 ± 1.50^{a}	7.73 ± 1.29^{a}	7.56 ± 3.07^{a}	7.13 ± 1.86^{a}	6.86 ± 1.53^{a}	6.7 ± 2.63^{a}
	OA+ Cd	7.93 ± 0.64^{a}	7.8 ± 0.7^{ab}	7.07 ± 0.67^{abc}	6.83 ± 0.59^{bc}	6.57 ± 0.35^{bc}	6.43 ± 0.21^{c}
Alkalinity (µmol kg^{-1})	OA	2004.86±5.76^{a}	1998.20±9.99^{ab}	1991.54±15.26^{abc}	1988.21±9.99^{abc}	1978.22±19.98^{bc}	1971.55±5.76^{c}
	OA+ Cd	2001.53±5.79^{a*}	1994.87±5.76^{ab*}	1988.21±9.99^{ab*}	1981.55±15.26^{bc*}	1968.22±9.99^{cd*}	1961.56±5.76^{d*}
TCO_2 (µmol kg^{-1})	OA	1650.68±14.19^{f}	1847.76±6.29^{e}	1908.30±5.46^{d}	1962.37±1.81^{c}	2010.96±12.10^{b}	2067.91±3.96^{a}
	OA+ Cd	1646.49±7.21^{f*}	1847.7±9.52^{e*}	1904.38±2.48^{d*}	1958.09±4.61^{c*}	2000.34±0.57^{b*}	2060.76±3.15^{a*}
$_{P}CO_2$ (µatm)	OA	216.25±13.59^{f}	677.94±51.24^{e}	1100.43±47.74^{d}	1761.49±77.20^{c}	2844.43±105.55^{b}	4456.88±193.12^{a}
	OA+ Cd	215.37±7.69^{f*}	694.43±56.30^{e*}	1099.17±82.55^{d*}	1799.82±124.99^{c*}	2832.22±198.86^{b*}	4539.66±215.33^{a*}
HCO_3^- (µmol kg^{-1})	OA	1407.81±26.06^{d}	1715.82±14.08^{c}	1800.47±2.09^{c}	1860.79±1.25^{b}	1896.26±13.74^{a}	1918.03±2.02^{a}
	OA+ Cd	1403.48±14.21^{f*}	1717.35±16.34^{e*}	1796.44±6.32^{d*}	1856.51±4.01^{c*}	1886.16±2.62^{b*}	1909.25±2.25^{a*}
CO_3 (µmol kg^{-1})	OA	236.63±12.37^{a}	112.38±9.57^{b}	76.08±5.44^{c}	50.76±3.60^{d}	32.65±2.53^{e}	21.31±1.52^{e}
	OA+ Cd	236.80±7.24^{a*}	110.34±8.51^{b*}	76.29±6.34^{c*}	48.76±5.96^{d*}	32.63±2.87^{e*}	20.64±1.38^{f*}
ΩCa	OA	5.74±0.29^{a}	2.72±0.23^{b}	1.84±0.13^{c}	1.22±0.08^{d}	0.79±0.06^{e}	0.51±0.03^{e}
	OA+ Cd	5.77±0.14^{a*}	2.67±0.19^{b*}	1.84±0.14^{c*}	1.20±0.1^{d*}	0.78±0.06^{e*}	0.50±0.03^{f*}
ΩAr	OA	3.76±0.20^{a}	1.78±0.15^{b}	1.20±0.08^{c}	0.80±0.05^{d}	0.51±0.04^{e}	0.33±0.22^{e}
	OA+ Cd	3.77±0.1^{a*}	1.75±0.12^{b*}	1.21±0.09^{c*}	0.79±0.07^{d*}	0.51±0.04^{e*}	0.33±0.02^{f*}

n = 6; mean ± SD; bars sharing different letters are considered significant at $p < 0.05$ among OA and OA+ Cd treatments; * indicates the significant difference between the respective pH of OA and OA+Cd as per paired sampled t-test. TCO_2, Total carbon dioxide; pCO_2, Partial carbon dioxide; HCO_3, Bicarbonate; CO_3, Carbonate; weight gain; SGR, ΩCa, Clacspar and ΩAr, aragonite.

Table 12: Two-way ANOVA results of ocean acidification and its interaction with Cd on the seawater properties

Parameters		**pH (1,96)**	**Cd (5,96)**	**pH+ Cd (5,96)**
pH	F	353.88	0.656	0.5
	Sig	0.000*	0.426	0.998
Alkalinity (µmol kg^{-1})	F	24.072	51182.99	5.553
	Sig.	0.000*	0.000*	0.002*
TCO_2 (µmol kg^{-1})	F	1052.384	88564.001	105.27
	Sig.	0.000*	0.000*	0.000*
pCO_2 µatm)	F	1487.605	937.607	111.799
	Sig.	0.000*	0.000*	0.000*
CO_3 (µmol kg^{-1})	F	757.936	948.245	61.076
	Sig	0.000*	0.000*	0.000*
Ω Ca	F	793.911	471.649	63.702
	Sig.	0.000*	0.000*	0.000*
Ω Ar	F	755.224	444.162	61.407
	Sig.	0.000*	0.000*	0.000*

* Indicated as significant effects

Table 13. Survival, growth and food indices of *S. serrata* exposed to acidified seawater at different pH

Parameter		pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Survival (%)	**OA**	100 ± 0.00^{a}	98.75 ± 2.50^{a}	98.75 ± 2.50^{a}	97.50 ± 2.88^{ab}	93.75 ± 2.50^{bc}	91.25 ± 4.78^{c}
	OA+ Cd	98.75 ± 2.50^{a}	97.50 ± 2.88^{a}	95.00 ± 0.00^{ab}	93.75 ± 2.50^{ab}	90.00 ± 4.08^{b}	90.00 ± 5.77^{b}
Initial Length (cm)	**OA**	0.7 ± 0.00^{a}	0.7 ± 0.00^{a}	0.7 ± 0.00^{a}	0.7 ± 0.00^{a}	0.7 ± 0.00^{a}	0.88 ± 0.00^{a}
	OA+ Cd	0.84 ± 0.21^{a}	0.88 ± 0.13^{a}	0.90 ± 0.07^{a}	0.74 ± 0.11^{a}	0.86 ± 0.11^{a}	0.84 ± 0.11^{a}
Final length (cm)	**OA**	2.52 ± 0.08^{a}	2.44 ± 0.05^{a}	2.02 ± 0.13^{b}	1.92 ± 0.16^{bc}	1.80 ± 0.15^{cd}	1.78 ± 0.19^{d}
	OA+ Cd	$2.20 \pm 0.20^{a*}$	$2.02 \pm 0.08^{b*}$	1.96 ± 0.11^{b}	1.76 ± 0.11^{c}	1.72 ± 0.13^{c}	1.62 ± 0.08^{c}
Length gain (cm)	**OA**	1.82 ± 0.08^{a}	1.74 ± 0.05^{a}	1.32 ± 0.13^{b}	1.22 ± 0.16^{bc}	1.10 ± 0.15^{cd}	1.02 ± 0.19^{d}
	OA+ Cd	$1.36 \pm 0.25^{a*}$	$1.14 \pm 0.19^{ab*}$	1.26 ± 0.11^{bc}	1.06 ± 0.11^{bc}	0.98 ± 0.13^{c}	0.92 ± 0.08^{c}
Initial weight (g)	**OA**	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}
	OA+ Cd	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}
Final weight (g)	**OA**	2.19 ± 0.34^{a}	1.91 ± 0.06^{b}	1.76 ± 0.14^{b}	1.49 ± 0.06^{c}	1.20 ± 0.06^{d}	1.11 ± 0.06^{d}
	OA+ Cd	1.85 ± 0.26^{a}	$1.51 \pm 0.22^{b*}$	1.41 ± 0.28^{bc}	$1.21 \pm 0.06^{cd*}$	$1.09 \pm 0.09^{de*}$	$0.92 \pm 0.06^{e*}$
Weight gain (g)	**OA**	2.09 ± 0.35^{a}	1.81 ± 0.06^{b}	1.66 ± 0.14^{b}	1.39 ± 0.06^{c}	1.10 ± 0.06^{d}	1.01 ± 0.06^{d}
	OA+ Cd	1.75 ± 0.26^{a}	$1.41 \pm 0.22^{b*}$	1.31 ± 0.28^{bc}	$1.11 \pm 0.06^{cd*}$	$0.99 \pm 0.09^{de*}$	$0.82 \pm 0.06^{e*}$
SGR (%/day)	**OA**	2.22 ± 0.12^{a}	2.13 ± 0.02^{b}	2.07 ± 0.05^{b}	1.95 ± 0.02^{c}	1.80 ± 0.04^{d}	1.74 ± 0.04^{d}
	OA+ Cd	2.10 ± 0.09^{a}	1.96 ± 0.09^{b}	1.90 ± 0.14^{bc}	$1.80 \pm 0.03^{cd*}$	$1.73 \pm 0.06^{d*}$	$1.60 \pm 0.04^{e*}$
Feed intake	**OA**	1.81 ± 0.36^{a}	1.70 ± 0.43^{ab}	1.67 ± 0.44^{abc}	1.61 ± 0.36^{bcd}	1.46 ± 0.56^{cd}	1.39 ± 0.43^{d}
	OA+ Cd	$1.47 \pm 0.63^{a*}$	$1.34 \pm 0.60^{ab*}$	$1.29 \pm 0.71^{ab*}$	$1.21 \pm 0.60^{ab*}$	$1.20 \pm 0.78^{ab*}$	$1.14 \pm 0.67^{b*}$
FCR	**OA**	0.86 ± 0.03^{b}	0.94 ± 0.20^{b}	0.99 ± 0.18^{b}	1.15 ± 0.21^{b}	1.31 ± 0.44^{a}	1.36 ± 0.35^{a}
	OA+ Cd	0.80 ± 0.20^{b}	0.92 ± 0.02^{b}	0.99 ± 0.32^{b}	1.14 ± 0.49^{b}	1.16 ± 0.68^{a}	1.29 ± 0.730^{a}

n= 60 for survival, moulting, FI, and FCR; n=15 for LG, WG and SGR; mean ± SD; bars sharing different letters are considered significant at $p < 0.05$ among OA and OA+ Cd treatments; * indicates the significant difference between the respective pH of OA and OA+Cd as per paired sampled t-test. LG, Length gain; WG, weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio.

Table 14: Two-way ANOVA: effect of ocean acidification and Cd exposure on the survival, LG, WG, moulting, protein, amino acid and haemocyte population in *S. serrata*

Parameters		**pH (1,96)**	**Cd (5,96)**	**pH+ Cd (5,96)**
Survival (%)	F	7.667	0.167	0.167
	Sig	0.000**	0.686	0.973
Length Gain (cm)	F	25.865	42.135	5.622
	Sig	0.000**	0.000**	0.000**
Weight Gain (gm)	F	44.315	37.14	0.956
	Sig	0.000**	0.000**	0.454*
Moulting (%)	F	3.015	7.973	0.384
	Sig	0.006**	0.008**	0.856
Protein (mg^{-1})	F	2101.9	3554.05	38.35
	Sig	0.000**	0.000**	0.000**
Amino acid (mg^{-1})	F	1391.27	108.28	37.2
	Sig	0.000**	0.000**	0.000**
Haemocytes ($Cells^{-1}$ $ml \times 10^6$)	F	35044.91.	11030.43	1064.94
	Sig	0.000**	0.000**	0.000**

* indicated as significant effects

Table 15. Biochemical constituents *S. serrata* exposed to acidified seawater at different pH

Parameter		pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Protein (mg^{-1})	**OA**	253.46 ± 4.48^{a}	246.86± 4.47^{b}	237.98 ± 02.58^{c}	217.57 ± 03.01^{d}	180.94 ± 04.38^{e}	170.50 ± 02.24^{f}
	OA+ Cd	224.44± 3.71^{a*}	202.76±01.51^{b*}	195.15 ± 02.44^{c*}	172.99 ± 02.97^{d*}	158.65 ± 03.18^{e*}	135.82 ± 01.97^{f*}
Carbohydrate (mg^{-1})	**OA**	91.03 ± 1.98^{a}	81.93 ± 02.69^{b}	77.15 ± 02.73^{b}	72.05 ± 05.18^{c}	62.13 ± 03.88^{d}	55.66 ± 04.94^{e}
	OA+ Cd	84.47 ± 4.87^{a}	81.94 ± 04.57^{b}	75.59 ± 05.40^{c}	61.41 ± 03.83^{c*}	59.54 ± 02.09^{c}	55.42 ± 02.95^{d}
Amino acid (mg^{-1})	**OA**	212.85 ± 4.26^{a}	170.90± 03.64^{b}	141.31 ± 03.89^{c*}	128.93 ± 04.64^{d}	111.95 ± 03.39^{e}	107.39 ± 04.79^{f}
	OA+ Cd	184.35± 4.06^{a*}	157.53±04.14^{b*}	139.29 ± 03.43^{c}	122.71 ± 04.14^{d*}	111.59 ± 04.43^{e*}	105.99 ± 02.25^{f*}
Lipid (mg^{-1})	**OA**	22.11 ± 3.35^{a}	18.86 ± 00.58^{b}	18.07 ± 00.84^{c}	16.82 ± 00.28^{d}	14.20 ± 00.48^{e}	11.24 ± 01.03^{f}
	OA+ Cd	21.86 ± 0.39^{a}	18.03 ± 03.19^{b}	16.01 ± 02.70^{b}	15.54 ± 03.39^{b}	11.35 ± 02.16^{c*}	09.80 ± 01.25^{c}

n = 3; mean ± SD; bars sharing different letters are considered significant at $p < 0.05$ among OA and OA+ Cd treatments; * indicates the significant difference between the respective pH of OA and OA+Cd as per paired sampled t-test.

Table 16. Antioxidant status and lipid peroxidation of *S. serrata* exposed to acidified seawater at different pH

Parameter		pH 8.2 (Control)	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
SOD (U mg $protein^{-1}$)	**OA**	03.00 ± 0.19^{f}	03.21± 0.02^{e}	03.32 ± 0.02^{d}	03.64 ± 0.05^{c}	04.36 ± 0.05^{b}	04.59 ± 0.05^{a}
	OA + Cd	03.33 ± 0.04^{f*}	03.76±0.05^{e*}	03.92 ± 0.05^{d*}	04.37 ± 0.05^{c*}	04.78 ± 0.05^{b*}	05.64 ± 0.09^{a*}
CAT (µ mol min^{-1} mg $protein^{-1}$)	**OA**	08.19 ± 0.11^{e}	08.25± 0.05^{e}	08.36 ±0.07^{d}	09.33 ± 0.05^{c}	11.51 ± 0.08^{b}	12.00 ± 0.06^{a}
	OA + Cd	09.13 ± 0.04^{f*}	09.87±0.05^{e*}	10.95 ± 0.07^{d*}	11.50 ± 0.24^{c*}	12.86 ± 0.10^{b*}	15.46 ± 0.27^{a*}
LPO (nano mol MDA^{-1} mg $protein^{-1}$	**OA**	01.30 ± 0.20^{e}	01.6 ± 0.37^{de}	01.78 ± 0.47^{cd}	01.99 ± 0.48^{c}	02.36 ± 0.15^{b}	03.54 ± 0.12^{a}
	OA + Cd	01.50 ±0.31^{c}	02.22±0.34^{b*}	02.25 ± 0.34^{b}	02.39 ± 0.35^{b}	02.47 ± 0.36^{b}	03.77 ± 0.19^{a*}

n = 3; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT). SOD: superoxide dismutase, CAT: catalyse, LPO: Lipid peroxidation.

Table 17. Metabolic enzymes of *S. serrata* exposed to acidified seawater at different pH

Parameter		pH 8.2 (Control)	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
GOT (U ml^{-1})	**OA**	14.13 ± 02.47^{d}	17.75± 03.65^{c}	19.97± 01.67^{c}	20.71± 05.13^{c}	26.86 ± 01.42^{b}	30.18 ± 02.43^{a}
	OA+ Cd	15.92 ± 02.48^{b}	28.89±02.86^{a*}	29.21±04.14^{a*}	29.42±04.25^{a*}	29.62 ± 02.74^{a}	31.81±03.31^{a}
GPT (U ml^{-1})	**OA**	43.10 ± 02.53^{c}	47.95 ± 0.69^{b}	48.88±01.34^{ab}	48.84±01.15^{ab}	50.18 ± 0.95^{a}	50.11 ± 02.11^{a}
	OA+ Cd	58.14 ± 0.74^{a*}	58.78 ± 0.96^{a*}	58.98±02.20^{a*}	59.12±02.29^{a*}	59.21 ± 0.79^{a*}	59.64 ± 0.97^{a*}

n = 3; mean ± SD; bars sharing different letters are considered significant at $p < 0.05$ among OA and OA+ Cd treatments; * indicates the significant difference between the respective pH of OA and OA+Cd as per paired sampled t-test. GOT: glutamic oxaloaceticate transaminase, GPT: glutamic pyruvic transaminase

Table 18. Haemocyte population of *S. serrata* exposed to acidified seawater at different pH

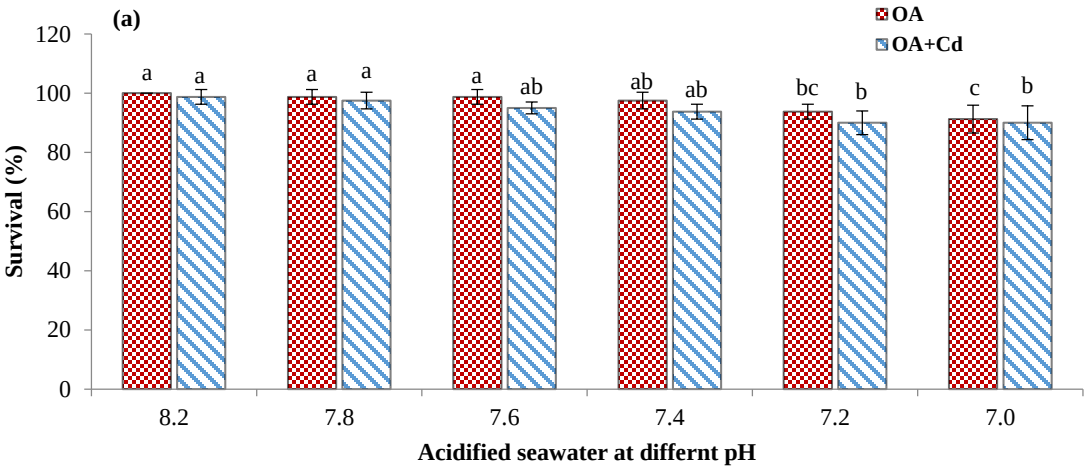
Parameter		pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Haemocyte count (cells ml^{-1} x 10^{6}	**OA**	12.25±0.01^{a}	9.98±0.01^{b}	9.89±0.02^{c}	8.36±0.01^{d}	8.26±0.01^{e}	7.34±0.01^{f}
	OA + Cd	11.16±0.01^{a*}	9.26±0.01^{b*}	8.36±0.01^{c*}	7.93±0.02^{d*}	7.64±0.01^{e*}	7.27±0.02^{f*}

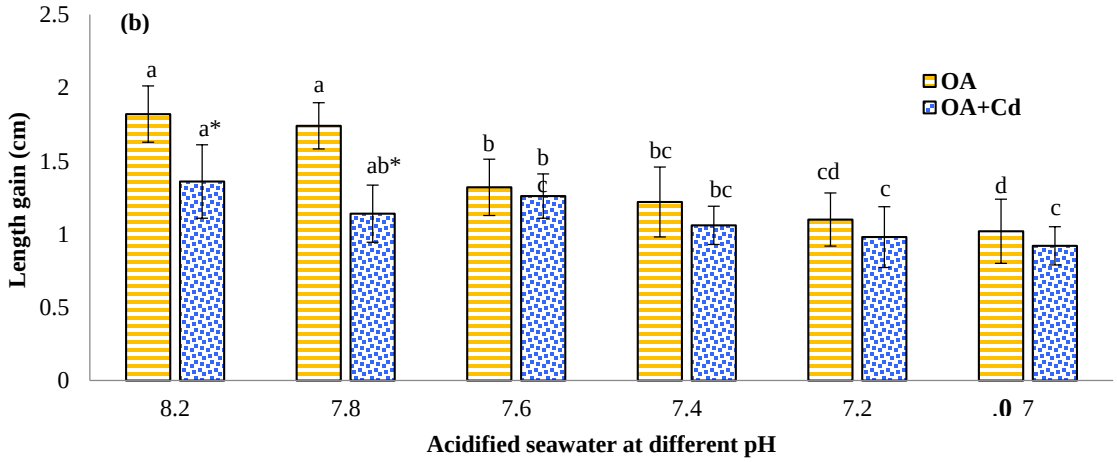
n = 3; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT).

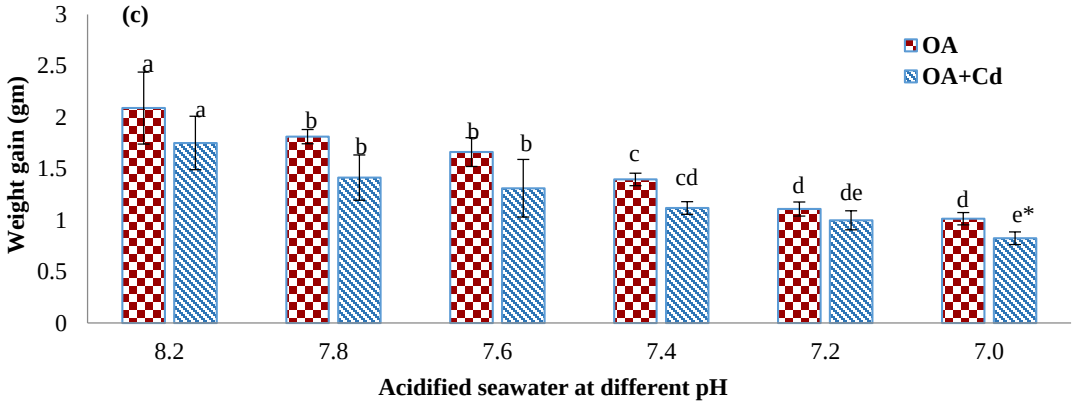
Table 19. Accumulation of Cd in seawater and *S. serrata* at different pH

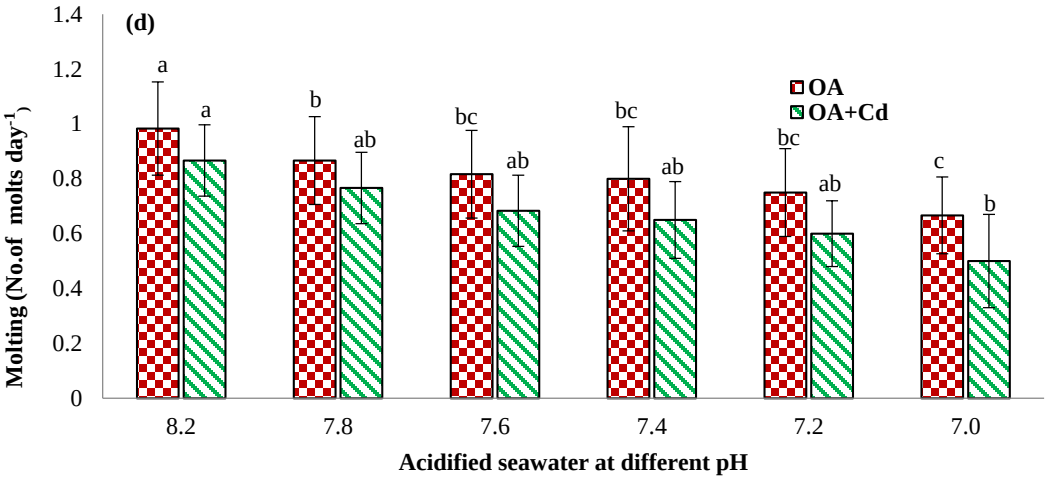
	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Normal Seawater (µg l^{-1})	0.22 ± 0.01^{a}	0.22 ± 0.00^{a}	0.23 ± 0.01^{a}	0.22 ± 0.01^{a}	0.22 ± 0.01^{a}	0.21 ± 0.00^{a}
OA + Cd (µg l^{-1})	10.13 ± 0.06^{a}	10.12 ± 0.02^{a}	10.14 ± 0.04^{a}	10.35 ± 0.29^{a}	10.18 ± 0.01^{a}	10.18 ± 0.00^{a}
Normal crab Tissue (mg kg^{-1})	0.12 ± 0.01^{d}	0.13 ± 0.01^{cd}	0.14 ± 0.00^{c}	0.16 ± 0.01^{b}	0.18 ± 0.01^{a}	0.19 ± 0.00^{a}
Acidified seawater crab tissue + Cd (mg kg^{-1})	0.47 ± 0.00^{b}	0.68 ± 0.01^{b}	0.70 ± 0.00^{b}	0.85 ± 0.00^{b}	1.05 ± 0.00^{b}	1.31 ± 0.00^{a}

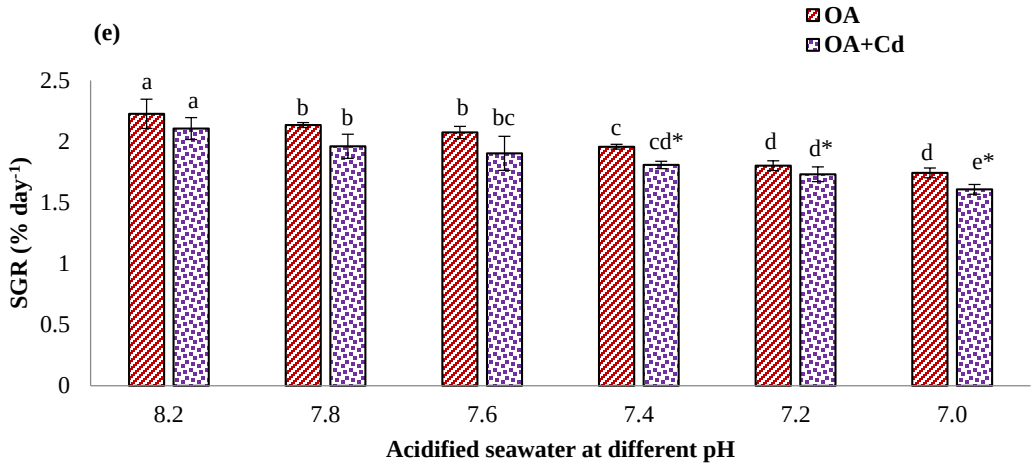
n = 3; mean ± SD; bars sharing different letters are considered significant at $p < 0.05$ among OA and OA+ Cd treatments; * indicates the significant difference between the respective pH of OA and OA+Cd as per paired sampled t-test.

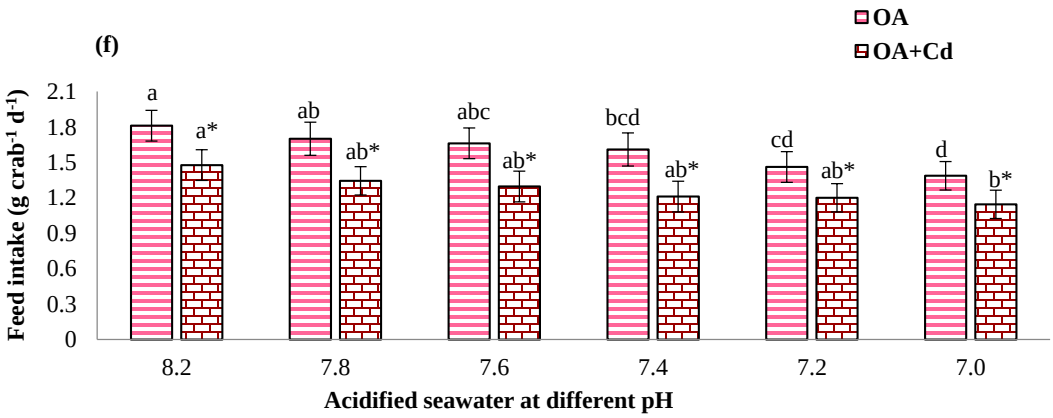

(a)
OA
OA+Cd
Survival (%)
120
100
80
60
40
20
0
a a a a a ab ab ab bc b c b
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at differnt pH


(b)
OA
OA+Cd
Length gain (cm)
2.5
2
1.5
1
0.5
0
a a* a ab* b b bc bc cd c d c
8.2
7.8
7.6
7.4
7.2
.0 7
Acidified seawater at different pH


(c)
OA
OA+Cd
Weight gain (gm)
3
2.5
2
1.5
1
0.5
0
a a b b b b c cd d de d e*
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at different pH


(d)
OA
OA+Cd
Molting (No.of molts day-1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a a b ab bc ab bc ab bc ab c b
8.2 7.8 7.6 7.4 7.2 7.0
Acidified seawater at different pH


(e)
OA
OA+Cd
SGR (% day-1)
2.5
2
1.5
1
0.5
0
a a b b b bc c cd* d d* d e*
8.2 7.8 7.6 7.4 7.2 7.0
Acidified seawater at different pH


(f)
OA
OA+Cd
Feed intake (g crab-1 d-1)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
a a* ab ab* abc ab* bcd ab* cd ab* d b*
8.2 7.8 7.6 7.4 7.2 7.0
Acidified seawater at different pH

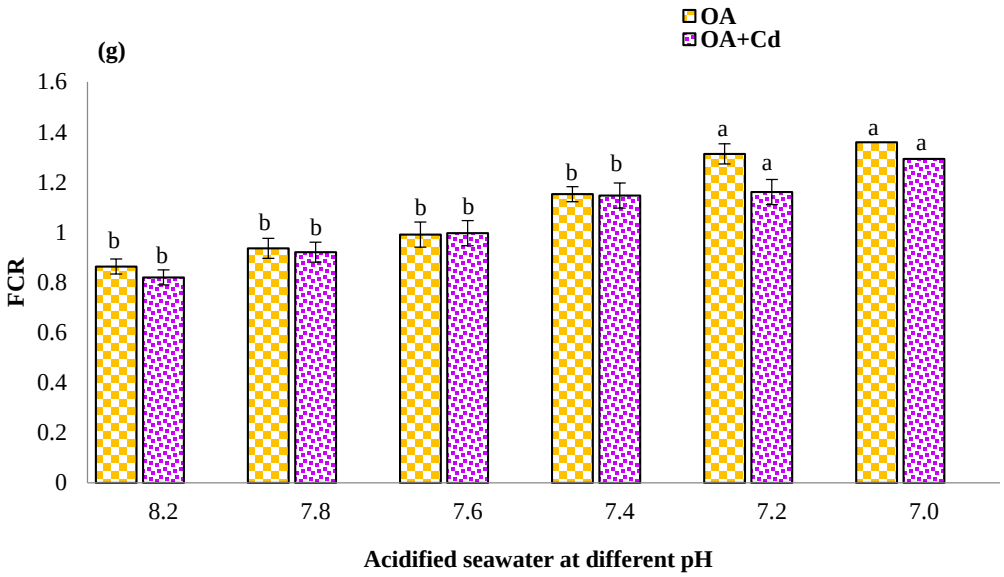
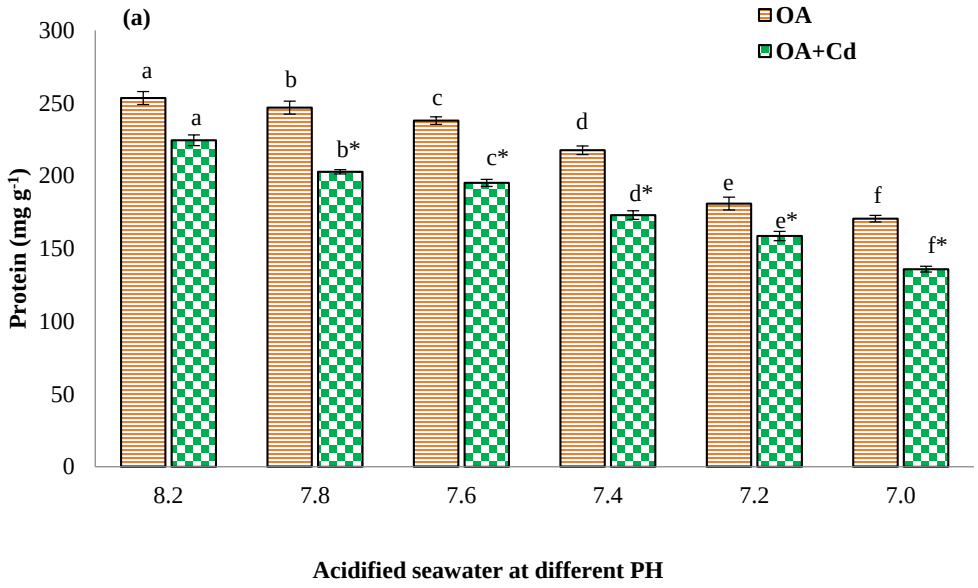
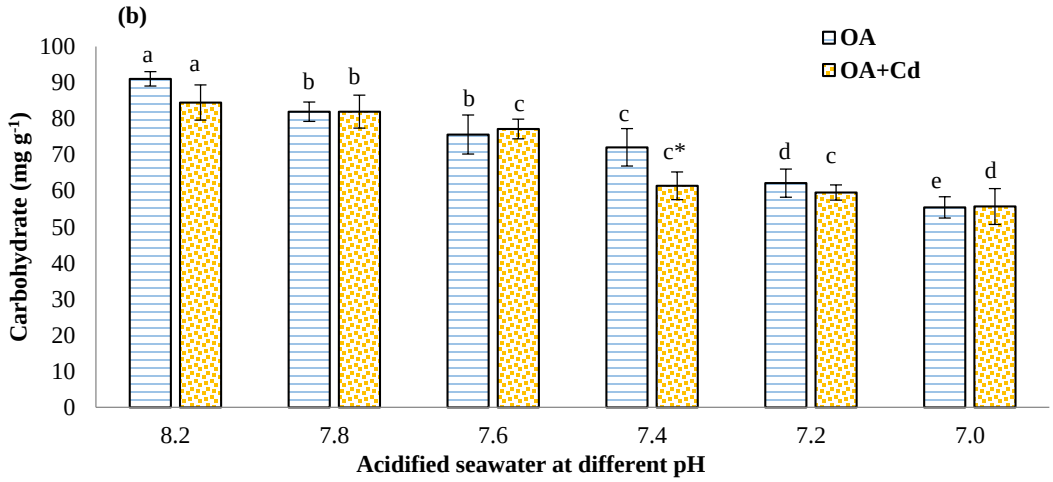


Figure 17. (a) Survival, (b) LG, (c) WG, (d) Molting rate, (e) SGR, (f) FI and (g) FCR of *S. serrata* exposed to CO_2 driven acidified seawater with and without Cd. n = 60 for survival, moulting, FI, and FCR; n = 15 for LG, WG and SGR; mean ± SD; bars sharing different letters are considered as significant at $p < 0.05$ among OA alone and OA+Cd treatments; * indicate the significant difference between the respective each pH of OA and OA+Cd as per paired sampled 't' test. LG, Length gain; WG, weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio.


(a)
OA
OA+Cd
Protein (mg g-1)
300
250
200
150
100
50
0
a
a
b
b*
c
c*
d
d*
e
e*
f
f*
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at different PH


(b)
OA
OA+Cd
Carbohydrate (mg g-1)
100
90
80
70
60
50
40
30
20
10
0
a
a
b
b
b
c
c
c*
d
c
e
d
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at different pH

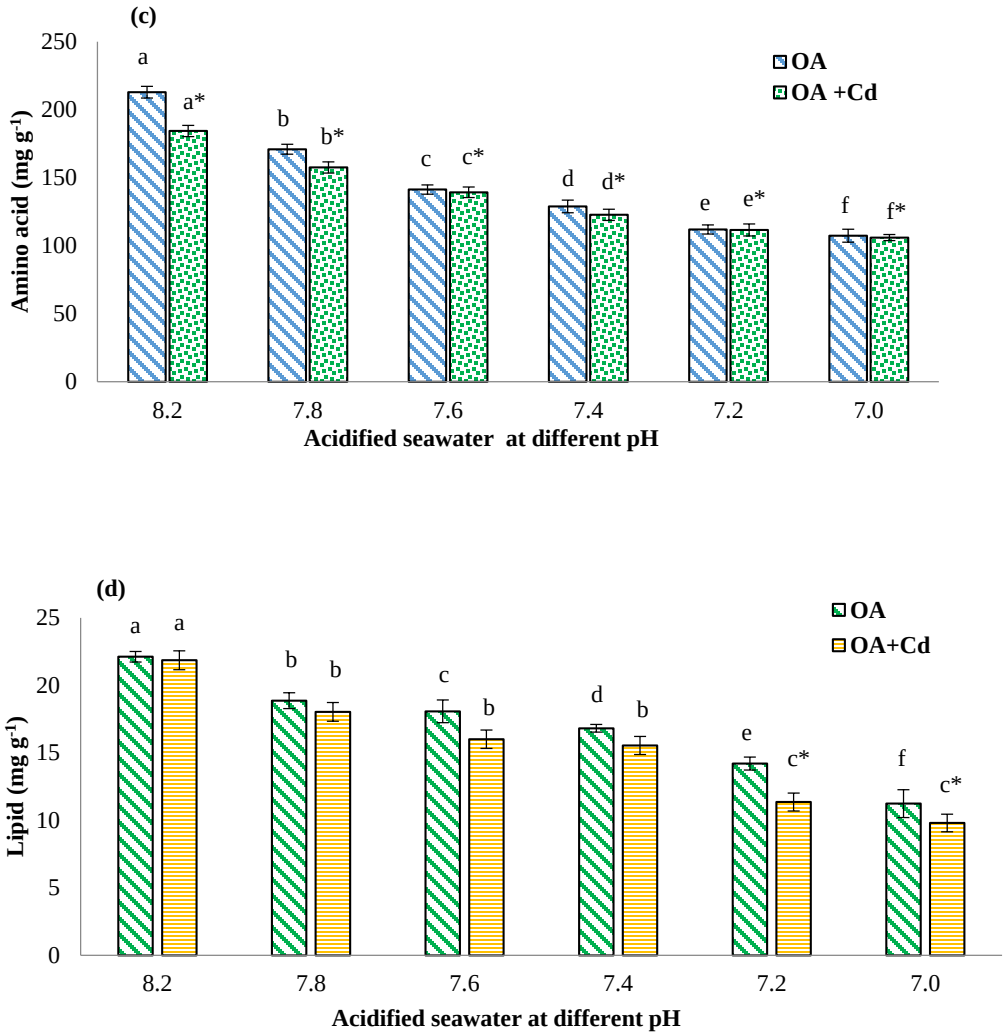


Figure 18: (a) Protein, (b) Carbohydrate, (c) Amino acid and (d) Lipid of *S. serrata* exposed to CO_2 driven acidified seawater with and without Cd. n = 9, mean ± SD; bars sharing different letters are considered as significant at $p < 0.05$ among OA alone and OA+ Cd treatments; * indicates the significant difference between the respective pH of OA and OA+ Cd as per paired sampled 't' test.

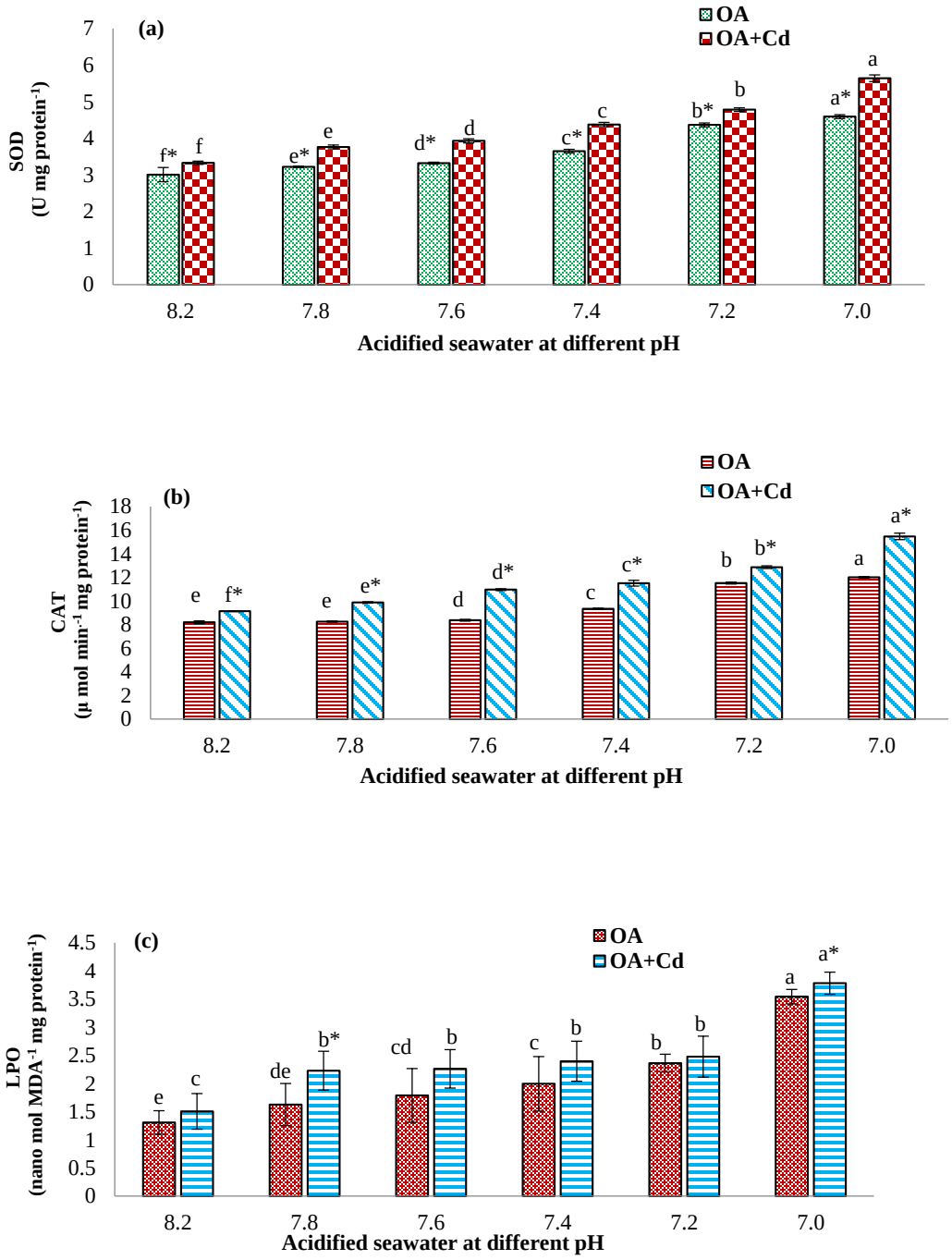
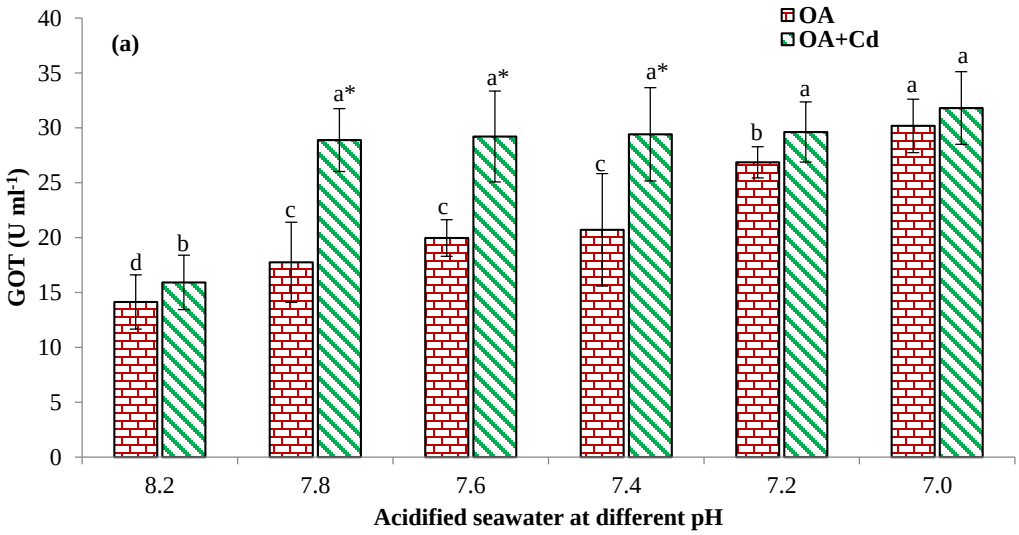
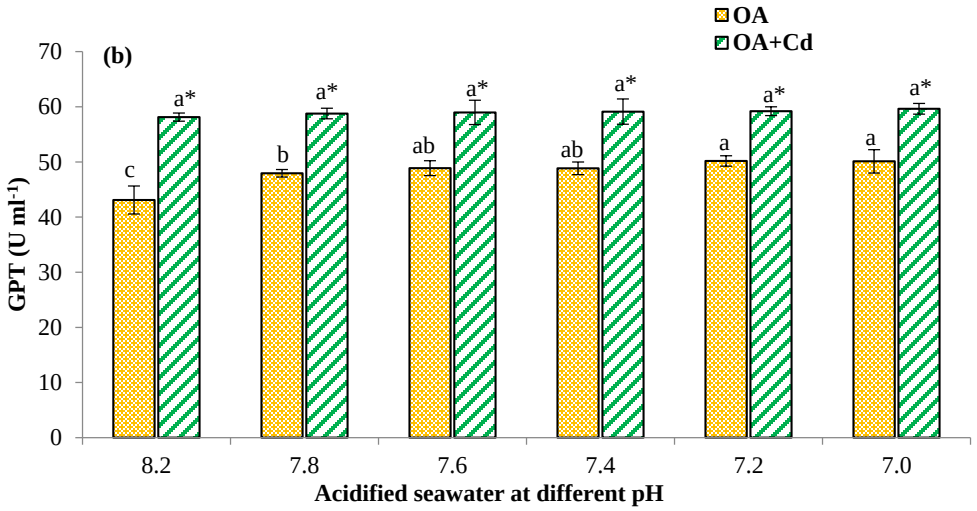
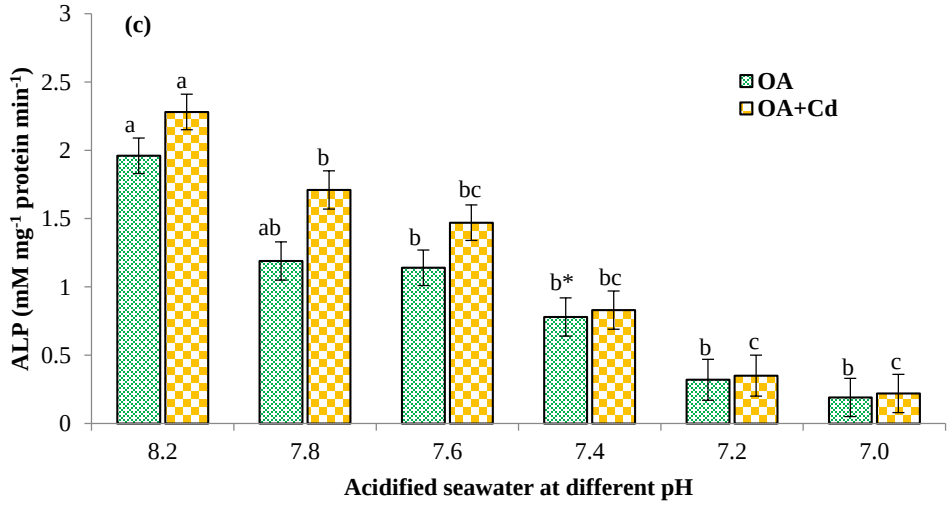
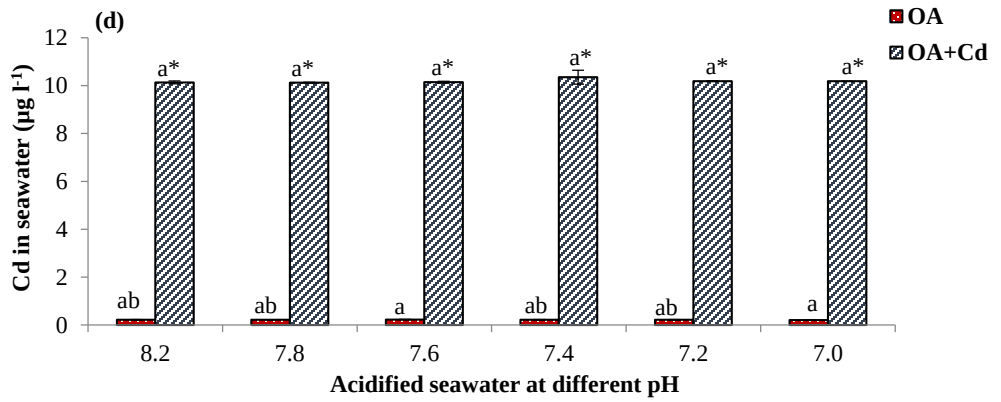


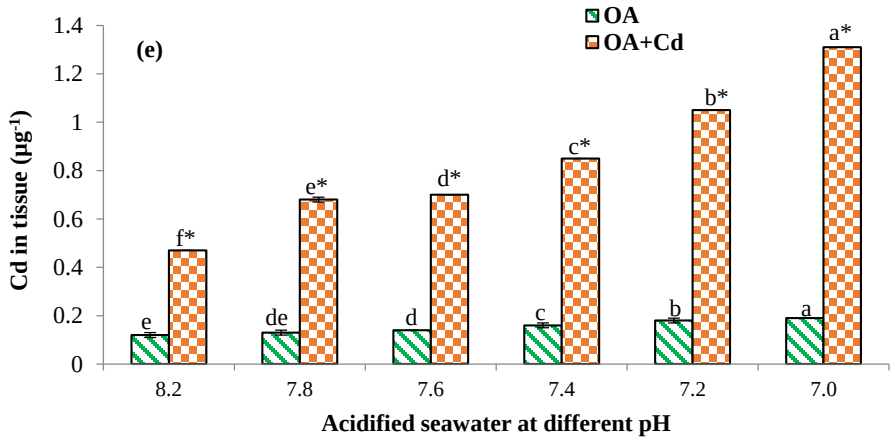
Figure 19: (a) SOD, (b) CAT, and (c) LPO of *S. serrata* exposed to CO_2 driven acidified seawater with and without Cd. n = 9, mean ± SD; bars sharing different letters are considered as significant at $p < 0.05$ among OA and OA+Cd treatments; * indicates the significant difference between the respective pH of OA alone and OA+Cd as per paired sampled 't' test. SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation.


(a)
OA
OA+Cd
GOT (U ml-1)
40
35
30
25
20
15
10
5
0
d
b
c
a*
c
a*
c
a*
b
a
a
a
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at different pH


(b)
OA
OA+Cd
GPT (U ml-1)
70
60
50
40
30
20
10
0
c
a*
b
a*
ab
a*
ab
a*
a
a*
a
a*
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at different pH


(c)
OA
OA+Cd
ALP (mM mg-1 protein min-1)
3
2.5
2
1.5
1
0.5
0
a
a
ab
b
b
bc
b*
bc
b
c
b
c
8.2
7.8
7.6
7.4
7.2
7.0
Acidified seawater at different pH





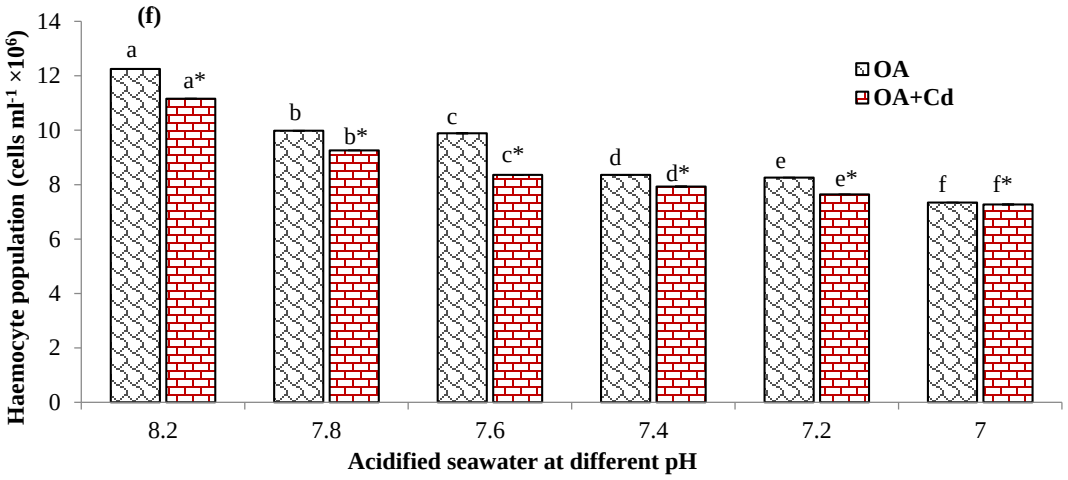


Figure 20: (a) GOT, (b) GPT, (c) ALP, (d) Accumulation of Cd in seawater, (e) Accumulation of Cd in tissue, and (f) Heamocyte population of *S. serrata* exposed to CO_2 driven acidified seawater with and without Cd. n = 9, mean ± SD; bars sharing different letters are considered as significant at $p < 0.05$ among OA alone and OA+Cd treatments; * indicates the significant difference between the respective pH of OA and OA+Cd as per paired sampled 't-test. GOT, Glutamic oxalic transaminase; GPT, Glutamic pyruvic transaminase catalase; ALP, Alkaline phosphate.

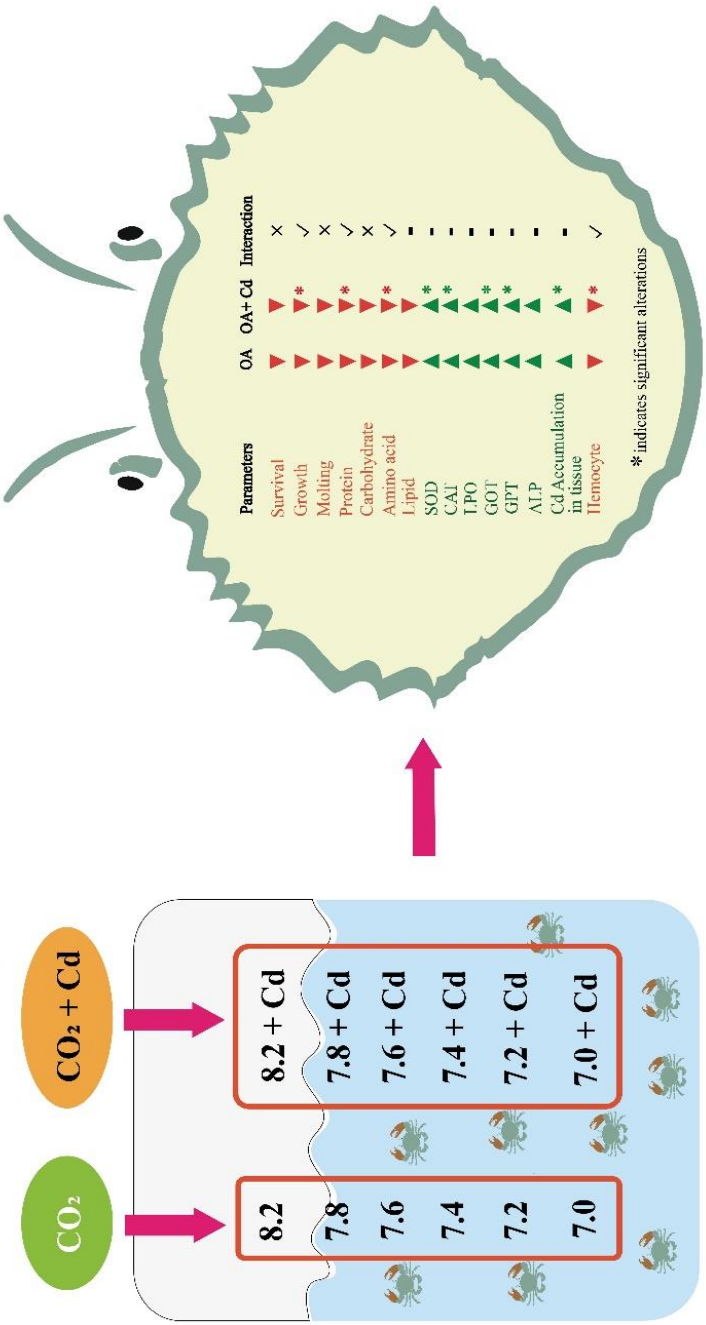


Figure 21: Schematic illustration of interactive effect of OA and the heavy metal Cd on *S. serrata*

8.4. Discussion

Seawater quality is crucial in maintaining their ambient physiological activities in marine life. The excess amount of CO_2 released into the atmosphere is one of the main factors to create ocean acidification, which disturbs the carbon content of the seawater that influences the water quality parameter, including dissolved inorganic carbon (DIC), pCO_2, aragonite, and calcite (Long et al., 2017; Thangal et al., 2022). Heavy metal pollution is another severe problem, which increases heavy metal availability in seawater and bioaccumulation in biotic organisms (Rezania et al., 2016). The increases in pollution, including ocean acidification and metal pollution, disturb the normal physiological activities of the crustaceans, which create ecological implications in the food web (Whiteley, 2011; Adeleke et al., 2020), followed by significant threats to higher tropic animals. In the present study, a significant decline in pH, pCO_2, total CO_2, carbonate, calcite, and aragonite of the all acidified seawater in OA experiments and OA + Cd experiments compared to the respective control (pH 8.2 and pH 8.2+ Cd) suggests that the CO_2 driven ocean acidification can alter the physicochemical properties of seawater. An insignificant variance in salinity and temperature of acidified seawater in OA and OA+ Cd experiments compared to the control indicates that CO_2-driven acidification did not produce any significant changes in these properties under laboratory conditions. Similar result alterations in the pCO_2, HCO_3-, ΩCa and ΩAr have been observed in the CO_2 navigated acidified seawater (Egilsdottir et al., 2009; Pedersen et al., 2014; Long et al., 2017; Anand et al., 2021). Seawater acidification experiments have recorded significant variations in salinity and temperature (Thangal et al., 2021; Thangal et al., 2022; Muralisankar et al., 2020; Shi et al., 2016; Cao et al., 2018).

Crustaceans are connecting links between benthic and pelagic species performing an important role in the marine ecosystem food chain. Many economically important commercial fishes and invertebrates depend on larval forms of crustaceans for their dietary needs. Particularly, *Decapods* are commercially vital for their delicious taste and easy assimilability. Crustaceans are affected by biotic (small plants, microbes and animals) and abiotic factors (salinity, temperature, alkalinity, dissolved oxygen etc.), which can influence their regular physiological activities. Therefore, crustaceans are considered bio-indicators to find the variation in aquatic ecosystems (Kumar et al., 2000;

Bastami et al., 2012; Szaniawska, 2018; Flint, 2021). Due to numerous anthropogenic activities, marine ecosystems are being polluted, which may lead to critical issues, including acidification and heavy metal contamination. A substantial decline in the survival, length, length gain, weight, weight gain, and SGR feed intake of the crabs in OA and OA + Cd of this current study indicated that the OA and OA + Cd could produce adverse effects in studied experimental crabs. In this context, the significant decreases in growth and feed intake of crab *S. serrata* under OA + Cd exposure revealed that the effect of OA with heavy metal Cd can produce more adverse effects on crabs than OA alone exposures. In this study, the increased feed conversion ratio of crabs in both OA treatments and OA + Cd treatments matched to ambient pH 8.2 (OA control ad OA+ Cd control) indicates the low nutrient utilization of crabs. However, insignificant differences recorded between OA and OA + Cd experiments indicated that both experiments had similar adverse effects on crabs. The digestive glands of aquatic animals are adversely affected by pH stress, which may cause consequences in appetite and feed intake (Liang et al., 2022). Also, animals under stress environments showed high metabolic demand, poor feeding, and assimilation, reducing growth (Hogstrand et al., 1996; Heydarnejad et al., 2013). The reduced hatching, survivorship, and growth in great spider crab *Hyas araneus,* Dungeness crab *Cancer magister*, and golden king crab *Lithodes aequispinus* when reared at pCO_2 (pH 7.5 for 127 days) rich seawater have been recorded earlier (Miller et al., 2016; Long et al., 2021). A notable decrease in the feeding rate of *Asterias rubens* was observed at 3500 µatm (Appelhans et al., 2012). Also, a commendable reduction in the survival rate, growth rate, and calcification of the Tanner crab *Chionoecetes bairdi* and juvenile red crab *Paralithodes camtschaticus* lived at pH 7.5 has been stated earlier by Long et al. (2013). Further, the poor survivorship, hatching rate and growth in great spider crab *Hyasaraneus,* Dungeness crab *Cancer magister*, and golden king crab *Lithodes aequispinus* when reared at pCO_2 rich (pH 7.1 for 45 days) seawater have been recorded earlier (Miller et al., 2016; Long et al., 2021).

Moreover, a study observed that acidified seawater and heavy metal toxicity decrease marine organisms' growth, survival and performance, such as Polychaete *Nereis diversicolor* and the Bivalve *Scrobicularia plana* in estuaries (Bonnard et al., 2009; Ivanina and Sokolova, 2015). Similarly, the combined effect of ocean acidification and

Cd exposure affected the survival of oyster *C. gigas* (Cao et al., 2018). Increased mortality in bivalves *Mytilus edulis* exposed to pH 6.8 with different heavy metals (Cd, Pb and Cu) was observed previously (Han et al., 2014). It indicates that the Cd, lead and copper heavy metals under acidified conditions can improve the accumulation of metallothionein in soft tissues, increasing the heavy metal toxicity for marine animals (Han et al., 2014). Heavy metals such as Cd, Cu, Pb, Zn, etc., harm the survival and reproduction capacity of marine fishes such as *Cyprinus carpio*, *Leuciscus idus*, and *Pagrus major* (Huang et al., 2010; Witeska et al., 2014; El-Greisy and El-Gamal, 2015).

Moulting is a vital process in arthropods, which donates to animal development, expansion, breeding and regeneration (Ghanawi et al., 2012). Both biotic factors (inhibit the moulting hormone and development of gonads) (Daoud et al., 2010; Gong et al., 2015) and abiotic factors (salinity, temperature, light, and nutrients) (Zanotto et al., 2002; Zhao et al., 2015) are affecting moulting in animals. In this investigation, the significant decrease in crabs' moulting suggests that the decreasing seawater pH can reduce the moult formation, followed by poor growth. Acidified seawater can reduce carbonates, decreasing $CaCO_3$, which is essential for forming calcareous shells. Besides, reductions in the moulting rate of crabs exposed to OA+ Cd compared to OA alone experiments indicate a more negative effect on studied crabs. OA can suppress chitinolytic enzymes (chitinase and N-acetyl β-D-glucosidase), adversely affecting crustaceans' moult regulation (Luo et al., 2015; Chen et al., 2019). Besides, Cd toxicity can stimulate the release of moult inhibition hormone in animals, including crustaceans, by affecting the moulting (Revathi et al., 2011; Ortega et al., 2022). Earlier studies also recorded the reduction in moulting in shrimp *L. vannamei* and hermit crab *Pagurus criniticornis* under pCO_2-rich seawater (pH 7.7 for 120 days) (Turra et al., 2019; Muralisankar et al., 2021). Also, Cd's moulting impairment in marine crab *Chasmagnathus granulate* has been reported (Moreno et al., 2003). Cryer et al. (2022) reported a notable decrease in the calcification of the tropical coral *Stylophora pistillata* was observed in the combined effect of high pCO_2 and Cu.

Biochemical properties have a vital role in the nutritional index of an animal, which influences the physiology and biology of the animal. In the present investigation, the notable drop in biochemical constituents like protein, amino acids, carbohydrates, and

lipids of crabs exposed to OA and OA+ Cd revealed that the production of biochemical elements might be negatively affected in crabs at OA and OA + Cd environments compared to normal (control) seawater environment. Moreover, the considerable decreases in biochemical elements in crabs exposed to OA+ Cd treatments compared to OA treatments alone showed that combining ocean acidification with Cd can produce more adverse effects on those biochemical productions. Proteins, carbohydrates, and lipids are the main constituents of animals, which play a significant role in energy metabolism and growth. Animals need high-energy demands under stressful environments that lead to more utilization of biochemical elements for maintaining regular physiological activities. Poor feeding and digestion under acidic and heavy metal stress (Heydarnejad et al., 2013; Liang et al., 2022) decreased protein, carbohydrate, and lipid in experimental crabs. A previous study by Turra et al. (2019) reported a noticeable decline in the lipid content of the hermit crab *Pagurus criniticornis* after being reared at pH 7.70 for 120 days. Similar reductions in total protein, amino acids, lipids, and carbohydrates have been observed in the shrimp *L. vannamei* and the brine shrimp *A. franciscana* when exposed to varying levels of acidified environments (pH 7.8 to 3.5) as reported by Muralisankar et al. (2021) and Thangal et al. (2021). Furthermore, Hsieh et al. (2021) reported a decrease in amino acid content in tiger shrimp *Penaeus monodon* after exposure to pH 7.5 for four weeks. The decreases in protein content in pearl oyster *Pinctada fucata* (pH 7.8 and 7.5 for 30 days) and shark, *Chiloscyllium platinum* (pH 7.2 for 50 days) reared in acidified seawater have been observed previously (Li et al., 2017). Furthermore, significant decreases in tissue lipid content have been noticed in the crab *Sinopotamon henanense* exposed to Cd toxicity (Yang et al., 2013). Decreases in protein, carbohydrate, and lipid content of prawn *Macrobrachium rosenbergii* have been observed earlier (Sowdeswari and Ananth, 2012).

Antioxidant has a special role in protecting cells. Remarkably, some marine animals can live in a polluted environment due to the shielding response against the scavenger free radicals delivered by the antioxidant cellular defence system (Rani et al., 2013). The antioxidant enzyme SOD converts into free radicle superoxide ion (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2) to protect cells (Jazayeri, 2012). Furthermore, H_2O_2 is converted into water (H_2O) and oxygen with the help of CAT. CAT effectively transforms hydrogen peroxide into H_2O and oxygen. Because of its capacity to eradicate

cytotoxic hydrogen peroxide and improve organism life span, it has been considered an important antioxidant enzyme (Arockiaraj et al., 2012). Lipid peroxidation (LPO) is a process that occurs when oxygen free radicals rob electrons from polyunsaturated fatty acids from cell membranes, which initiate a self-replicating chain reaction that is harmful to the viability of cells. It is a sign of free radicals in cells and tissues or oxidative stress (Salaenoi et al., 2015). Antioxidant defence systems of animals could alter by pH and Cd stress and stimulate the production of reactive oxygen species, which cause oxidative stress by reacting with cellular macromolecules like lipids, proteins and DNA that lead to apoptosis and membrane lipid peroxidation (Valko et al., 2006; Liu et al., 2011; Wang et al., 2011). In the present investigation, significant elevation of CAT and SOD of *S. serrata* under acidified seawater suggested that the more amount of antioxidants produced to reduce the toxic reactive oxygen species that produced by acidic stress and Cd stress. Moreover, the substantial elevation of LPO in crabs under OA and OA+ Cd, when matched to control pH 8.2, showed cell membrane damage. H^+ ions are essential for the production of ATP from ADP and inorganic phosphate (Pi) by the enzyme ATP synthase. Under an acidic environment, more availability of H^+ ions enters body fluids through gills. These excess H^+ ions may inhibit the active ion transport of cells that disrupts Na^+/H^+ and Cl^-/HCO^{3-} exchanges, thus disturbing the acid-base balance followed by the development of blood acidosis in aquatic animals (Ultsch et al., 1981; Heisler, 1982) and altering the regular cellular metabolism. This altering cellular metabolism might produce more free radicals, elevating crabs' antioxidants. Earlier studies reported that the elevation of SOD, CAT and LPO in shrimp *L. vannamei* (Muralisankar et al., 2020), bivalves *M. coruscus* (Huang et al., 2018) and brine shrimp *A. franciscana* (Thangal et al., 2021) exposed in the acidified seawater. The considerable elevation in antioxidants SOD and CAT of crabs exposed to OA+ Cd indicates that the seawater acidification with the heavy metal Cd had produced more adverse effects on crabs than on crabs exposed to OA treatments. Similarly, a significant elevation in SOD and CAT has been observed in the Chinese mitten crab, *Eriocheir sinensis*, exposed in the acidified seawater (pH 7.8, 7.3, and 6.5) along with Cd (1 mgL^{-1}) for 7, 14, and 21 days (Zhao et al., 2021). Cao et al. (2018) noted the increment of LPO level in the oyster *C. gigas* exposed to pH 7.8 and 7.6 along with 10mg/l cadmium. The interactive effect of

OA and OA+ Cd on the antioxidants like CAT, glutathione S-transferases, and glutathione peroxidase in the smooth scallop *Flexopecten glaber* has been studied earlier (Nardi et al., 2018). The usage and metabolism of nutrients, such as protein and carbohydrates, depend greatly on metabolic enzymes such as GOT and GPT. Massive amounts of transaminase are released into the hemolymph and stressors by tissue cells under liver injury during toxic stress. The increases in the concentration of heavy metals like Cu and Cd and decreased water pH can cause energy transformations in animals, including crustaceans, to manage toxic stress, thereby changes in GPT and GOT activity (Tang et al., 2000; Das et al., 2006; Hu et al., 2015; Kim et al., 2021; Thangal et al., 2022). In the OA trial, commendable elevation was noted in crabs subjected to pH 7.8 to 7.0 in both OA alone and OA+ Cd treatments once matched to the ambient pH 8.2, which reveals the negative impact of these stressors on the hepatopancreas of crabs. Further, significant improvements in GOT and GPT of crabs treated with OA+ Cd compared to OA alone treatment show these two stressors' synergistic effect on hepatopancreas. The previous studies publicized that the increment of GOT and GPT activity in the brine shrimp *A. franciscana,* mussel *M. corruscus* and shrimp *L. vannamei* exposed to the pCO_2-rich seawater (7.8, 7.7, 7.6, 7.4, 7.2 and 7.0 for 30 days) (Muralisankar et al., 2020; Thangal et al., 2021; Khan et al., 2021). Heavy metals like Fe, Mn, Zn, Cu, Pb, and Cd negatively affect the GOT and GPT activity of *Tilapia zillii*, *Clarias gariepinus* and *Mugil ccephalous* (Ibrahim et al., 2005). The juvenile rohu carp *Labeo rohita* exposed to the heavy metal (144 µg/l) showed a significant elevation of GOT and GPT in the liver (Humtsoe et al., 2007). The combined effect of ocean acidification and heavy metal reports is fragmentary. Therefore, the present study will give the possible effect of the combined effect of ocean acidification and Cd heavy metal on the digestive enzyme activity of *S. serrata.*

Alkaline phosphatases are present in several living organisms. They are essential for several vital processes, including DNA synthesis, protein synthesis, carbohydrate metabolism, bone calcification, and the reduction of inflammatory responses by the innate immune system. They also hydrolyze various physiological substances, including phosphate (Zaher et al., 2020). Alkaline phosphatase (ALP) is strongly active in the cells of mineralized tissue and is essential for developing hard tissue (Vimalraj, 2020).

In the current study, a significant decrease in ALP at OA and OA+ Cd treatments when matched to the control pointed out the unfavourable impact of OA and Cd on c, leading to poor physiological activities like decreased acid-base balance biomineralization, metabolism, innate immune system and growth. A similar result was observed in crab *Cardiosoma armatum* and *Tachypleus tridentatus* exposed to the acidified seawater (Lawal-Are et al., 2021; Liu et al., 2022). A considerable decline in ALP activity was noticed in the mud crab *S. serrata* and fiddler crab *Uca annulipes* under Cd exposure (Dhavale and Masurekar, 1986; Suresh et al., 2016).

Seawater acidification may alter heavy metals' toxicity and increase toxic metal accumulations (Campbel et al., 2014). In the present study, the gradual increase of Cd accumulation in carbs under all OA and OA+Cd indicates that the OA can improve the bioaccumulation of Cd in *S. serrata*. Exclusion of Cd is a more energy-consuming process in aquatic animals. OA can hinder Cd exclusion by organisms due to high energy demand for physiological maintenance under stress conditions (Roberts et al., 2013). A similar result was observed earlier in crab *Dotilla fenestrate* exposed to the OA+ Cd at pH 7.2, 7.4 and 7.6 for 96 hours (Adeleke et al., 2020). Accumulation of Cd in the gills and digestive glands of three bivalve species, *M. edulis, Tegillarca granola,* and *Meretrix meretrix,* was observed previously at pH 7.8 and 7.6 for 30 days (Shi et al., 2016). Also, the accumulation of Cd in the copepod *Tigriopus japonicas* under acidified (1000 μatm pCO_2) environment has been noticed by Wei et al. (2021). Cd is a soluble heavy metal and is easily accumulated by organisms. This property of the Cd results from toxic heavy metal poisoning; hence, Cd consider a high environmental threat heavy metal (Pascal et al., 2010).

Haematological indicators like total blood cells can be used to determine an organism's physiological and immune state (Ahmed et al., 2020). Three types of blood cells are present in green crabs' haemolymph: hyaline cells and semi and large-granule haemocytes. Hyaline cells are naturally small and have a high nucleocytoplasmic ratio and thin cytoplasmic granule content. Semi and large granule haemocytes are distinguished by the dominance of small and large electron-thick granules in their cytoplasm (Clare et al., 1994). The haemocytes of crustaceans play a critical function in host immune responses, including identifying pathogens, melanization, phagocytosis, cytotoxicity and cell communication (Johansson et al., 2000). In the present investigation,

the considerable declining level of haemocyte populations of studied crabs in OA+ Cd reveals the detrimental toxic effect compared to crabs reared in OA alone experiment. It has been assumed that acidosis may occur in the crabs' haemolymph under a low pH environment, which seems to alter the physiology of haemocytes and cell death by apoptotic processes (Meseck et al., 2016).

Furthermore, degranulation and lysis of haemocytes can occur when the crustacean is exposed to endotoxin (Smith and Söderhäll, 1983), decreasing the blood cell population. This present outcome study is consistent with previous studies in *L. vannamei*, which showed that the count of blood cell populations was reduced once shrimp were reared in CO_2-driven acidic seawater (pH 7.8, 7.6, 7.4, 7.2 and 7.0) for 60 days (Huang et al., 2018, Muralisankar et al., 2021). An earlier study denotes that OA, acidification, and Cd exposure in Pacific oyster *Crassostrea gigas* have decreased the number of haemocytes in lower pH (7.8 and 7.6 for 31 days) compared to the control (Cao et al., 2018). Significant decrease in haemocytes of American lobsters *Homarus americanus* recorded under acidified (pH 7.6) environment (Harrington and Hamlin, 2019). A study revealed slower phagocytosis capacity in the *M. edulis* exposed to pH 6.2, along with Cd and led heavy metal (Han et al., 2014).

8.5 Conclusion

The present study revealed the detrimental effect of OA (pH 7.8 to 7.0) with Cd (0.01 mg/l) on growth, biochemical muscle elements, and hemocytes population with signs of considerable improvements in stress biomarkers (antioxidants and metabolic enzymes) and Cd accumulation in *S. serrata*. Besides, the interaction effects of OA and Cd significantly affect the growth, protein, amino acid, and haemocyte population of *S. serrata,* that the synergistic activities of these two stressors on the studied crab species (Fig. 21). Hence, the present study suggests the toxicity of Cd can be more in the mud crab *S. serrata* instars under decreased ocean pH. Nonetheless, further studies are required to know the biochemical and molecular pathway of the interactive effect of OA and Cd in *S. serrata*.

Summary and Conclusion

9. SUMMARY AND CONCLUSION

Ocean acidification (OA) is the decline of oceanic pH due to increasing levels of atmospheric CO_2 globally. Chapters I to IV from the present study, the effect of acidified seawater in the edible crab *Scylla serrata* using a multi-cell cage-based experimental system. The *S. serrata* instars were exposed to OA at pH 8.2 (control), 7.8 (IPCC predicted pH at the end of the 21st century), 7.6, 7.4, 7.2, and 7.0 for 60 days. This study revealed that the significant reduction in survival (pH 7.6 to 7.0), feed intake, growth, moulting, digestive enzymes activities, tissue biochemical constituents, the content of minerals including aminoacid and fatty acid composition (pH 7.8 to 7.0), chitin (pH 7.4 to 7.0), and alkaline phosphatase (pH 7.2 and 7.0) in crabs exposed to OA. Compared to control pH, a considerable increase in enzymatic antioxidants, lipid peroxidation, and metabolic enzymes was noticed in crabs treated in all acidified seawater, which shows crabs were under oxidative stress by free radicals, cell membrane damage, and metabolic pressure. Hence, our findings encountered that CO_2-induced ocean acidification can cause adverse effects on *S. serrata* instars.

Besides, the effect of OA on the toxicity of cadmium (Cd) in the crab *S. serrata* was evaluated in Chapter V of the present study. These crab instars were subjected to pH 8.2, 7.8, 7.6, 7.4, 7.2, and 7.0 with and without 0.01 mg/l of Cd for 60 days. We notice a significant decrease in growth, moulting, protein, carbohydrate, amino acid, lipid, alkaline phosphatase, and haemocytes of crabs under OA+Cd compared to OA treatment alone. In contrast, OA, Cd, and its interactions significantly affected the growth, protein, amino acid, and haemocyte level (OA+Cd). However, antioxidants, lipid peroxidation, metabolic enzymes, and bioavailability of Cd in crabs were considerably elevated in OA+Cd treatments compared to OA alone treatments. The present investigation showed that the effect of Cd toxicity might be raised under OA on *S. serrata*. Our study demonstrated that ocean acidification significantly affects the biological indices and oxidative stress responses of *S. serrata* exposed to Cd toxicity. Hence, the present study suggests the toxicity of Cd can be more in the mud crab *S. serrata* instars under decreased ocean pH. Nonetheless, further studies are required to know the biochemical and molecular pathway of the interactive effect of OA and Cd in *S. serrata*.

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Annexure

ANNEXURE

CHAPTER- II

1. Activities of digestive enzymes

The activities of digestive enzymes, such as protease, amylase and lipase were assayed in initial and final test crabs.

Extraction (Preparation of crude enzyme extract)

1) The whole crab was taking the assays but remove the eyestalk, exoskeleton and legs.
2) It placed on a cold table (4℃) / ice tray.
3) Homogenize the flesh in 5 volumes (v/w) of ice cold distilled water using tissue homogenizer.
4) Centrifuge the contents at 4℃ at 10000 rpm.
5) Use the supernatant for the determination of enzyme activity.

A) Determination of total protease enzyme activity (Furne et al., 2005)

Principle

One unit of enzyme activity represents the amount of enzyme required to liberate one µg of tyrosine min^{-1}under assay conditions.

Reagents used

1) 0.1M Phosphate buffer, pH 7.6:

   Add 16 ml of 0.2M NaH_2PO_4 to 84 ml of 0.2M Na_2HPO_4 and make the volume to 200 ml using distilled water and adjust the pH to 7.6.

   For preparing 0.2M Na_2HPO_4 dissolve 3.56 g Na_2HPO_4 in 100 ml distilled water

   For preparing 0.2M NaH_2PO_4 dissolve 3.12 g NaH_2PO_4 in 100 ml distilled water)

2) 8% Trichloroacetic acid (w/v)

   Dissolve 8 g of TCA in distilled water and make the volume to 100 ml.

3) 1% Casein (w/v)

   10 mg casein / 1ml of distilled water add some drops of NaoH and adjust pH until 7 with HCl.

4) Standard solution of L-Tyrosine 0.03 % (w/v): 0.03 g L-Tyrosine in 100 ml of distilled H_2O.

Procedure

Take the 250 µl of the substrate (1% Casein) solution, 250 µl of 0.1M phosphate buffer (pH 7.6), 100 µl of crude enzyme extract (homogenate) in test tube. Shake and incubate the tubes at 37℃ for 1 hr. After 1 hr stop the reaction by adding 600 µl of 8% TCA solution and then kept in ice for 1 hr. After 1 hr the sample was centrifuged 4000 rpm for 10 minutes. Use the supernatant for the determination of enzyme activity and it was read at 280 nm.

Determine the soluble protein from the enzyme extract by Lowry's method.

Calculate enzyme activity as follows:

Protease enzyme activity (µg/g/hr) = OD of assay× total volume of assay/ volume of enzyme× time (min) × volume used in colorimetric determination.

Specific protease enzyme activity (tyrosine liberated/ U/ mg protein)

= Enzyme activity (µg/g/hr) / Protein (mg/g).

Preparation of standard curve

Dissolve 30 mg of tyrosine in distilled water and make the volume to 100 ml to obtain the standard tyrosine solution of 3 µg/ml. Prepare the other dilution in the range of 50-300 µg were taken and treated in a similar manner along with the blank containing only the reagent.

B) Determination amylase enzyme activity (Hidalgo et al., 1999)

Principle

Amylase enzyme activity is assayed according to the Bernfeld (1955) in which the increase in reducing power of buffered starch solutions is measured.

Reagents used

1) Starch solution (1%)

   Dissolve 1 g of starch in warm distilled water and make the volume to 100 ml.

2) NaCl Solution (1%)

   Dissolve 1g of sodium chloride in distilled water and make the volume to 100 ml.

3) 3,5 Dinitrosalicylic acid reagent

   Dissolve 1 g of 3, 5 dintrosalicylic acid, 30 g sodium-potassium tartarate and 1.6 g NaOH and make the volume to 100 ml. Store in brown bottle.

4) 0.1M phosphate buffer (pH 7).

   Add 16 ml of 0.2M NaH_2PO_4 to 84 ml of 0.2M Na_2HPO_4 and make the volume to 200 ml using distilled water and adjust the pH to 7.6.

   For preparing 0.2M Na_2HPO_4 dissolve 3.56 g Na_2HPO_4 in 100 ml distilled water.

   For preparing 0.2M NaH_2PO_4 dissolve 3.12 g NaH_2PO_4 in 100 ml distilled water)

5) Standard Maltose Solution: Dissolve 100 mg of maltose monohydrate in distilled water and make the volume up to 100 ml.

Procedure

Take 1 ml of 1% starch solution (substrate), 1 ml of 0.1M phosphate buffer (pH 7.0), 1 ml of 1% NaCl and 1 ml of enzyme extract solution in test tube. Incubate the test tube for 1 h at 37℃. After 1 hr, stop the reaction by the addition of 0.5 ml of 3, 5 dinitrosalicylic acid. Note the absorbance at 540 nm. Deduce the value from standard curve prepared by using maltose monohydrate. Determine soluble protein (mg/g) from the enzyme the extract by Lowry's (1951) method.

Amylase enzyme activity (µg/g/hr) = OD of assay× total volume of assay/ volume of enzyme× time (min) × volume used in colorimetric determination.

Specific amylase enzyme activity (maltose liberated/U/ mg protein)

= Enzyme activity (µg/g/hr) / Protein (mg/g).

Preparation of standard curve

Dissolve 100 mg of maltose monohydrate in distilled water and make the volume to 100 ml to obtain standard maltose solution of 1 mg/ml concentration. From that the range of 200-1000 µg were taken and treated in a similar manner along with the blank containing only the reagent.

C) Determination of lipase enzyme activity (Furne et al., 2005)

Principle

The amount of free fatty acid released per unit time is estimated by the amount of NaOH required to maintain pH constant. The lipase activity is measured as milli equivalents of alkali consumed.

Reagents used

1) Alcohol-Acetone Solution (1:1).
2) Phenolphthalein Solution 1% (w/v) in ethanol.
3) Standard Solution of lipase 0.02% (w/v): 0.02 g lipase / 100 ml of distilled water.
4) Olive oil-PVA solution (emulsion):
    - Dissolve 10 g polyvinyl alcohol (PVA) in 1 liter of distilled water + 5 ml HCl 0.1N. Mix by mechanic agitation.
    - Incubate 75-85℃ in 1 h.
    - Cool at room temperature.
    - Filter and adjust pH to 8 with NaOH 0.1 N.
    - Take 100 ml and add 10.8 ml of olive oil.
    - Emulsify shaking for 5minutes.
5) Buffer Mcllvaine:
    - Solution A: 0.1 M Citrate – 19.21 g of citrate in 1L of distilled H_2O.
    - Solution B: Na_2HPO_4 – 28.4 g of Na_2HPO_4 in 1L of distilled H_2O.
    - Mix A and B until reach pH to 8.

Procedure

Take 500 µl of sample and incubate 100℃ for 5-10 minutes. 1000 µl of Ao-PVA (emulsion) and 500 µl of Mcllvaine buffer. Incubate with constant shaking 4 hr 37℃. And then add 3ml of alcohol-acetone 1:1 and 5 drops of Phenolphthalein (1% in ethanol). As the pH drops by about 0.2 unit, add 0.1N NaOH to bring the pH back to 7.0 and tritrate against 0.01N NaOH till pael pink colour was appeared. Repeat titration for 30 minutes period and note the volume of NaOH consumed. Estimate the protein content in the enzyme extract according to Lowry's method.

Calculate enzyme activity as amount of enzyme required to release one milliequivalent of free fatty acid/ minute/ g of sample and specific as equivalents/minute/ mg of protein.

Lipase activity (meq/minute/g of sample)

= Volume of NaOH consumed × 0.1 (normality of NaoH)/ volume of sample× time (minutes)

Specific lipase activity (lipase liberated / U/ mg protein)

= Enzyme activity (mg/g/hr) / Protein (mg/g).

Preparation of standard curve

Dissolve 20 mg of lipase in distilled water and make the volume to 100 ml to obtain standard lipase solution of 200 µg ml^{-1} concentration. Prepare the other dilution in the range of 50-200 µg were taken and treated in a similar manner along with the blank containing only the reagent.

2. Biochemical constituents

A) Estimation of total protein (Lowry et al., 1951)

Principle

Protein reacts with Folin ciocalteus reagent to give a coloured complex. The colour formed is due to the reaction of alkaline copper with the protein at the reduction of phosphomolybdate tyrosine and tryptophan present in the protein. The intensity of the colour depends upon the amount of these aromatic acids present and will thus vary for different proteins.

Reagents

1) **80% ethanol:** 80 ml of ethyl alcohol was dissolved in 20 ml distilled water.
2) **NaOH (0.1N):** 400 mg of NaOH was dissolved in 100 ml of distilled water.
3) **NaOH (1N):** 4 g of NaOH was dissolved in 100 ml of distilled water.
4) **Solution A:** 2 g of sodium carbonate was dissolved in 100 ml of 0.1N NaOH.
5) **Solution B:** Solution B was prepared by dissolving 500 mg of copper sulphate in 1% sodium potassium tartarate (1 g of sodium potassium tartarate in 100 ml of D. H_2O).
6) **Solution C:** Solution C was prepared by mixing 50 ml of solution A with 1 ml of solution B.
7) **Folinciocalteus reagent:** Folin ciocalteus reagent was prepared by mixing 1 ml of Folin ciocalteus reagent with 1 ml of distilled water.
8) **Blank:** 5 ml of solution C, 0.5 ml of 1N NaOH and 0.5 ml of Folin ciocalteus reagent served as the blank solution.
9) **Standard:** Bovine serum albumin (BSA) at the concentration of mg/ml and different dilutions from this stock solution served as the standard.

Procedure

From each group, 0.1 g of crab tissue sample from each experimental group was taken and it was homogenized well using 2 ml of 80% ethanol. Then it was centrifuged at 5000 rpm at 4℃ for 15 min. The precipitate was dissolved in 1N NaOH and made up to 5 ml. From this, 0.5 ml was taken and then 5 ml of the solution C was added and incubated for 20 min. Finally, 0.5 ml of Folin ciocalteus reagent was added and the intensity of the colour developed was read at 660 nm in a Spectrophotometer.

Calculation

Protein present in the sample (mg/g) = OD of the sample/ OD of the standard × Conc. of the std. (mg)

B) Estimation of Amino acid (Moore and Stein, 1984)

Principle

When amino acids are heated with ninhydrin, they undergo deamination and a beautiful blue or purple colour developed, which is of value in both qualitative and quantitative determination of amino acids. The ketoacid formed in the oxidative deamination is decomposed by heat into an aldehyde and carbon dioxide.

Ninhydrin + amino acid Hydrantin +aldehyde +CO_2 +NH_3 Hydrantin reacts with some of the ninhydrin to form Ruhemans purple.

Reagents

1) **10% Sodium tungstate:** Prepared by dissolving 10 g Sodium tungstate in 100 ml distilled water.
2) **2/3N.H_2SO_4:** 6.6 ml of concentrated sulphuric acid was diluted to 350 ml with distilled water.
3) **Ninhydrin reagent:** It was prepared by dissolving 0.5 g of ninhydrin in 12.5 ml ethanol and stored in the refrigerator.
4) **1% Leucine solution (standard solution):** 100 mg leucine was dissolved in 10 ml of 80% ethanol.

Procedure

From each group, 0.5 g of crabs' tissue was accurately weighed and homogenized individually with 2 ml distilled water, to this 1 ml of sodium tungstate and 1 ml 2/3N H_2SO_4 were added. This mixture was then centrifuged at 3000 rpm for 10 minutes and the supernatant was collected. Three test tubes were taken and labelled as blank, test, and standard. 0.5 ml supernatant was added to the test tube 'test', 0.5 to 'standard' and 4.5 ml distilled water was added to both test tubes. 5 ml of distilled water was added to the blank. 0.5 ml ninhydrin was pipetted to all test tubes and were cotton plugged. The tubes were kept in a boiling water bath until blue colour developed. The tubes were cooled and the intensity of the colour developed was measured with UV spectrophotometer at 540 nm.

Calculation

Amino acid present in the sample (mg/g) = OD of the sample/ OD of the standard × Conc. of the std. (mg)

C) Estimation of carbohydrate (Roe et al., 1955)

Principle

Sulphuric acid hydrolyzes the di and oligosaccharides into monosaccharides and converts the monosaccharides into furfural or furfural derivatives, which react with anthrone and produces a complex coloured product.

Reagents

1) **80% ethanol:** 80 ml of ethanol was dissolved in 20 ml of distilled water.
2) **Anthrone reagent:** 200 mg of anthrone powder was dissolved in 50 ml cold concentrated sulphuric acid. To this, 0.5 ml of thiourea was added to stabilize the colour.
3) **Standard:** 100 mg of D-glucose was dissolved in 100 ml of saturated benzoic acid and different dilutions from this stock solution served as a standard.

Procedure

From each group, 0.5 g of crab tissue sample was taken and it was homogenized well using 2 ml of 80% ethanol. Then it was centrifuged at 5000 rpm for 15 min. at 4℃. To the clear supernatant (0.5 ml), 4 ml of anthrone reagent was added and the test tubes were kept in a boiling water bath for 15 minutes. The test tubes were taken out and kept in a dark room for 10 min. and finally, the colour developed was measured at 620 nm in a spectrophotometer.

Calculation

Carbohydrate present in the sample (mg/g) = OD of the sample/OD of the standard ×Conc. of the std. (mg)

D) Estimation of lipid (Folch *et al*., 1957)

Principle

The quantitative determination of lipid by sulphophosphovanillin method depends on the reaction of lipids extracted from the sample using chloroform-methanol, with sulphuric acid, phosphoric acid and vanillin to give a red complex.

Reagents

1) **Chloroform-methanol (2:1):** This reagent was prepared by mixing 200 ml of chloroform and 100 ml of methanol.
2) Sodium chloride (0.9%): 900 mg of NaCl was dissolved in 100 ml distilled water.
3) **Sulphophosphovanillin reagent:** 800 ml of Orthophosphoric acid was added to 200 ml of distilled water. To this, 2 g of vanillin powder was added and mixed well.
4) **Stand**ard: 10 mg of olive oil was dissolved in 10 ml chloroform-methanol mixture (2:1) and different dilutions from this stock solution served as the standard.
5) **Blank:** Vanillin reagent was used as a blank solution.

Procedure

From each group, 0.5 g of crabs' tissue sample was taken and homogenized well with 4 ml of the chloroform-methanol mixture. After mixing well, 0.2 ml of 0.9% sodium chloride was added and the mixture was kept undisturbed overnight. The lower layer of lipid was collected carefully and dried in a vacuum desiccator. The dried lipid content was dissolved in concentrated sulphuric acid (0.5 ml) and kept in a boiling water bath for 10 min. From the lipid sample, 0.2 ml was taken in a test tube and 5 ml of sulphophosphovanillin reagent was added, shaken well and kept undisturbed for 30 minutes. The intensity of red colour was measured at 520 nm in a spectrophotometer.

Calculation

Lipid present in the sample (mg/g) = OD of the sample/ OD of the standard × Conc.

E) Estimation of ash and moisture

Principle

The wet tissue was allowed to dry by kept in desiccator. The difference between the wet weight of the tissue and its dry weight give the amount of water present in the fresh tissue. On heating the dry material to higher temperature all the organic constituents were burnt leaving only the inorganic constituents in the form as ash.

Procedure

Known amount of wet tissue sample was taken individually on previously weighed concave glass and they were kept in a desiccator, maintaining 0.5% relative humidity. Dry the tissues in the desiccator till they reached a constant weight. Then the dried materials were transferred individually in silica crucible and kept in a muffle furnace and heated at 550-600℃ for 4 h. Finally the ash formed was weighed.

Calculation

$$\text{Ash (\%)} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample taken (g)}} \times 100$$

$$\text{Moisture (\%)} = \frac{\text{Wet weight (g)} - \text{Dry weight (g)}}{\text{Wet weight (g)}} \times 100$$

CHAPTER III

1. Determination of amino acids

Procedure

Sample digestion

The powdered crab samples of each 100 mg were weighed accurately in an electronic balance and transferred into labelled test tubes. 1 ml of 6N Hydrochloric acid solution was added to the samples in specified test tubes. These test tubes were sealed at the top under vacuum by high temperature gas flame, conducted triplicates of samples. All the sealed tubes were kept in a hot-air oven at 110℃ for 48 hr continuously.

Test solution preparation

After completion of digestion, broken the tubes at the top and transferred the digest into beakers individually, rinsed the tubes 5 times with distilled water. The acid in the digest was evaporated to core dry under vacuum using Roto-vac evaporator. The residual content was dissolved with distilled water and made-up to 6 ml in a centrifuge tubes. This solution contains 41.6 µg dried raw sample in 1 µl distilled water and used as test solution for amino-acid profile analysis by HPTLC technique.

HPTLC analysis

Standards

Group I	Group II	Group III	Group IV
Asparagine	Aspartic acid	Lysine	Histidine
Glutamine	Glutamic acid	Glycine	Arginine
Serine	Alanine	Threonine	Cystine
Proline	Valine	Tyrosine	Tryptophan
Methionine	Phenyl alanine	Isoleucine	Leucine

Sample and Standard amino acid loading

1.2 µl of each test solutions were loaded as 6mm band in pre-coated Silica gel $60F_{254}$ TLC plate (13 cm × 10 cm) using 100 µl Hamilton syringe and CAMAG-LINOMAT 5 instrument. 2 µl of each Group I, II, III & IV standards were loaded in the plate for analysis as separate tracks.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber with respective mobile phase (Amino acids), 20 min for Chamber saturation. After chamber saturation, the plate was developed in respective mobile phase up to 90 mm (Double run with the same mobile phase).

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was documented using Photo-documentation chamber (CAMAG-REPROSTAR 3) at Daylight, UV 220 nm and UV 366 nm mode.

Derivatization

The plate was sprayed with respective spray reagent (Amino-acids) and dried at 110℃ in a hot air oven. After derivatization, the plate was documented at daylight using CAMAG-REPROSTAR 3.

Scanning

Finally, the plate was fixed in scanner stage and scanned at 500 nm using CAMAG-TLC SCANNER 3. The Rf value and peak area of each track were observed for quantification study.

Calculations

Sample concentration **:** 100 mg dried raw material in 6 ml distilled water.

Loaded volume of test solution **:** 20 µl.

Individual amino acid content (%) : Conc. of amino acid in µg / 50 µg ×100.

2. Determination of fatty acids using GC

Folch Extraction of Fatty Acids from crab tissue

Crab tissue (0.5 g) was extracted with $CHCl_3$: MeOH (2:1, vol/vol) containing C13:0 as the internal standard, according to the method of Folch et al. (1956), using a Brinkmann polytron at room temperature. The extraction mixture was then filtered through a scintered glass filter, and replicate aliquots were pipetted into a 16 × 125 mm screw-cap Pyrex culture tube and washed with 0.02% aqueous $CaCl_2$. The organic phase was dried with Na_2SO_4 and K_2CO_3 (10:1, wt/wt), and the solvent was subsequently removed under N at 55°C. The fatty acid composition of the FAME was determined by capillary GC on a SHRT-2560, 100 m × 25mm × 0.25 μm capillary column (Supelco) installed on a Shimadzu GC-2014 chromatograph, a flame ionization detector, and split injection. The initial oven temperature was 140°C, held for 5 min, subsequently increased to 240°C at a rate of 4°C min^{-1}, and then held for 20 min. Nitrogen was used as the carrier gas at a flow rate of 0.5 mL min^{-1}, and the column head pressure was 280 kPa. Both the injector and the detector were set at 260°C. The split ratio was 30:1. Fatty acids were identified by comparing their retention times with the fatty acid methyl standards described previously.

CHAPTER IV

1. Antioxidant activity

A) Estimation of Super oxide dismutase (SOD) (Marklund and Marklund, 1974)

Principle

The degree of inhibition of auto oxidation of pyrogallol at an alkaline pH superoxide dismutase is used as a measure of the enzymatic activity.

Reagents

1. **Tris-HCI buffer**: 0.1 M, pH 8.2 containing 2 mM of di ethylene tri amine penta acetic acid.
2. **Tris- HCI:** 0.05 M, pH 7.4.
3. **Pyrogallol stock solution:** 25.2 mg of pyrogallol was dissolved in 1 ml of 0.05 M Tris HCI buffer, pH 7.4 in a test tube stoppered and wrapped with an aluminium foil.
4. **Pyrogallol working solution:** At the time of assay 0.5 ml was diluted to 50 ml with 0.05 M Tric HCl buffer, pH 7.4 to give a 2 mM solution and shielded from exposure to light.
5. **Absolute ethanol.**
6. **Chloroform.**

Procedure

Partially purified SOD was prepared as described by McCord and Fridovich (1969). To 1 ml of the tissue homogenate, 0.25 ml of absolute ethanol and 0.15 ml of chloroform was added. After 15 min of centrifugation at 13,000 rpm, the suspension was centrifuged and the supernatant obtained constituted the enzyme extract. The reaction mixture for autoxidation consisted of 2 ml of the buffer containing DETAPAC, 0.5 ml of 2 rnM pyrogallol and 1.5 ml water. Initially, the rate of autoxidation of pyrogallol was noted at an interval of one minute to three minutes. The assay mixture for the enzyme contained 2 ml of 0.05 M Tris-HCI buffer, 0.5 ml pyrogallol, aliquots of the homogenate and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol autoxidation

after the addition of the enzyme was noted. Iron accelerates pyrogallol autoxidation even in trace amounts. DETAPAC acts as a chelator and thus prevents the interference from Fe^{2+} as well as from CU^{2+} and Mn^{2+}. The enzyme activity was expressed in terms of unit/mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50%.

B) Estimation of Catalyse (CAT) (Sinha, 1972)

Principle

The catalyse enzyme preparation was allowed to split hydrogen peroxide for different period of time. The reaction was stopped at specified time intervals by adding dichromate/acetic acid mixture. The dichromate in acetic acid is reduced to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of per chloric acid as an unstable intermediate. The chromic acetate thus produced is measured at 620 nm. Since dichrornate has no absorbance in this region, the presence of this compound in the assay mixture does not interfere with the calorimetric determination of chromic acetate.

Reagents

1. **Stock dichromatic acetic acid reagent:** This reagent was prepared by mixing a 5% solution of potassium dichromate with glacial acetic acid (1:3) by volume.
2. **Working dichromatic acetic acid reagent:** The stock was diluted (1:s) with water to make the working dichromatic acetic acid solution.
3. **Hydrogen peroxide (0.2 M):** 1.0 ml of 30% hydrogen peroxide was made up to 45.0 ml with water.
4. **Phosphate buffer:** 0.01 M, pH 7.0

Procedure

Tissue homogenate was prepared by using phosphate buffer (0.01M, pH 7.0). The reaction mixture contained 0.5 ml of hydrogen peroxide, 1.0 ml of buffer, 0.4 ml of water and 0.1 ml of diluted homogenate (1: 10). After 15, 30 and 60 seconds of incubation, 2.0 ml of dichromatic acetic acid reagent was added. To the control tube, the

enzyme was added after the addition of acid reagent the tubes were then heated and the colour developed was read at 610 nm. The activity of catalase was arrived at from the amount of hydrogen peroxide consumed and was expressed as poles of hydrogen peroxide consumed/min/mg protein.

C) Estimation of Lipid peroxidation (LPO) (Sinha, 1972)

Thiobarbituric acid reactive substances (TBARS)

The levels of lipid peroxidation in tissues was determined by the method of Ohkawa et al. (1979).

Principle

Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) are quantitated by their reactivity with thiobarbituric acid (TBA) in acidic conditions. The reaction generates a pink coloured chromophore, which can be read in a colorimeter at 535 nm.

Reagents

1) **Trichloroacetic acid**, TCA-5%.
2) **Thiobarbituric acid:** TBA-0.375% in hot distilled water.
3) **Hydrochloric acid (HCL-0.25N).**
4) **TCA+TBA+HCL reagent:** Solutions a, b and c were mixed freshly in the ratio of 1:1:1.
5) **Stock Standard:** 4, 4 mol/l solution of stock was prepared from 1,1,3,3 - tetra methoxy propane in distilled water.
6) **Working Standard:** The Stock solution was diluted to get a concentration of 50 nmol/ml.

Procedure

To 1.0 ml of the tissue homogenate, 2.0 ml of TCA-TBA-HCL reagent was added and mixed thoroughly. The mixture was kept in a boiling water bath for 15 min. After cooling, the tubes were centrifuged at 100 × g for 20 min and the colour developed in the

supernatant was measured in a colorimeter at 535 nm against a reagent blank. A series of standard solutions in the concentration of 2-10 µ moles were treated in a similar manner. Values were expressed as µ moles of malondialdehyde (MDA)/ mg protein.

2. Metabolic enzymes activity

A) Estimation of Glutamic oxaloacetic transaminase (GOT)

The GOT activity was determined according to the method of Reitman and Frankel (1957) using kit (HiMedia Laboratories Pvt. Ltd. Mumbai, Maharashtra, India).

Principle

GOT (AST) catalyses the following reaction: α- ketoglutarate + L- Aspartate L-Glutamate + oxaloacetate oxaloacetate so formed is coupled with 2,4-Dinitrophenylhydrazine (2,4- DNPH) to give the corresponding hydrozone, which gives brown colour in alkaline medium.

Reagents

Reagent 1 - Buffered aspartate α ketoglutarate substrate, pH 7.4

Reagent 2 - DNPH colour reagent

Reagent 3 - Sodium hydroxide, 4N

Reagent 4 - Working Pyruvate standard, 2mM

Procedure

Exactly, 100 mg of test animals were homogenized in sucrose of 0.25 M and centrifuged at 6000 rpm for 20 minutes in a high-speed cooling centrifuge at 4℃. Buffered aspartate-/-ketoglutarate substrate (250 µl; pH 7.4) was added in marked test tubes. Centrifuged sample supernatant (20 µl) was added to each of the buffered substrates, mixed well, and incubated at 37ºC for 1 hr. After 60 min incubation, 250 µl of 2, 4- dinitrophenyl hydrazine colour reagent was added to each of the incubated mixtures, mixed well, and allowed to stand at room temperature for 20 min. After 20 min, 3 ml of 0.4 M sodium hydroxide solution (freshly prepared) was added to each of the incubated mixtures, mixed well. OD of the colour developed in the incubated mixtures was read at 505 nm in a spectrophotometer within 15 min. The enzyme activities were expressed in micromole of pyruvate formed U/ml.

B) Estimation of Glutamic pyruvate transaminase (GPT)

GPT activity was determined according to the method of Reitman and Frankel (1957) using kit (HiMedia Laboratories Pvt. Ltd. Mumbai, Maharashtra, India).

Principle

GPT (ALT) catalyses the following reaction: α- ketoglutarate + L- Alanine L-Glutamate + pyruvate pyruvate so formed is with 2, 4- Dinitrophenylhydrazine (2, 4-DNPH) to give the corresponding hydrozone, which gives brown colour in alkaline medium.

Reagents

Reagent 1- Buffered alanine α ketoglutarate substrate pH 7.4

Reagent 2- DNPH colour reagent

Reagent 3- Sodium hydroxide, 4N

Reagent 4 – Working Pyruvate standard, 2mM

Procedure

100 mg of test animals were homogenized in sucrose of 0.25 M and centrifuged at 6000 rpm for 20 minutes in a high-speed cooling centrifuge at 4℃. Bufferedalanine/ketoglutarate substrate (250µL; pH 7.4) was added in marked test tubes. Centrifuged sample supernatant (20 µl) was added to each of the buffered substrates, mixed well, and incubated at 37℃ for 30 min. After 30 min incubation, 250 µL of 2, 4- dinitrophenyl hydrazine colour reagent was added to each of the incubated mixtures, mixed well, and allowed to stand at room temperature for 20 min. After 20 min, 3 ml of 0.4 M sodium hydroxide solution (freshly prepared) was added to each of the incubated mixtures, mixed well. OD of the colour developed in the incubated mixtures was read at 505 nm in a spectrophotometer within 15 min. The enzyme activities were expressed in micromole of pyruvate formed U/ml.

C) Estimation of Alkaline phosphatase activity (ALP)

Alkaline phosphatase (ALP) activity was estimated according to Kind and King's (1954) method. In short, the whole crab, excluding carapace, eyestalk, and legs, was homogenized in 1ml of 50mM ice-cold citrate buffer and centrifuge at 9961×g at 4 °C for

10 min. Four test tubes were taken as blank (B), standard (S), control (C) and test (T). Precisely, 1.05 and 1 ml double distilled water were added in B and the rest of the three tubes (S, C, and T), respectively. Further, 1000 µl of buffer reagent and 100 µlml of disodium phenyl phosphate substrate reagent were added to all tubes, followed by mixed well and allowing to stand at 37 °C for 3 min. The extracted sample (50 µl) was added into T, and 50 µlof phenol solution was added into S as standard. These tubes were mixed well, followed by allowing to stand for 15 min at 37 °C. The colour reagent (1000 µl) (Phenol + 4-aminoantipyrine) was added in all tubes, and 50 µl of sample was added in C alone. Finally, the absorbance of each tube (B, S, C, and T) was measured at 510 nm in a UV-visible spectrophotometer against distilled water.

Experimental setup of the proposed work

Publications

PUBLICATIONS

1. **Thangal, S. H.,** Muralisankar, T., Anandhan, K., Gayathri, V., Yogeshwaran, A. 2022. Effect of CO_2 driven ocean acidification on the mud crab *Scylla serrata* instars. Environ. Pollut. 312, 119995. https://doi.org/10.1016/j.envpol.2022.119995 (**Impact factor: 9.98**).
2. **Thangal, S. H.,** Nivetha, M., Muttharasi, C., Anandhan, K., Muralisankar, T. 2021. Effects of acidified seawater on biological and physiological responses of *Artemia franciscana*. Mar. Pollut. Bull. 169, 112476. https://doi.org/10.1016 /j.marpolbul.2021.112476 (**Impact factor: 7.001)**
3. Muralisankar, T., Kalaivani, P., **Thangal, S. H.,** Santhanam, P. 2021. Growth, biochemical, antioxidants, metabolic enzymes and hemocytes population of the shrimp Litopenaeus vannamei exposed to acidified seawater. Comp. Biochem. Physiol. Part - C: Toxicol. 239, 108843. https://doi.org/10.1016 /j.cbpc.2020.108843 (**Impact factor: 4.53)**
4. Anandhan, K., Tharini, K., **Thangal, S. H.,** Yogeshwaran, A., Muralisankar, T. 2022. Occurrence of microplastics in the gastrointestinal tracts of edible fishes from South Indian Rivers. Bull. Environ. Contam. Toxicol. 109, 1023–1028. https://doi.org/10.1007/s00128-022-03595-3 (**Impact factor: 2.80)**
5. Muttharasi, C., Muralisankar, T., Uthayakumar, V., Gayathri, V., **Thangal, S.H.** 2019. Utilization of marine fisheries wastes for the production of the freshwater fish *Cyprinus carpio*. Trop. Anim. Health Prod. 2305-2313. doi: 10.1007/s11250-019-01938-7. 7 **(IF: 1.96)**
6. **Thangal, S. H.,** Nandhinipriya, R., Vasuki, C., Gayathri, V., Anandhan, K., Yogeshwaran, A., Ramesh, M., Rajaram, R., Santhanam, P., Muralisankar, T. 2023. The impact of ocean acidification and cadmium toxicity in the marine crab *Scylla serrata*: Biological indices and oxidative stress responses. Chemosphere, Manuscript No. CHEM120809 (under review).
7. Muralisankar, T., **Thangal, S. H.,** Rajaram, R. 2023. Changes in amino acides and fatty acids in the edible shrimp Litopenaeus vannamei reared in CO_2 driven ocean acidification. Aquaculture, Manuscript No. AQUACULTURE-D-23-00960 (under review).

Paper presentation

International Conference

Thangal, S. H., Muralisankar, T. Oral presentation on "Effects of pH change due to CO_2 elevation on survival, growth, biochemical constituents and antioxidant status of Mud crab *Scylla serrata* in SERB, DST-PURSE, DRDO, AND MPEDA" sponsored International Conference on Recent Biotechnological Innovation in Aquaculture (RBIA) -2020 held on 27-28/02/2020, Bharathiar University, Coimbatore

Thangal, S. H., Muralisankar, T. Oral presentation on "Effect of different pH on survival, growth, biochemical constituent and Antioxidant status of Brine shrimp (*Artemia franciscana*) in RUSA" sponsored International Conference on Molecular physiology, Therapeutics and Experimental Medicine- 2019 held on 25-26/07/2019, Alagappa University, Karaikudi.

Thangal, S. H., Muralisankar, T. Poster presentation on "Effect of CO_2 driven ocean acidification on the survival, growth and biochemical constituents of mud crab *Scylla serrata*" in DBT sponsored International Conference on recent progresses in biological sciences (ICRPBS)- 2022 held on 4 and 5- 03-2022, Ayya Nadar Janaki Ammal College, Sivakasi, Coimbatore.

National Conference

Thangal, S. H., Muralisankar, T. Paper presentation on "Effect of CO_2 driven ocean acidification on the toxicity of cadmium in the mud crab *Scylla serrata* instars" in Kerala Sasthra Sahithya Parishath (KSSP) and Kerala institute of Local Administrative (KILA) sponsored Young Scholar Congress on Resilience to Climate Change Impacts on Ecosystems and Livelihood of Kerala, held on 5 and 6-01-2023, KILA Campus, Thrissur, Kerala.

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Workshop/ training attended

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Effect of CO_2 driven ocean acidification on the mud crab *Scylla serrata* instars☆

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ABSTRACT

The decreasing ocean pH seems to adversely affect marine organisms, including crustaceans, which leads to potential threats to seafood safety. The present investigation evaluated the effect of seawater acidification on the edible marine mud crab *Scylla serrata* instars. The experimental setup was designed using a multi-cell cage based system assembled with 20 pre holed PVC pipes containing 20 individual crabs to avoid cannibalism. The crab instars were exposed to CO_2 driven acidified seawater at pH 7.8 (IPCC forecast pH at the end of the 21st century), 7.6, 7.4, 7.2, and 7.0 for 60 days. The crabs reared in seawater without acidification at pH 8.2 served as control. The present study revealed a notable decrease in survival, feed intake, growth, molting, tissue biochemical constituents, minerals, chitin, and alkaline phosphatase in *S. serrata* instar reared in acidified seawater, denotes the adverse effect of seawater acidification on crabs. The significant elevations in antioxidants, lipid peroxidation, and metabolic enzymes in all acidified seawater compared to ambient pH indicates the physiological stress of the crabs' instars. The changes in the metabolic enzymes reveal the metabolism of protein and glucose for additional energy required by the crabs to tolerate the acidic stress. Hence, the present study provides insight into the seawater acidification can adversely affect the crab *S. serrata*.

1. Introduction

The emission of CO_2 into the atmosphere is gradually increasing yearly due to anthropogenic activities. This elevated level of atmospheric CO_2 can produce a severe issue in the marine environment regarding ocean acidification, the atmospheric CO_2 combined with seawater form carbonic acid (H_2CO_3). The carbonic acid releases the hydrogen (H^+) ions, leading to bicarbonate (HCO_3-) ions forming. Then the released H^+ ions combined with carbonate ions led to bicarbonate formation (Hoegh-Guldberg, 2011; Figuerola et al., 2021). These changes in water chemistry lead to a scarcity of carbonate ions to bind with calcium (Ca) for the formation of calcium carbonate ($CaCO_3$), which is highly essential for the calcareous species in marine environments (Frölicher et al., 2015; Wit et al., 2018; NOAA, 2020a,b). As per the forecast of the "Intergovernmental Panel on Climate Change" (IPCC, 2007), the ocean pH could alter to pH 7.8 during the year 2100 because of the continuous release of CO_2 by various anthropogenic influences such as the burning of fossil fuels, oil and gas industries, deforestation, improper chemical waste management, etc. (Caldeira and Wickett, 2005). The alterations in ocean pH will create a potentially adverse effect on marine animals, especially calcifiers like Crustacea, Echinoidea, Cnidaria, Bivalvia, Gastropoda, Annelida, etc. (Bierbower and Cooper, 2010; Carter et al., 2013; Mollica et al., 2018; Melzner et al., 2020; Anand et al., 2021), and also economically valuable marine organisms (Branch et al., 2013; Muralisankar et al., 2021). Moreover, CO_2 driven acidification interacts with other stressors like toxic metals, organic pollutants, temperature, hypoxia, etc., creating detrimental effects on survival, growth, larval development, reproduction, antioxidants, metabolism, haemocytes and phagocytosis of the organisms (Wu et al., 2017; Kong et al., 2019; Wang et al., 2020; Shang et al., 2020; Khan et al., 2021; Yang et al., 2022).

Crustaceans perform high in demand in the global seafood trade due to their rich nutrients, delicious taste, and increased market value. Marine crustaceans contributed 9.4 million tons (USD 69.3 billion) to the world aquaculture and fisheries economy (FAO, 2020). The variations in the ocean environment seem to be instabilities in regulating

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acid-base equilibrium, shell formation, molting, and immunity that cause vulnerability to several abiotic and biotic substances and deprived survivorship in crustaceans (Small et al., 2010; Rehman et al., 2021). Ocean acidification adversely affects the survival, feed intake, growth, behaviour, reproduction, immune response, exoskeleton formation, haemolymph pH, oxidative stress, metabolic stress, genotoxicity, etc., of crustaceans, including crabs like *Cancer pagurus*, *Necorapuber, Paralithodes camtschaticus, Metacarcinus magister*, and *Lithodes aequispinus* (Wang et al., 2018; Small et al., 2010; Long et al., 2013 Bednaršek et al., 2020; Long et al., 2021), shrimps, (Kurihara et al., 2008; Furtado et al., 2015; Furtado et al., 2017; Muralisankar et al., 2021), lobsters (Nagle et al., 2018; Gravinese et al., 2020), brine shrimps (Monteiro, 2021; Thangal et al., 2021), copepods (Campoy et al., 2020; Halsband et al., 2021) and sea urchin (Anand et al., 2021).

The mud crab (*Scylla serrata*) has high economic valuable seafood in the international market (Bhuiyan et al., 2022) due to its rich nutrients. Southeast Asian nations such as Vietnam, the Philippines and Indonesia are the major mud crabs production countries, with an average production of 65,463, 18,100, and 15,000 tons, respectively (Yxtung, 2020). However, marine crabs are highly susceptible to climate changes (Bednaršek et al., 2020), including ocean acidification. In addition, crabs showed poor survival, growth, development, and immune response in the acidified environments (Walther et al., 2010; Long et al., 2013; Miller et al., 2016; Meseck et al., 2016). Nonetheless, the impact of seawater acidification on the mud crab *S. serrata* has not yet been reported. Hence, it is essential to evaluate the possible effect of ocean acidification on this economically important crab species to ensure seafood safety. Therefore, the present investigation focused on assessing the potential impact of CO_2 driven ocean acidification on survival, growth, molting, food indices, biochemicals, chitin, minerals, antioxidants, lipid peroxidation, metabolic enzymes and alkaline phosphatase of *S. serrata* using a multi-cell cage-based experiment to conduct laboratory experiments to prevent cannibalism.

2. Material and methods

2.1. Procurement of and acclimatization of mud crab instars

The mud crab *S. serrata* 1st instar was selected for this experiment due to its high sensitivity to changing environments and higher metabolic rate during the larval stages than adults. Crab's instars were purchased from Rajiv Gandhi Center for Aquaculture (RGCA-MPEDA) Sirgali, Tamilnadu, India. A total of 400 crab instars were transported in a five-litter transparent polythene cover filled with oxygenated natural seawater and hideouts to prevent cannibalism. Instars were accommodated in the ambient laboratory environment for one week in a fibre-reinforced plastic (FRP) tank (500 L) with natural seawater. During the acclimatized period, instars were fed 100%/body mass (Alava et al., 2017) of frozen brine shrimps twice daily (40% in the morning and 60% in the evening). Nearly 20% of seawater from the stocking tank was renewed, and the unfed feed and feces were removed during the renewing process. The detailed information for acclimatization of crabs is provided in the supplementary materials (SM) section 2.1.

2.2. Seawater acidification

The pH manipulation setup was planned as per the earlier method of Riebesell et al. (2007) with slight modifications (Fig. 1). The experimental system consisted of six aquaria with control pH 8.2, 7.8 (IPCC expected ocean pH by the year 2100), 7.6, 7.4, 7.2, and 7.0 were the pCO_2 level of 267.81, 588.03, 970.67, 1521.71, 2480.39, and 3731.44 µatm respectively. Water quality factors, such as salinity, dissolved oxygen, ammonia, and total alkalinity were evaluated based on standard methods (APHA, 2005). The carbonates (CO_3^{2-}), bicarbonates (HCO_3^-),

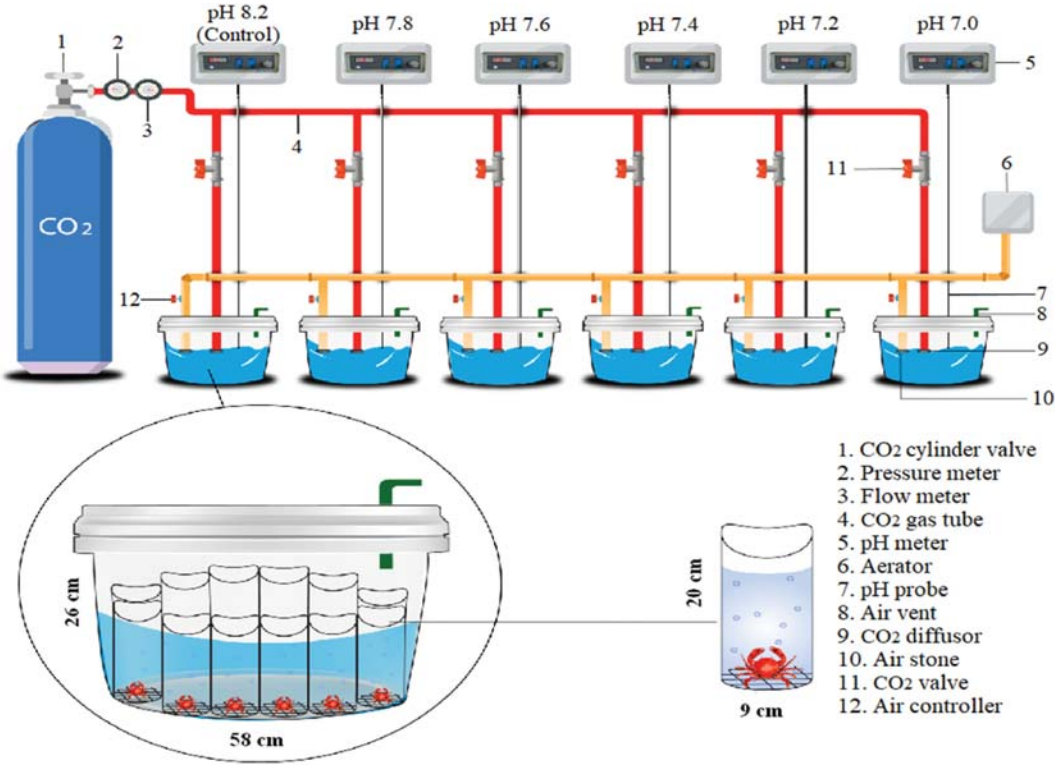

Fig. 1. Design of CO_2 experimental setup with multi-cell cage.

partial pressure of CO_2, saturation of calcium carbonate for calcspar (ΩCa) and aragonite (ΩAr), and total carbon dioxide (TCO_2) were determined as prescribed by Robbins et al. (2010) during the experimental period (Table S1 supplementary material). The detailed information for the seawater acidification process is provided in SM section 2.2.

2.3. Experimental setup on crabs

After seven days of acclimatization, a total number of 120 crabs (0.6 ± 0.05 cm length and 0.05 ± 0.01 g weight) were transferred into six aquaria for six different desired pH exposures (pH 8.2, 7.8, 7.6, 7.4, 7.2, and 7.0) in triplicate (120 × 3 = 360). Each aquarium was partitioned with 20 pre-holed PVC pipes as multi-cell cages, and individual crabs were placed in each cell to prevent cannibalism (Fig. 1). The desired pH of each investigational aquaria was employed and sustained bubbling of CO_2 (as detailed in the seawater acidification part) during the 60 days experimental duration. The crab instars were fed with frozen brine shrimp (*Artemia franciscana*) biomass for 15 days, and for the remaining 45 days, chopped white leg shrimp (*Litopenaeus vannamei*) muscle meat twice per day (Ganesh et al., 2015). Nearly 25% of aquarium water was renewed daily to maintain adequate water quality. The detailed information for the experimental crab setup is provided in SM section 2.3.

2.4. Survivorship, growth, molting, and food indices

At the end of 60 days of the experiment, the survivorship, growth parameters, like length and weight gain, specific growth rate, molting rate, and feed indices like feed intake and feed conversion ratio were determined using standard formula (Tekinay and Davies, 2001). The detailed information for this section is provided in SM section 2.4.

2.5. Analysis of biochemical elements

The total soluble tissue protein content of *S. serrata* was estimated by the method suggested by Lowry et al. (1951) using ethanolic precipitated tissue samples, and the bovine serum albumin was used as standard. The total amino acid was analyzed by the ninhydrin method using sulfuric acid and sodium tungstate extracted tissue samples with leucine as per the standard method of Moore and Stein (1948). The content of carbohydrates was estimated in tissue samples extracted with TCA by glucose as standard (Roe, 1955). Crab's lipid content was analyzed in tissue samples extracted using a chloroform-methanol mixture (Folch et al., 1957; Barnes and Blackstock, 1973), and virgin olive oil was used as standard. The moister and ash levels of crabs were determined by the standard AOAC (1995) methods.

2.6. Quantitative and qualitative determination of chitin

The level of chitin in the crab's shells was determined according to Pandharipande and Bhagat (2016). About 0.50 mg of crab chitin was analyzed in Fourier transform infrared (FT-IR) spectrometer with an absorption range between 4000 and 400 cm^{-1} to determine the IR bands. The detailed procedure for analysis of chitin is provided in SM section 2.5.

2.7. Assessment of minerals

The mineral contents like calcium (Ca), sodium (Na), and potassium (K) in the crab tissue in each pH treatment were determined using a flame photometer (Jeffery et al., 1989). Briefly, 1 g of crab tissue from each pH experimental was heated in 80 °C of 10 ml of triacid using HNO_3, H_2SO_4, and $HClO_4$ at the ratio of 9:2:1 for 3 h, followed by cooled at room temperature and filtered through a nylon filter (0.8μ) before mixing with double-distilled water. Determination of minerals was performed against Na, K, and Ca filtered by injecting the digested clear solution into the flame. The concentration of each mineral was expressed as mg kg^{-1} based on the digested sample weight.

2.8. Determination of antioxidants and lipid peroxidation

After 60 days, each pH treatment crab was anaesthetized with ice. The tissue of crabs from each pH was extracted in ice-cold tris buffer and centrifuged for 20 min at 2415×g at 4 °C. The supernatant was separated. The antioxidants such as superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (LPO) were analyzed using the supernatant. SOD activity was evaluated by pyrogallol autoxidation in tris buffer (Marklund and Marklund, 1974). CAT activity of crab was measured by the method of Sinha (1972) using hydrogen peroxide (H_2O_2) as substrate in phosphate buffer. LPO of the crab was assessed according to Ohkawa et al. (1979) by the creation of thio barbituric acid reactive substances. The detailed information for analysis of SOD, CAT, and LPO are provided in SM section 2.6.

2.9. Metabolic enzymes and alkaline phosphatase activity analysis

The metabolic enzyme activities, including glutamic oxaloacetate transaminase (GOT) and glutamic pyruvate transaminase (GPT) in the tissues of crabs exposed to acidified seawater were assessed as prescribed by Reitman and Frankel (1957). In these measurements, L-aspartic and L-alanine were utilized as the substrates for GOT and GPT activities, respectively, and sodium pyruvate was used as a calibrator. Alkaline phosphatase (ALP) activity was estimated according to Kind and King's (1954) method. The detailed information for analysing GOT, GPT, and ALP are provided in SM section 2.7.

2.10. Statistical analysis

The significant variations of obtained data among pH treatments were compared by Duncan's multiple range test (DMRT) using one-way analysis of variance (ANOVA) in SPSS (16.0) software, and the significant variation was denoted when $p < 0.05$. All the data were communicated as mean ± SD. The survival data, specific growth rate, moister, and ash were arcsine transformed before subject to one-way ANOVA.

3. Results

3.1. Physiochemical properties of experimental seawater

In the present investigation, all acidified experiments showed notable decreases ($p < 0.05$) in physicochemical characteristics of seawater, including pH, dissolved oxygen, CO_3^{2-}, calcite, and aragonite compared with the control pH. Nonetheless, an insignificant variation ($p > 0.05$) was observed in salinity, temperature, and alkalinity of all acidified seawater compared to the control. In this context, TCO_2, $_PCO_2$, and HCO_3^- were significantly raised ($p < 0.05$) in all acidified seawater compared to the control (Table S1 supplementary material).

3.2. Survivorship, growth, molting and food directories

The level of survival in *S. serrata* was decreased ($p < 0.05$) in acidified seawater, particularly the pH of 7.6 to 7.0 than that of pH 8.2 (control). The crabs exposed to pH 7.6, 7.4, 7.2 and 7.0 decreased their survival by 7, 12, 25 and 30%, respectively, with respect to the control. Nonetheless, an insignificant difference was recorded between the crab group reared in pH 7.8 and 8.2 ($p > 0.05$). The length, length gain, weight, and weight gain of the *S. serrata* exposed to all pH treatments indicated significant decreases ($p < 0.05$) compared to ambient pH 8.2. While the control crabs increased five times their initial length, those of the pH treatments increased 4.3, 4, 3.5, 3.3, and 3 times respectively. Besides the weight gained by the crabs, those with pH 7.8, 7.6, 7.4, 7.2, and 7.0 reduced their gain by 33, 45, 58, 70, and 75% with respect to the

control, respectively. However, crabs treated at pH 7.8 and 7.6 indicated negligible variance ($p > 0.05$) in these measurements. In contrast, a remarkable ($p < 0.05$) decline in molting rate was observed in crabs treated with all acidified seawater than in control. The crabs reared at pH 7.0 observed a maximum level of decrease (22%) in molting rate with respect to control. Nevertheless, crabs' moulting between pH 7.8, 7.6 and pH 7.2 and 7.0 was insignificant ($p > 0.05$) (Fig. 2).

The feed intake of crabs was reduced by 50% in all acidification experiments in respect of control, while crabs treated at pH 7.8 to 7.2 indicated negligible ($p > 0.05$) variation. The specific growth rate of crab revealed a significant difference ($p < 0.05$) reared in the pH 7.6 to 7.0 than that of control, and it showed 0.92, 0.88, 0.81, and 0.77 times lower in pH 7.6, 7.4, 7.2, and 7.0 respectively in respect of control. However, an insignificant variance in the specific growth rate of crabs was noticed at pH 7.8 compared to that at pH 8.2. A significant upsurge ($p < 0.05$) in feed conversion ratio was observed in crabs treated with pH 7.0 (increased 75% with respect to control) rather than pH treatments (8.2–7.2). Nevertheless, the feed conversion ratio was insignificantly affected at pH 7.8 when compared to the control (Fig. 2).

3.3. Biochemical constituents

The biochemical components like protein, carbohydrate, and amino acid were remarkably decreased ($p < 0.05$) in *S. serrata* reared in all acidified seawater compared to control. The protein level decreased by 25, 31, 37, 50, and 55% in crabs at pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, in respect of control. While, crabs decreased their carbohydrate level by 36, 49, 55, 59 and 61% in pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, with respect to ambient pH 8.2. The level of amino acids declined by 12, 18, 20, 27, and 36% in crabs exposed to pH 7.8, 7.6, 7.4, 7.2, and 7.0, respectively, regarding the control. The lipid level was noted to be significantly decreased in the crabs reared at pH 7.4, 7.2, and 7.0 with 4, 8, and 13% reductions, respectively with respect to the control crabs. Whereas *S. serrata* treated in pH 7.8 and 7.6 produced negligible variation ($p > 0.05$) in lipid content when compared to control pH. The moister and ash were increased and declined respectively in the crabs treated in all acidification experiments rather than in control. The crabs attained maximum decrease and increase in ash (74%) and moisture (15%) contents exposed to pH 7.0 with respect to control (Table 1).

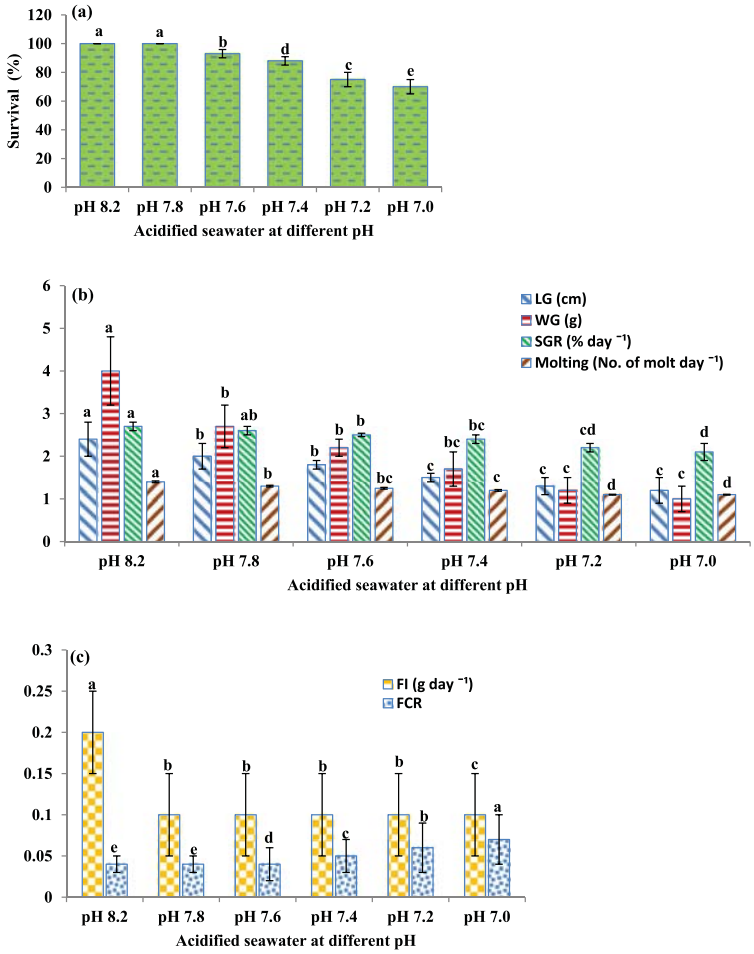

Fig. 2. (a) Survival, (b) LG, WG, SGR and molting and (c) FI and FCR of *S. serrata* exposed to CO_2 driven acidified seawater. n = 60 for survival, molting, FI, and FCR; n = 15 for LG, WG and SGR; mean ± SD; bars sharing different letters in eachparameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups. LG, Length gain; WG, weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio.

Table 1
Biochemical constituents, chitin, and minerals contents of *S. serrata* exposed to acidified seawater at different pH.

Parameter	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Protein (mg g^{-1}) *	228.01 ± 6.81^{a}	171.38 ± 9.32^{b}	156.22 ± 14.80^{c}	144.47 ± 15.96^{d}	114.47 ± 8.10^{e}	101.88 ± 4.09^{f}
Carbohydrate (mg g^{-1}) *	85 ± 10^{a}	54 ± 6.3^{b}	43 ± 8^{c}	38.4 ± 2.6^{d}	35 ± 1.1^{d}	33 ± 2.6^{d}
Amino acid (mg g^{-1}) *	204 ± 14^{a}	180 ± 13^{b}	167 ± 10^{bc}	164 ± 7^{cd}	149 ± 17^{d}	130 ± 27^{e}
Lipid (mg g^{-1}) *	24 ± 0.6^{a}	24 ± 0.6^{a}	24 ± 0.5^{a}	23 ± 1.2^{b}	22 ± 0.6^{c}	21 ± 0.4^{d}
Moisture (%) *	65 ± 1^{e}	67 ± 1.5^{d}	69 ± 0.5^{c}	71 ± 0.5^{c}	73 ± 0.5^{b}	75 ± 1^{a}
Ash (%) *	8.7 ± 1.2^{a}	3.7 ± 1.2^{b}	3.7 ± 0.6^{b}	3 ± 0.6^{b}	3 ± 0.6^{b}	2.3 ± 0.6^{b}
Chitin (mgg^{-1})$^{\#}$	0.94 ± 0.01^{a}	0.94 ± 0.01^{a}	0.93 ± 0.01^{ab}	0.92 ± 0.01^{bc}	0.92 ± 0.01^{c}	0.9 ± 0.01^{d}
Na (mgkg^{-1}) *	185 ± 4.3^{a}	168 ± 0.2^{b}	159 ± 0.7^{c}	98 ± 0.5^{d}	84 ± 0.2^{e}	78 ± 0.5^{f}
K (mgkg^{-1}) *	28 ± 0.2^{a}	25 ± 0.2^{b}	23 ± 0.4^{c}	22.5 ± 0.4^{c}	22 ± 1^{c}	17 ± 0.4^{d}
Ca (mgkg^{-1}) *	51 ± 0.3^{a}	31 ± 0.3^{b}	27 ± 0.2^{c}	21 ± 0.5^{d}	20.5 ± 0.3^{e}	20 ± 0.6^{f}

#n = 60; *n = 3; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$. Na, Sodium; K, Potassium; Ca, Calcium.

3.4. Quantitative and qualitative analysis of chitin

S. serrata treated in the acidified seawater at pH 7.4 to 7.0 exhibited a significant ($p < 0.05$) decrease in chitin levels compared to ambient pH. The crabs decreased nearly one fold of their chitin content exposed to pH 7.4 to 7.0. Whereas crabs treated at pH 7.8 and 7.6 exhibited no significant variation ($p > 0.05$) in chitin level compared to control crabs (Table 1). The chitin FT-IR spectra of crab reared at various ocean acidification (pH 8.2 to 7.0) revealed the pressure of O–H and NH groups occurring in the range 3491–3235 cm^{-1} and 3265-3116 cm^{-1}, respectively. The spectrum at 2888 cm^{-1} represents stretching vibrations of C–H, and the peak at 1074 cm^{-1} indicates the occurrence of C–O–C bands. The main sign in the chitin spectrum was the presence of peaks at 1500 and 1319 cm^{-1} with characteristics stretching vibrations of amide II and III bands, respectively. Two amide I peaks were observed at 1627 and 1649, indicating that the chitin structure obtained from the crab shells indicates the α-form (Fig. 3).

3.5. Mineral salts

The sodium, potassium, and calcium minerals were reduced remarkably ($p < 0.05$) in crabs treated in all acidified seawater experiments compared to pH 8.2. Nonetheless, an insignificant change was recorded in crabs' potassium from pH 7.4 to 7.0 (Table 1). Moreover, 9, 14, 47, 55, and 58% of reduction in sodium, 11, 18, 20, 21, and 39% reduction in potassium, and 39, 47, 59, 60, and 61% reduction in calcium were noticed in crabs exposed to pH 7.8, 7.6, 7.4, 7.2, and 7.0 respectively with respect to control pH 8.2.

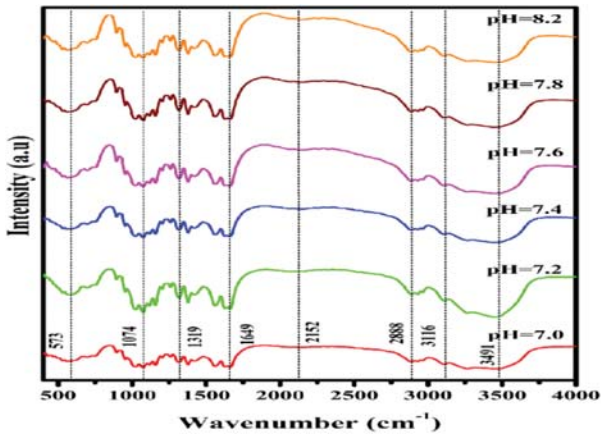

Fig. 3. FT-IR analysis of chitin extracted from *S. serrata* shells.

3.6. Activities of antioxidants, LPO, metabolic enzymes, and ALP

In this study, SOD, CAT, and LPO levels in the crab *S. serrata* reared in all acidified seawater exhibited a higher elevation ($p < 0.05$) than in the control pH of 8.2. Moreover, the gradual elevation in SOD, CAT, and LPO were noticed in crabs at pH 7.8 to 7.0 (Fig. 4). Moreover, the crabs

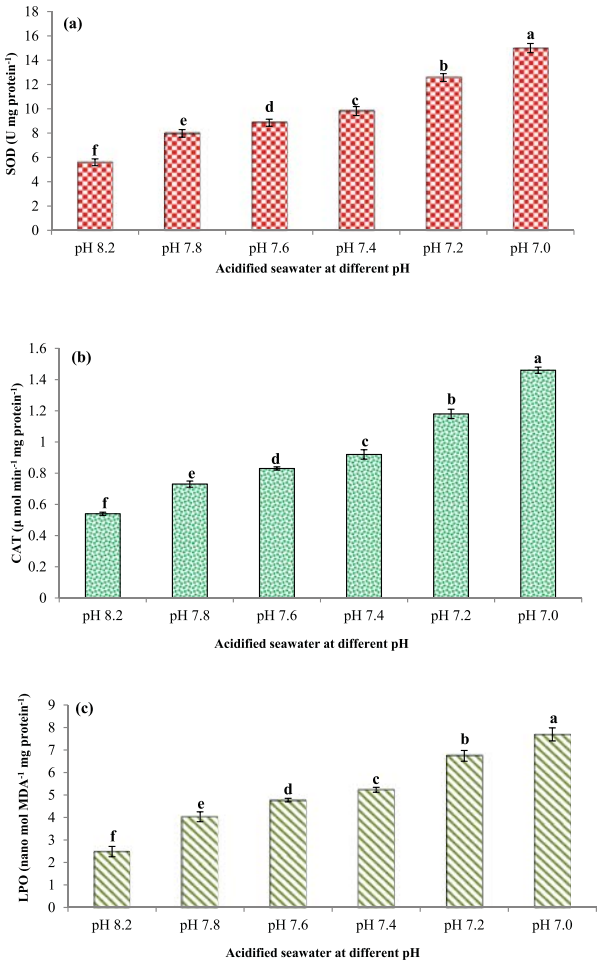

Fig. 4. (a) SOD, (b) CAT, and (c) LPO of *S. serrata* exposed to CO_2 driven acidified seawater. n = 3, mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups. SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation.

attained a 1.42, 1.58, 1.75, 2.24, and 2.68 fold increase in SOD, 1.35, 1.53, 1.70, 2.18, and 2.68 fold increases in CAT, and 1.62, 1.92, 2.10, 2.71, and 3.10 fold increases in LPO at pH 7.8, 7.6, 7.4, 7.2 and 7.0 respectively with respect to the control.

The level of metabolic enzymes, namely GOT and GPT were significantly improved ($p < 0.05$) in *S. serrata* treated in all acidified seawater experiments than that of control pH 8.2. Amidst pH exposures, *S. serrata* exposed to pH 7.0 produced a maximum level of studied metabolic enzymes followed by pH 7.2 to 7.8 (Fig. 5). The GOT of crabs increased by 1.06, 1.22, 1.29, 1.30, and 1.44 times and GPT of crabs elevated 1.25, 1.26, 1.29, 1.32, and 1.51 in pH 7.8, 7.6, 7.4, 7.2, and 7.0 respectively, with respect to the control. Alkaline phosphatase activity was significantly decreased ($p < 0.05$) in *S. serrata* treated at pH 7.2 and 7.0 compared to crabs reared at pH 8.2. While the level of ALP decreased 0.58, 0.58, 0.41, 0.16, and 0.09 times in pH 7.8, 7.6, 7.4, 7.2, and 7.0 respectively in respect of control pH. However, crabs treated at pH 7.8 to

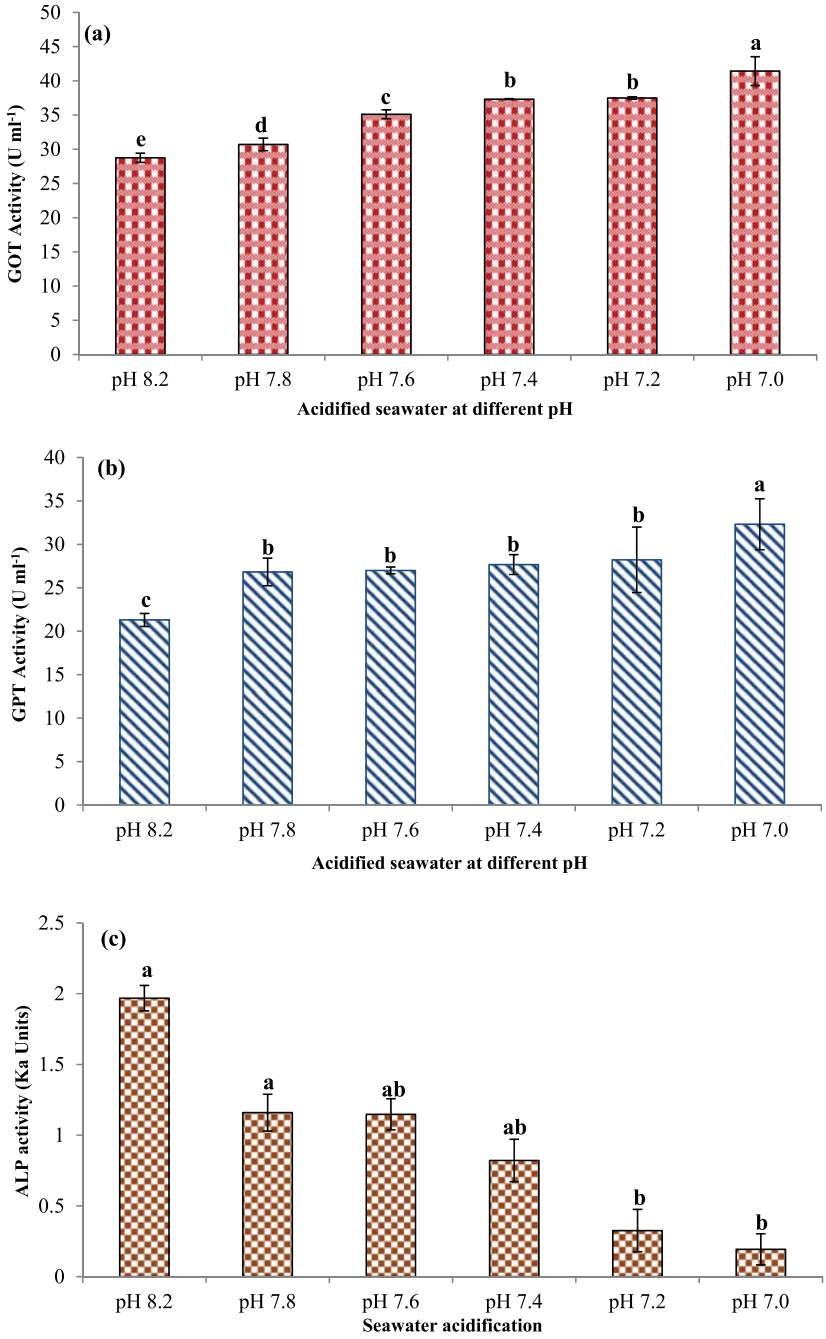

Fig. 5. (a) GOT, (b) GPT and (c) ALP of *S. serrata* exposed to CO_2 driven acidified seawater. n = 3, mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups. GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvate transaminase; ALP, Alkaline phosphatase.

7.4 produced an insignificant level of ALP compared to that in control (Fig. 5).

4. Discussion

Oceans absorb nearly 30% of the CO_2 from the atmosphere (NOAA, 2020a,b). The level of CO_2 in the atmosphere increasing by various man made activities like burning fossil fuels, deforestation, industrial wastes, vehicle emissions, etc., which raise the pCO_2, followed by decreased pH and carbonate ions in the marine environment. The decreases in bicarbonate levels negatively affect the formation of calcium carbonate in the shell and skeletons of calcareous species. Moreover, decreasing ocean pH also affects the feeding behaviour, reproduction, and physiological regulations in invertebrates and vertebrates (Dixson et al., 2014; Pansch et al., 2018; Zlatkin and Heuer, 2019). The continuous emission of CO_2 to the atmosphere is projected to decrease 0.3 or more pH units in the ocean by 2100 (IPCC, 2007; Penman et al., 2014). The decreasing ocean pH in future will lead to severe consequences in the marine ecosystem directly (behaviour, morphology, and physiology of organisms) and indirectly (reduction of small prey seems to affect higher tropical animals in the food chain). Besides, the changes in seawater chemistry can affect the production of edible marine organisms, including fishes and shellfishes, which adversely impacts seafood safety (Birchenough et al., 2017).

Marine organisms' normal biological and physiological activities are mainly based on the properties of seawater. The alteration of seawater chemistry by the acidification process can affect the health of marine organisms. In the current study, salinity, temperature, and alkalinity were insignificantly influenced in all pH experiments demonstrating that CO_2 induced seawater acidification did not produce significant fluctuation in the above-studied factors during the 60 days experiment. Further, a significant reduction in dissolved oxygen of acidified seawater might be due to an alteration of the crabs' metabolism under acidic stress, which led to an increase in the oxygen consumption from the surrounding environment to tolerate the stress. Elevation of pCO_2 leads to a rise in TCO_2 and HCO_3^- levels in the experimental seawater and reduces saturated calcite and aragonite levels. As a result, the lack of calcium and bicarbonate ions makes the calcareous species struggle for shell formation. Similarly, ocean acidification experiments have observed significant alterations in pCO_2, bicarbonates, calcite, and aragonite levels (Pedersen et al., 2014; Anand et al., 2021). Further, the alterations in the physicochemical characteristics of seawater by high pCO_2 have been recorded (Egilsdottir et al., 2009).

The growth progress, survivorship, and consumption of foods are essential factors that affect the production of crustaceans. In this study, a significant reduction in survival, weight gain, length gain, molt rate, feed intake, and specific growth rate of *S. serrata* treated in acidified seawater indicates the detrimental effect on *S. serrata*. Amidst pH treatments, pH 7.4 to 7.0 revealed extreme negative impacts on these parameters in *S. serrata.* The variations in seawater chemistry could affect the physiology like shell formation, molting, foraging, key enzymes (digestive enzymes and metabolic enzymes), haemolymph pH, formation of ROS, and cell membrane damage which seem to more stress on *S. serrata*, followed by poor survival and growth. A harmful impact on the growth and survival percentage of the juvenile Tanner crab *Chionoecetes bairdi* and red crab *P. camtschaticus* exposed to elevated pCO_2 (pH 7.5) has been observed (Long et al., 2013). Also, previous findings stated that the reduced survival, growth rate, and hatching in crab *Cancer magister* (pH 7.5 and 7.1), *Hyas araneus* (pH 7.2), and *Lithodes aequispinus* (pH 7.5) when cultured at CO_2 enriched seawater (Miller et al., 2016; Long et al., 2021). In the current investigation, a significant decline in *S. serrata* moulting under acidified seawater indicated that ocean acidification had adverse effectson the crabs' development. Ocean acidification can reduce the chitinolytic enzyme activities like chitinase and N-acetyl β-D-glucosidase (β-NAGase), which negatively affects molting in crustaceans (Spindler Barth et al., 1990; Luo et al., 2015; Chen et al., 2019). Liu et al. (2022) noticed a significant decrease in chitinase and β-NAGase enzymes, followed by an adverse impact on the molting of horseshoe crab *Tachypleus tridentatus* under acidified environment (pH 7.3). Likewise, the Dungeness crab *C. magister* exposed to pH 7.2 produced adverse effects on molting (Reinhardt, 2020).

Protein, lipid, amino acids, and carbohydrates are considered essential biochemical elements that play a pivotal role in an organism's physiological and biological processes. In the present investigation, the considerable decreases in biochemical components of *S. serrata* exposed to all CO_2 induced acidified seawater indicated that the low pH harmed the biochemical constituents in *S. serrata*, which indicates the detrimental effect of ocean acidification on the physiological activities of experimental crabs. The decrease in food utilization like feed intake in the crab *S. serrata* exposed to pH 7.6, 7.4, 7.2, and 7.0 for 60 days resulted in substantial reductions in protein and carbohydrate, lipid, and amino acids productions. A stronger decrease in the lipid content of hermit crab *Pagurus criniticornis* reared at pH 7.7 for 120 days was reported earlier (Turra et al., 2020). Similarly, the reductions in biochemical elements (total protein, amino acids, lipid, and carbohydrate) in the shrimp *L. vannamei* and the brine shrimp *A. franciscana* treated under different acidified environments (pH 7.8 to 3.5) have been noticed (Muralisankar et al., 2021; Thangal et al., 2021). The reduction in amino acid content in the tiger shrimp *Penaeus monodon* exposed to a pH of 7.5 for four weeks was observed earlier (Hsieh et al., 2021).

Chitin is a linear chain polysaccharide of the amino sugar in the extracellular matrix of invertebrates (Moussian, 2019), including crustaceans. Chitin offers maximum support to the exoskeleton, shape of the organism, and defence from predators (Hendriks et al., 2015). In this study, the significant decrease in crab's chitin in acidified seawater (pH 7.0) suggests that the acidified environment had created a severe effect on the chitin biosynthesis. Chitin synthetases and chitinolytic enzymes play a chief role in arthropods' chitin synthesis (Merzendorfer and Zimoch, 2003). In this study, reductions in chitin level and molting interference of crabs explicit the ocean acidification could disturb the enzymes responsible for chitin biosynthesis, followed by poor shell formation and molting. The loss of the production of the exoskeleton in the marine organism causes loss of survival, escape from predators, loss of hardness of the claw, etc. The reduction in the chitin levels was observed earlier in tanner crab *C. bairdi* (7.8 and 7.5) and white leg shrimp *L. vannamei* treated in acidified seawater (Mustafa et al., 2015; Muralisankar et al., 2021; Dickinson et al., 2021).

The chitin FTIR spectrum of this study is similar to the earlier reports on crustaceans, including crabs (Fernando et al., 2016; Mohan et al., 2021). Moreover, FT-IR analysis of the current study revealed that the chitin structure of crab *S. serrata* reared in acidified environments produced considerable structural changes compared to *S. serrata* raised in control pH 8.2. Still, all spectra of crab's chitin are similar in control and experimental groups; however, the spectral band for CH ring at 855 cm^{-1} and amide I at 1649 cm^{-1} were broadening in crabs reared in pH 7.4 to 7.0 when matching with control. Moreover, the spectra of C–O–C at 1074 cm^{-1}, amide III at 1319 cm^{-1}, amide II at 1500 cm^{-1}, N–H stretching at 3116 cm-1, and O–H stretching at 3419 cm^{-1} were also notably broadened *S. serrata* chitin at pH 7.0 compared to control and other pH treatments. These changes suggest the effect of acidified environment on crabs' chitin quality. A similar finding has been observed in the cuticle of the carapace in crab, *C. bairdi* exposed to pH 7.5 (Dickinson et al., 2021).The changes in the FT-IR spectrum of shell powders of sea urchin *Salmacis virgulata* exposed to pH 7.8 and 7.6 have been noticed compared to the control pH of 8.2 (Anand et al., 2021).

Minerals are essential for several biological and physiological functions of living organisms (Lall, 2003). Among minerals, calcium, potassium, and sodium are necessary for the absorption of nutrients, muscle contraction and transmission of nerve signals, and maintaining fluid balance respectively in animals, including crustaceans. The present study reveals that the reduction of essential minerals in *S. serrata* reared in all pH environments indicates the acidification process could inhibit

the consumption of minerals salts from water and diets (Liu et al., 2020). Further, the decline in calcium in the form of calcium carbonate in the surrounding environment can cause poor calcification levels in the crabs in an acidified environment. Lack of availability of essential minerals negatively affects the organism, such as hyponatremia, weakness and fatigue, and skeleton strength (Karppanen et al., 2005). The red king crab *P. camtschaaticus* (pH 7.7), snow crab *C. bairdi* (pH 7.8 and 7.5) and Atlantic crab *Panopeus herbstii* (pH 7.86, 6.98 and 6.97) indicated a considerable reduction in calcification treated in seawater with low pH (Long et al., 2013; Swiney et al., 2016; Dodd et al., 2021). A reduction in calcium and magnesium in the decorator crabs *Pelia tumida* (pH 7.74) has been observed under acidified water (Rankin et al., 2019). Changes in pH due to combining of CO_2 alter the sodium, potassium and calcium characteristics of the mineralized cuticle of crab *P. camtschaticus* and blue crab *Paralithodes platypus* (pH 7.8 and 7.5) have been studied earlier (Coffey et al., 2017).

Antioxidants are elements that defence cells against the free radicles to prevent injuries. SOD and CAT are the major antioxidant defence enzymes needed for protecting cells from superoxide (O_2^-) anion radicals created by the oxidative process, phagocytosis, and tissue damage after regular body metabolism (Rathore et al., 1998; Fang et al., 2002; Ighodaro and Akinloy, 2018). LPO is a free radical-mediated chain of reaction (Hampel et al., 2016) that can cause oxidative degradation of polyunsaturated fats and cell membranes are the primary target parts of a biological system. In the present investigation, the considerable rise of SOD and CAT in the mud crab *S. serrata* reared in CO_2 induced seawater acidification than that of control reveals the excess input of these studied antioxidant enzymes against oxygen-free radicles created by pH stress. Moreover, the pH strain can boost the cell membrane injury in *S. serrata* with the evidence of elevated LPO under acidic pressure. Likewise, the crab, *T. tridentatus* exposed to acidified seawater (pH 7.3) showed significant elevation in glutathione peroxidase, SOD, CAT, and malondialdehyde (Liu et al., 2022). Also, the alterations in SOD, glutathione S-transferase (GST), and LPO in the Chinese crab *Portunus trituberculatus* have been observed exposed to a high oceanic CO_2 environment (Lin et al., 2020). Elevation of GST, CAT, and SOD in the shrimp *L. vannamei* (pH 7.6–3.5) and brine shrimp *Artemia sinica* (pH 7.8 and 7.6) and *A. franciscana* (pH 7.8 and 6.8) exposed in the acidic seawater has been reported (Furtado et al., 2015; Zheng et al., 2015; Muralisankar et al., 2021; Thangal et al., 2021).

Metabolic enzymes are essential to the organism for their digestion, cellular respiration, transcription, energy storage, etc. The remarkable variations in metabolic enzymes denote liver impairment. The alterations in GOT and GPT of *S. serrata* reared in acidic seawater indicate that ocean acidification can produce metabolic stress on crabs. Consequently, it suggests that variations in seawater pH may be toxic to crabs. Increasing the metabolic enzymes of the organism can directly affect the growth, survival, biochemical, and mineral contents of organisms. Likewise, the deep-sea hermit crab *Pagurus tanneri* showed an increment in the metabolic enzymes under acidic stress (pH 7.6 and 7.1) (Kim et al., 2016). The elevation in GOT and GPT under acidified conditions was observed earlier in copepod *Pseudo calanusacuspes* and *Ruditapes philippinarum* (Ross et al., 2011). Besides, the crab *Petrolisthes cinctipes* and shrimp *L. vannamei* produced a substantial rise in GOT and GPT levels under acidic stress (Carter et al., 2013; Muralisankar et al., 2021).

Alkaline phosphatase is a membrane-bound metalloenzyme consisting of isoenzymes that play a key role in processing metabolites and the biomineralization process (Szabo and Ferrier, 2014). This enzyme is a suitable indicator for evaluating the physiological condition of an animal under acidic stress (Zambonino-Infante et al., 2008). In marine invertebrates, the activity of ALP is decreased by nearly 50% due to the low pH (Norris and Rao, 1935). In this study, the significant decrease in ALP in crabs exposed to pH 7.2 and 7.0 showed adverse effects of these extreme acidic stress on the ALP activity of *S. serrata*, which explicit the poor nutrient assimilation and digestion for conserving energy to regulate intracellular acid-base balance (Michaelidis et al., 2005; Rosa et al., 2014). Likewise, the reduction in ALP activity was observed in shrimp (*Ancylocaris brevicarpalis*) and sea anemone (*Stichodactyla haddoni*) under acidified conditions (Prakash et al., 2022). The decrease in ALP has also been observed in horseshoe crab *Tachypleus tridentatus* under acidified environment (pH 7.3) (Liu et al., 2022).

5. Conclusion

These findings publicized the adverse effect of ocean acidification on the growth, food indices, biochemical elements, minerals, chitin, and alkaline phosphatase level of *S. serrata* with the evidence of stress biomarker enzymes like antioxidants and metabolic enzymes. The negative effect of CO_2 induced ocean acidification on economically important edible crab *S. serrata* instar reveals the alarming to seafood safety. Moreover, the larval stages of crabs play a significant role in the marine food web to transfer the energy to higher tropic animals, hence, ocean acidification can also affect the balancing of the ecosystem.

Authorship contribution

Thirunavukkarasu Muralisankar: Conceptualization, Funding acquisition, Project administration and Writing an original draft. Said Hamid Thangal: Investigation, Sample Collection, Methodology, Culture, Software and Formal analysis. Krishnan Anandhan: Reviewing and Editing. Velusamy Gayathri: Data Curation, Statistical Analysis. Arumugam Yogeshwaran: Formal Analysis, Reviewing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119995.

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Baseline

Effects of acidified seawater on biological and physiological responses of *Artemia franciscana*

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ABSTRACT

Ocean acidification is becoming a potential threat to marine animals. The present study investigated the effect of seawater acidification on *Artemia franciscana*. *A. franciscana* cysts were allowed to hatch at different pH levels of pH 8.2 (control), 7.8, and 6.8. After 48 h incubation, the hatching percentage was significantly reduced in acidified seawater compared to that in control. Further, the hatched *Artemia* nauplii from each pH treatment were transferred to freshly acidified seawater for chronic study for 15 days. At the end of the experiment, survival, growth, and biochemical constituents were significantly decreased in *Artemia* at pH 7.8 and 6.8 compared to that in control, which indicates the adverse effects of acidified seawater on *Artemia*. The antioxidants, lipid peroxidation, and metabolic enzymes were significantly elevated in *A. franciscana* exposed to acidified seawater compared to that in control, which shows oxidative and metabolic stress on *A. franciscana* under acidified environment.

The marine environments are one of the vast ecosystems on the earth, and they consist of more than 15% global species diversity, which plays an important role in ecosystem functioning (Grosberg et al., 2012). Pollution of marine environments is considered a potential threat to marine ecosystems and biodiversity, which affects the quality and productivity of the marine environment. Among various pollutants, the elevated level of atmospheric CO_2 has been considered a serious threat to the marine environment as it can stimulate the ocean acidification process. Oceans absorb about 30% of carbon dioxide from the atmosphere; consequently, seawater pH reduces, and it forms carbonic acid and releases hydrogen ions into seawater (Feely et al., 2004; Fabry et al., 2008; De Wit et al., 2018). According to the prediction of the Intergovernmental Panel on Climate Change (IPCC, 2007), the seawater pH could change to pH 7.8 by 2100 due to future emission of CO_2 and other factors (Caldeira and Michael, 2005). Acidification of seawater could affect the biological and physiological properties, including antioxidants and metabolic enzymes that are essential for various cellular functions such as defense mechanism against reactive oxygen species, homeostasis, cellular respiration, nutrient digestion, and energy storage. Acidification could also affect an instant response of various marine invertebrates, specifically the calcifying animals such as shrimps, sea urchins, corals, mollusks and bivalves, which in turn affect the entire food chain (Whiteley, 2011; Mollica et al., 2018; Melzner et al., 2020; Wang and Wang, 2020) due to environmental changes. Moreover, seawater acidification can interact with other stressors such as hypoxia, temperature, toxic metals, and ultraviolet radiation and produce severe adverse effect on the biological response of organisms from primary producers to tertiary consumers (Kong et al., 2019; Khan et al., 2020; Shang et al., 2020; Gao et al., 2020; Wang et al., 2020; Khan et al., 2021).

Artemia is an aquatic crustacean also called brine shrimp that is distributed all over the inland saltwater lakes. *Artemia* plays a pivotal role in the aquatic food chain due to the presence of high nutrients such as proteins, fatty acids, amino acids, antioxidants, and vitamins (Hamre and Harboe, 2008; Manickam et al., 2020). The majority of the commercial aqua hatcheries are depending on *Artemia* nauplii as a sole live feed for the larviculture of fish and shellfishes (Sorgeloos et al., 2001). However, acidified seawater causes adverse effects on the hatching efficiency, survival, and reproduction of *Artemia* species including *A. parthenogenetica*, *A. franciscana*, and *A. sinica* (Salma et al., 2012; Sui et al., 2014; Zheng et al., 2015). Nevertheless, the information on the effect of seawater acidification on the biochemical constituents, minerals, and metabolic enzymes of *Artemia* is limited, hence, the present

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study was focused to evaluate the impact of seawater acidification on the hatching, survival, growth, biochemical constituents, antioxidants, lipid peroxidation, and metabolic enzymes of *A. franciscana.*

In the present study, seawater without pH manipulation served as a control (pH 8.2), and the desired concentration of seawater acidification such as pH 7.8 (IPCC-predicted ocean pH by 2100) and pH 6.8 was obtained by mixing with 1 N HCl and 1 N NaOH to decrease and increase the seawater pH, respectively (Riebesell et al., 2010). For the acidification experiment on *A. franciscana*, 0.25 g of *Artemia* cysts (Ocean Star International, Inc., OSI Pro 80) were disinfected and hydrated using 4% aqueous solution of sodium hypochlorite *w/v* in 500 ml of marine water for 1 h in nine Erlenmeyer flasks and washed in seawater until all traces of chlorine was removed. The cysts were transferred into three different acidified seawater such as pH 8.2 (control), 7.8 (IPCC-predicted seawater pH by 2100) and 6.8 in aquaria (5 l capacity) in triplicate. The setup was provided with gentle aeration using an air tube controller and 2000 lx light intensity in 28 °C for 48 h for incubation. After complete hatching of the cysts into nauplii, they were harvested using a plankton net of mesh size <150 μm, and 200 nauplii from each pH was transferred to fresh acidified seawater in triplicate for culture studies for 15 days. The *Artemia* nauplii were fed with 1 ml of dried *Arthrospira platensis* powder (2% in distilled water) twice per day. The pH of each aquarium was monitored every 6 h per day, and the desired pH was adjusted using 1 N HCl and 1 N NaOH. The physicochemical properties of experimental seawater analyzed according to the standard procedures of APHA (2005), and the level of carbonate ions, calcium carbonate saturation state for calcite and aragonite were calculated according to Robbins et al. (2010) and depicted in Table 1.

After 48 h of incubation, the hatching of *Artemia* cysts was determined by counting the average number of nauplii by sucking top, middle, and bottom part of each aquarium using a 1 ml transparent pipette and placing to a petri dish three times per aquaria and the hatching percentage was calculated using the following formula (Kulasekarapandian, 2003)

$$\text{Hatching percentage}\ (\%) = \frac{\text{Number of nauplii}}{\text{Total no. of cysts}} \times 100$$

At the end of the 7th and 15thday of acidification experiment, the survival of *A. franciscana* was determined as survival (%) = no. of live nauplii/ Total no. of nauplii × 100. The growth (length) of *A. franciscana* was determined using Tucson inverted microscope and calibrating using software T capture, and the length gain was calculated as length gain (mm) = final length- initial length of *A. franciscana.*

Total protein, amino acids, carbohydrates, and lipid content were estimated in the whole body of *A. franciscana* at the end of 15 days of acidification experiment. Briefly, the total protein content was estimated using the ethanolic precipitated sample with bovine serum albumin as a standard (Lowry et al., 1951), and the total amino acid level was estimated using sodium tungstate and sulfuric acid extracted samples with standard leucine (Moore and Stein, 1948). The carbohydrate content was determined in trichloroacetic acid extracted sample using glucose as standard (Roe, 1955), and the total lipid was estimated in chloroform-methanol extracted samples (Folch et al., 1957) using cholesterol as a standard (Barnes and Blackstock, 1973).

After 15 days of acidification experiment, the whole body of *A. franciscana* from each acidification experiment was extracted in ice-cold Tris buffer, which served as an enzyme source for antioxidants and lipid peroxidation assays. The superoxide dismutase (SOD) activity of *A. franciscana* was measured according to the procedure of Marklund and Marklund (1974) by the process auto reduction of pyrogallol in tris buffer, and the catalase (CAT) activity was determined using the substrate hydrogen peroxide in phosphate buffer prescribed by Sinha (1972). Lipid peroxidation (LPO) in *A. franciscana* homogenates was measured by the formation of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) in nanomoles (Ohkawa et al., 1979). Metabolic enzyme activities such as glutamic oxaloacetate transaminase (GOT) and glutamic pyruvate transaminase (GPT) of *Artemia* were determined according to the previous procedure of (Reitman and Frankel, 1957) using sucrose-extracted samples.

Duncan's multiple range test (DMRT) was performed for data analysis using one-way analysis of variance (ANOVA) in SPSS (16.0) software to compare the significant differences among treatments, and the significance level was considered at $P = 0.05$. Data of all parameters are expressed as mean ± SD (standard deviation). Principal component analysis (PCA) was also conducted to determine the relationships of different pH among biological and physiological indexes using PAST software 3.0 version.

The properties of seawater play an important role to regulate the physiological process of marine animals. In the current study, salinity, temperature, dissolved oxygen, ammonia, and total alkalinity were insignificantly ($P > 0.05$) altered in pH-manipulated seawater (pH 7.8 and 6.8) compared to control pH, which suggests that the acidification of seawater did not significantly change these properties under the laboratory condition. The pH was significantly ($P < 0.05$) reduced in the pH-manipulated seawater, which indicates that the desired pH in the respective aquarium was maintained during the experiment. Further, the carbonate and calcium carbonate saturation state for calcite and aragonite level was significantly increased in acidified seawater compared to that in control, suggesting that acidification led to significant changes in the seawater properties (Table 1). The changes in seawater chemistry including pH, dissolved oxygen, ammonia, carbonates, calcium carbonate saturation state for aragonite and calcite due to acidification has also been reported earlier (Egilsdottir et al., 2009; Furtado et al., 2015; Muralisankar et al., 2020).

In the present study, the hatching percentage of *A. franciscana* was found to be significantly ($P < 0.05$) reduced in cysts exposed to pH 7.8 and 6.8 compared to cysts exposed to pH 8.2 (control). However, an insignificant difference ($P > 0.05$) was noted in the hatching percent of *A. franciscana* cysts exposed to pH 7.8 and 6.8. The hatching of cysts of aquatic animals is based on favorable environmental conditions. The

Table 1
Water quality parameters of experimental seawater.

Parameters	pH 8.2	pH 7.8	pH 6.8
pH (Measured)	8.20 ± 0.11^{a}	7.80 ± 0.10^{b}	6.80 ± 0.10^{c}
Salinity (ppt)	35.0 ± 0.22^{a}	35.0 ± 0.12^{a}	35.0 ± 0.14^{a}
Temperature (^{o} C)	27.0 ± 0.12^{a}	27.0 ± 0.18^{a}	27.0 ± 0.15^{a}
Dissolved oxygen (mg/l)	4.0 ± 0.30^{a}	4.0 ± 0.20^{a}	4.0 ± 0.10^{a}
Ammonia (mg/l)	0.50 ± 0.02^{a}	0.50 ± 0.08^{a}	0.50 ± 0.05^{a}
Total Alkalinity (ppm)	1856.99 ± 3.20^{a}	1782.05 ± 1.52^{b}	1481.66 ± 20.40^{c}
CO_3^{2-} (μmol/kg)	236.71 ± 40.68^{a}	113.27 ± 22.42^{b}	10.13 ± 1.22^{c}
Ω Ca	5.71 ± 0.97^{a}	2.73 ± 0.53^{b}	0.26 ± 0.06^{c}
Ω Ar	3.78 ± 0.64^{a}	1.81 ± 0.36^{b}	0.17 ± 0.04^{c}

$n = 3$ (three individual samplings); mean ± SD; Mean values within the same row sharing the same superscript are not significantly different ($P > 0.05$). CO_3^{2-}, carbonate ions; Ω Ca and Ω Ar, calcium carbonate saturation state for calcite and aragonite.

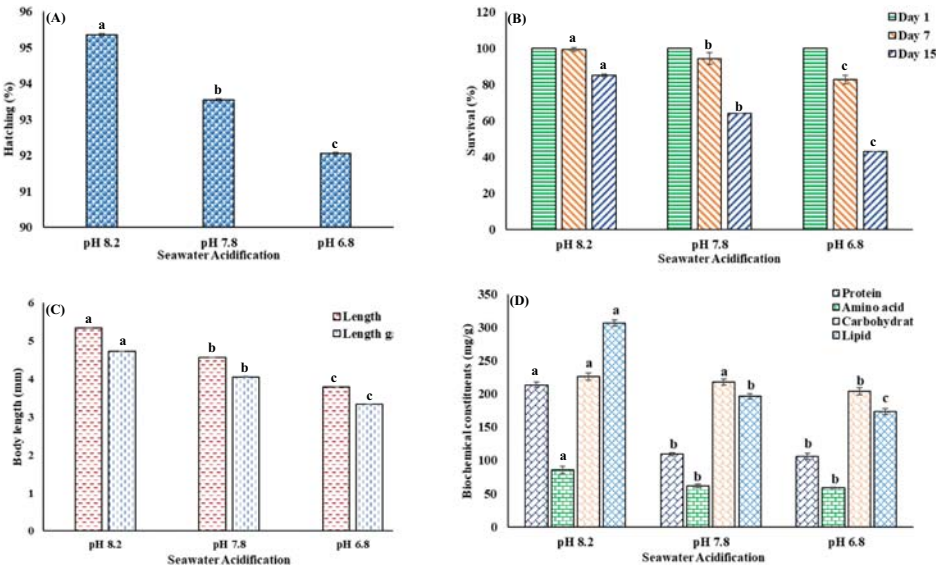

Fig. 1. Hatching percent (A), survival (B), growth (C), and biochemical constituents (D) of *A. franciscana* exposed to acidified seawater. $n = 3$ (three individual samplings), mean ± SD; bars with different letters indicate significant differences ($P < 0.05$) among pH treatments.

poor hatching of *A. franciscana* cysts exposed to pH 7.8 and 6.8 of this study showed that these acidified seawater did produce adverse effects on *Artemia* cysts (Fig. 1A). These finding agreed with the earlier study of Salma et al. (2012) who reported a negative effect of acidified seawater (pH 7.6, 7.3, and 7.0) on the hatching of *A. franciscana* cysts. Similarly, the impact of seawater acidification (pH 7.8 and 7.6) on the hatching ability of *A. sinica* has been reported by Zheng et al. (2015). Further, the adverse effects of seawater acidification with pH 7.3, 7.2, and 6.9 on the egg production and hatching of copepods (*Acartia spinicauda* and *Centropages tenuiremis*) have been reported earlier (Dajuan et al., 2011). Moreover, significantly decreased fertilization and increased embryo deformation were observed in the hard-shelled mussel *Mytilus coruscus* and blue mussel *Mytilus edulis* exposed to acidified seawater (pH 7.7 and 7.3) and seawater acidification with hypoxic condition (Kong et al., 2019; Wang et al., 2020).

Assessment of survival and growth plays a pivotal role to determine the health status of any organism. In this study, a significant ($P < 0.05$) decrease in survival on both the 7th and 15th day of observations, and poor growth of *A. franciscana* was recorded in acidified seawater (pH 6.8 and 7.8) compared to that in control (pH 8.2). In this context, acidified seawater at pH 6.8 showed significantly poor performance in survival and growth of *Artemia* compared to that at pH 7.8, which indicates the detrimental effects of increasing acidification of seawater on *A. franciscana* (Fig. 1B & C).These poor survival and growth of *A. franciscana* under acidified seawater might be due to the compensation of maintaining normal physiological processes. Previously, poor survival was reported in *A. parthenogenetica* and *A. franciscana* reared at low pH (pH 6 and 5) for 18 days (Sui et al., 2014). *A. sinica* also showed a significant reduction in survival and growth exposed to acidified seawater such as pH 7.8 and pH 7.6 for 14-day experiment (Zheng et al., 2015). A significant decrease was observed in the survival and growth of the whiteleg shrimp *Litopenaeus vannamei* exposed to pH 7.8, 7.6, 7.4, 7.2, and 7.0 for 7 weeks, the shrimp *Palaemon pacificus* exposed to pH 7.6 for 30 weeks, and the Dungeness crab *Cancer magister* exposed to pH 7.5 and 7.1 for 45 days (Kurihara et al., 2008; Miller et al., 2016; Muralisankar et al., 2020). Kong et al. (2019) and Wang et al. (2020) observed that both *M. coruscus* and *M. edulis* showed poor growth rate in terms of shell length exposed to pH 7.7 and 7.3 alone and with hypoxic environment.

The physiological and health status of animals can be assessed by the determination of biochemical elements such as proteins, carbohydrates, and lipids. A significant decrease in these biochemical elements indicates the poor health status of an organism. In the present findings, the contents of protein, amino acid, and lipid were significantly ($P < 0.05$) reduced in *A. franciscana* exposed to pH 7.8 and 6.8 compared to that in control pH 8.2. Further, a significant ($P < 0.05$) reduction was also noted in the lipid content of *Artemia* nauplii at pH 6.8 compared to pH 7.8. Nonetheless, an insignificant difference ($P > 0.05$) was noted in the carbohydrate content of *A. franciscana* exposed to pH 8.2 and 7.8. In this context, the carbohydrate content was significantly ($P < 0.05$) decreased in *A. franciscana* reared at pH 6.8 rather than control and other pH exposure. This decreased level of biochemical constituents indicates the adverse effects of acidified seawater in *A. franciscana*, which led to poor health status (Fig. 1D). Similarly, *L. vannamei* exposed to acidified seawater (pH 7.8 to 7.0) for 7-week experiment showed a significant decrease in muscle protein, amino acid, carbohydrate, and lipid contents (Muralisankar et al., 2020). The decreased amino acid level in the Dungeness crab, *Carcinus maenas* and the starfish *Luidia clathrata* exposed to acidified seawater at pH 7.8, 7.4, 6.6, and 6.3 has been observed earlier (Schram et al., 2011; Hammer et al., 2012). The alteration in protein, carbohydrate and lipid levels in the red coral *Corallium rubrum* exposed to pH 7.8 for 314 days has also been noted (Bramanti et al., 2013).

Antioxidants like SOD and CAT are significant primary defense enzymes for protecting against free radicals (Ighodaro and Akinloye, 2017). Free radicals mediated chain reactions that lead to oxidative degradation of polyunsaturated fats is known as lipid peroxidation, which mainly targets cell membranes of an organism. Unfavorable surrounding environments such as alterations in pH, temperature, salinity and pollutions are responsible for the LPO reactions and production of antioxidants in organisms. In the current study, antioxidants such as SOD and CAT, and LPO were significantly ($P < 0.05$) elevated in *A. franciscana* exposed to pH 6.8, followed by pH 7.8 compared to control pH 8.2 (Fig. 2A). These results suggested that the acidification of seawater might be responsible for an increased level of antioxidants and lipid peroxidation, which indicates the oxidative stress in *A. franciscana.*

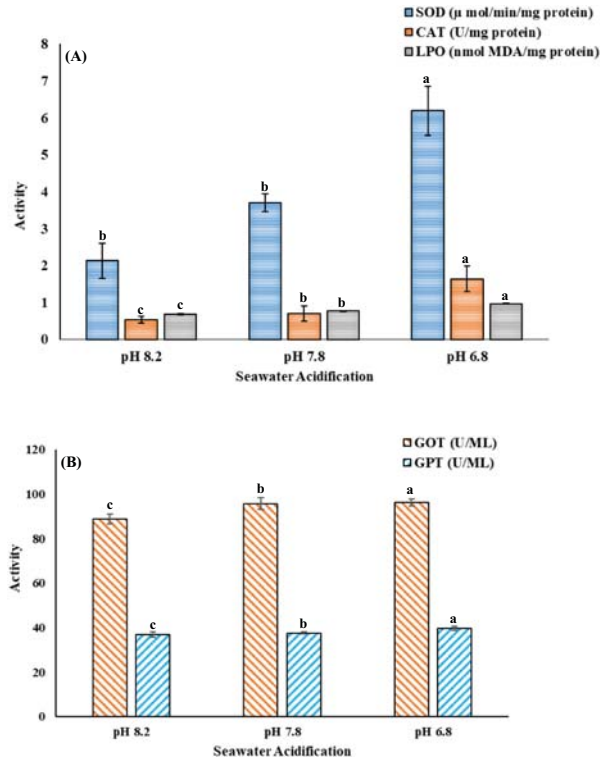

Fig. 2. Antioxidants and lipid peroxidation (A), and metabolic enzymes (B) activities of *A. franciscana* exposed to acidified seawater. $n = 3$ (three individual samplings), mean ± SD; bars with different letters indicate significant difference ($P < 0.05$) among pH treatments. SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation; GOT, glutamic oxaloacetate transaminase; GPT, glutamic pyruvate transaminase.

Moreover, *A. franciscana* may reduce the damage to the body caused by the increase of oxygen free radicals by improving the antioxidant defense performance of enzyme activities. Similarly, the elevated level of antioxidants (SOD and CAT) and LPO have been observed in the crustaceans such as *A. sinica* and *L. vannmei*, and the scallop *Patinopecten yessoensis* exposed to low pH between pH 7.8 to 3.5 (Furtado et al., 2015; Zheng et al., 2015; Liao et al., 2019; Muralisankar et al., 2020). Significant increases in the activity of SOD, CAT, glutathione (GSH), glutathione peroxidase (GPx) and production of malondialdehyde (MDA) has been observed in gills and digestive glands of the hard-shelled mussel *M. coruscus* exposed to low pH, such as pH 7.7 and 7.3 alone and also combined with elevated temperature during the 14-day experiment (Hu et al., 2015). The SOD, CAT, GSH, GPx, and MDA levels in *M. coruscus* exposed to acidified seawater with hypoxic condition and elevated temperature were significantly increased and decreased at 15th and 30th day sampling, respectively (Khan et al., 2021). Furthermore, significant alterations in antioxidants (SOD, CAT, and GST) and LPO activities were also recorded in fish (*Atherina presbyter* and *Solea senegalensis*) reared under acidified environments (Pimentel et al., 2015; Silva et al., 2016).

Metabolic enzymes are essential for the metabolism of nutrients in animals, and these enzymes are significantly elevated during any stress condition to recompense the energy required for tolerating such stress. In this study, GOT and GPT were increased significantly in *A. franciscana* exposed to acidified seawater at pH 6.8 and pH 7.8 compared to pH 8.2; this showed that the metabolic stress produced by acidified seawater to *A. franciscana* (Fig. 2B). Likewise, the significant increase in GOT and GPT level in the whiteleg shrimp *L. vannamei* exposed to acidified

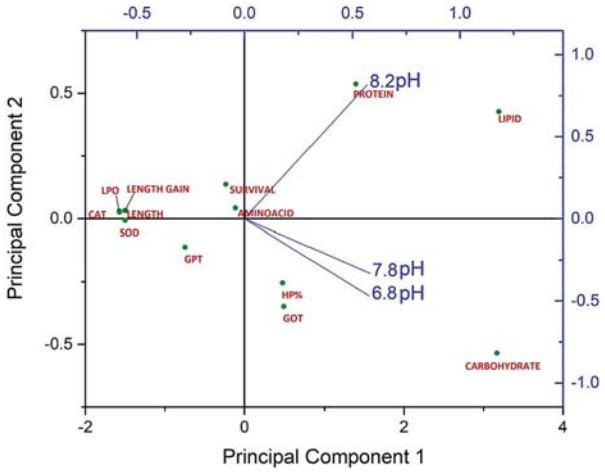

Fig. 3. Principal component analysis of *A. franciscana* exposed to acidified seawater for 15 days. HP, Hatching percent; SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation; GOT, glutamic oxaloacetate transaminase; GPT, glutamic pyruvate transaminase.

seawater has been observed earlier by Muralisankar et al. (2020). Alterations in metabolic enzymes such as GOT and GPT in the gills and hepatopancreas of the scallop *P. yessoensis* exposed to low pH 7.5 for 45 days has been reported previously (Liao et al., 2019). Hu et al. (2015) also observed significant improvements in GPT, alkaline phosphatase (AKP), acid phosphate (ACP) in the gills and digestive glands of the hard-shelled mussel *M. coruscus* exposed to acidified seawater at pH 7.7 and 7.3 and combined with elevated temperature for 14 days. Similarly, Khan et al. (2021) reported significant elevations in AKP, ACP, and GPT activities in *M. coruscus* reared under acidified seawater alone and combined with hypoxia and increased temperature during 15-day exposure period.

The PCA analysis indicated that the dataset of influence of different pH values on the biological and physiological indexes generated three PCs (Fig. 3), in which, PC1 accounted for 97.16%, followed by PC2 (2.72%), and PC3 (0.12%) of the total variance. According to the PCA, four parameters such as protein, lipid, survival, and amino acid are significantly affected with variation in pH 8.2. Three parameters, viz. hatching percentage (HP), GOT, and carbohydrate, are significantly affected in pH 6.8. Not all other parameters are affected considerably by the changes in the pH values.

In conclusion, the results from this study indicate that the acidified seawater can produce an adverse impact on cyst hatching, survival, growth, and biochemical constituents in *A. franciscana*. Moreover, significant increases in antioxidants, lipid peroxidation, and metabolic enzymes suggest that acidified seawater is responsible for oxidative and metabolic stress on *A. franciscana.* Therefore, the present study indicates that the acidification of seawater can produce a detrimental effect on *Artemia* species.

CRediT authorship contribution statement

S.H. Thangal: Investigation, Writing original draft. M. Nivetha: Investigation, Writing original draft. C. Muttharasi: Software & Formal analysis. A. Anandhan: Resources; T. Muralisankar: Conceptualization, Funding acquisition, Project administration, Writing - review & editing.

Declaration of competing interest

None declared.

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Growth, biochemical, antioxidants, metabolic enzymes and hemocytes population of the shrimp *Litopenaeus vannamei* exposed to acidified seawater

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ABSTRACT

Acidification in the marine environment has become a global issue that creates serious threats to marine organisms. In the present study, we evaluated the effect of CO_2 driven acidification on the shrimp *Litopenaeus vannamei* post-larvae (PL). *L. vannamei* PL were exposed to six different CO_2 driven acidified seawater, such as 8.2 (control), pH 7.8 (IPCC-predicted ocean pH by 2100), 7.6, 7.4, 7.2 and 7.0 with corresponding pCO_2 level of 380.66, 557.53, 878.55, 1355.48, 2129.46, and 3312.12 μatm for seven weeks. At the end of the acidification experiment, results revealed that the survival, growth, feed index, biochemical constituents, chitin, minerals (Na, K, and Ca), and hemocyte populations of shrimps were found to be significantly decreased in CO_2 driven acidified seawater which indicates the negative impacts of acidified seawater on these parameters in *L. vannamei*. Further, the level of antioxidants, lipid peroxidation, and metabolic enzymes were significantly higher in the muscle of shrimps exposed to acidified seawater suggests that the *L. vannamei* under oxidative stress and metabolic stress. Among the various acidified seawater tested, pH 7.6 to 7.0 produced a significantly adverse effect on shrimps. Hence, the present study concluded that the elevated level of seawater acidification can produce harmful effects on the biology and physiology of the commercially important shrimp *L. vannamei* PL.

1. Introduction

The marine environment provides enormous resources to humans including nutritional security and employment improving the socio-economic status of humans. However, in recent years, marine environments have been facing serious threats due to the overexploitation of resources and pollutions by various anthropogenic activities (Norse, 2001). Marine environments are highly vulnerable to various pollutants including heavy metals, hydrocarbons, microplastics, greenhouse gases, etc. (Chitrakar et al., 2019). Among divergent pollutants, CO_2 plays a significant role in marine pollution in terms of changes in seawater chemistry and warming which affect the biology and physiology of marine organisms, followed by undesirable alterations in food webs (Meehl et al., 2007; Halpern et al., 2008; Portner and Farrell, 2008; Brown et al., 2010; Hoegh-Guldberg, 2011). As per the report of Union of Concerned Scientists (2019), the top three CO_2 emitted countries were China, USA, and India with 9056.8MT, 4833.1MT, and 2076.8MT respectively during 2016 by burning of fossil fuels which includes coal, oil, and natural gas as the primary energy source. One third of this atmospheric CO_2 has been absorbed by the oceans which lead to an increase in seawater temperature and acidification (Feely et al., 2004; Frolicher et al., 2015; NOAA, 2020). The Intergovernmental Panel on Climate Change (IPCC) predicts that the ocean acidity could increase by 150% (pH 7.8 or beyond) by 2100 (IPCC, 2007) based on the extent of future emissions CO_2 by anthropogenic activities and other factors. These small changes in pH of seawater are highly vulnerable to marine calcifiers species, such as mollusks, crustaceans, reef-forming corals, etc., which form skeletons or shells out of calcium carbonate (Hofmann et al., 2010). A rapid overturn of pCO_2 in seawaters leads to acidification and lacking carbonate ions which can cause biological and physiological stresses of various calcareous organisms (Knoll et al., 1996; Kurihara and Shirayama, 2004; Gazeau et al., 2007; Kurihara et al., 2008; Ries et al., 2009; Bierbower and Cooper, 2010; Carter et al., 2013; Mollica et al., 2018; Melzner et al., 2020) and commercially important fish species which are the main seafood source for humans (Branch et al., 2013).

Crustaceans play a significant role in world aquaculture trade due to their higher market value. According to FAO (2018), marine crustaceans contributed 9.82% of the total annual production of the world during 2016. India has contributed 35.99 lakh tonnes of crustaceans in 2016. On the other hand, crustaceans are very sensitive to biotic and abiotic factors, hence, they have been used as environmental

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bioindicators. Crustaceans are highly sensitive to changes in ocean carbon chemistry also known as ocean acidification (Whiteley, 2011). The alterations in carbon chemistry of the surrounding environment can produce the fluctuations in regulations of acid-base balance, calcification, molting, and immunity which leads to poor survival, growth, and easily susceptible to various biotic and abiotic factors (Small et al., 2010; Whiteley, 2011; Roleda et al., 2012; Taylor et al., 2015). The effect of seawater acidification on marine crustaceans, such as copepods (Walton et al., 1982; Weydmann et al., 2012), brine shrimp (Salma et al., 2012; Sui et al., 2014), shrimps (Mustafa et al., 2015; Furtado et al., 2017), lobsters (Keppel et al., 2012), and crabs (Appelhans et al., 2012) have been studied earlier.

The Pacific white shrimp, *Litopenaeus vannamei* is one of the most dominant cultivable shrimp species in the world due to their fast growth, better survival in high densities culture, and also rich nutritional properties, such as protein, essential amino acids and fatty acids, pigments, minerals, and vitamins. *L. vannamei* has been introduced and extensive cultural practices have been carried out in many coastal areas for the past few years. Presently it is dominated by 90% of the total shrimp culture due to their fast growth rate compared to other Penaeid species. The global production of *L. vannamei* is gradually increased from 2012 to 2016 with the total production of 4156 metric tonnes in 2016 (FAO, 2018). However, the shrimp culture is highly vulnerable to vagaries of climate change (Muralidhar et al., 2012) including temperature and acidification (Furtado et al., 2017; Abdelrahman et al., 2018). Also, shrimps including *L. vannamei* can be used as model organisms to assess the aquatic pollutions (Kathyayani et al., 2019; Neto et al., 2019; Duarte-Restrepo et al., 2020). Therefore, the present study was focused to investigate the possible detrimental effects of CO_2 driven seawater acidification on the Pacific white-leg shrimp *L. vannamei* to understand the possible impacts of ocean acidification on the marine shrimp species.

2. Materials and methods

2.1. Procurement of L. vannamei

The experimental shrimp *L. vannamei* post-larvae (PL30) was obtained from Aqua Nova Hatcheries Private Limited, Chennai. PLs were brought to the laboratory using polythene bags with oxygenated hatchery seawater and acclimatized to ambient laboratory conditions for 14 days in FRP tank (500 L) with natural seawater [salinity (34 ± 0.32 ppt), temperature (25 ± 0.11 °C), pH (8.21 ± 0.08), dissolved oxygen (5.76 ± 0.4 mg L^{-1}), total alkalinity (260 ± 5.3 mg L^{-1}), and NH_3 (0.20 ± 0.01 mg L^{-1})]. During the acclimatization, shrimp PLs were fed with commercial pellet feeds (TNT 400 to 600 micron pellet size, late post-larval feed with the composition of 38% protein, 12% moisture, 9.5% fat and 3% fiber) alternately two times (at 06:00 h and 18:00 h) per day. The adequate level of seawater (about 60%) in the acclimatization tank was renewed on a daily basis to keep the healthy environs.

2.2. Seawater manipulation

The seawater pH manipulation system was designed according to Riebesell et al. (2010) with some modifications. In brief, the pH control system consisted of seven parts, namely CO_2 cylinder, pressure gauge, flow meter, CO_2 valve, pH meter, aquarium (50 L), and aerator (Fig. 1). Six different pH, such as 8.2 (control), pH 7.8 (IPCC-predicted ocean pH by 2100), 7.6, 7.4, 7.2, and 7.0 were manipulated in a respective aquarium with triplicate. The pCO_2 level of pH 8.2, 7.8, 7.6, 7.4, 7.2, and 7.0 were 380.66, 557.53, 878.55, 1355.48, 2129.46, and 3312.12 μatm respectively (Table 1). For the manipulation of seawater pH, the pure (99.9%) CO_2 from the cylinder was released manually by respective CO_2 valve through an insulated rubber tube to bubble in respective aquaria until the desired pH was reached. The changes in pH were monitored by individual pH meters which pre-connected with respective aquaria. Every 4 h the pH of each aquarium was monitored and maintained manually. During the exposure experiment, water parameters (salinity, temperature, ammonia, dissolved oxygen, and total alkalinity) were analyzed as per the standard methods of APHA (2005) and the partial pressure of CO_2, bicarbonate ions (HCO_3^-), carbonate ions (CO_3^{2-}), calcium carbonate saturation state for aragonite and calcite (ΩCa and ΩAr), and total CO_2 were calculated using CO_2 calculator (Robbins et al., 2010).

2.3. Experimental setup

For each acidification treatment, the stocking density was 30 post-larvae (stage PL-20) of *L. vannamei* with 2.81 cm and 0.1 g of average length respectively were assigned for each aquarium. The seawater quality, such as salinity (34 ppt), temperature (25 °C), dissolved oxygen (5.76 mg L^{-1}), total alkalinity (260 mg L^{-1}), and NH_3 (0.20 mg L^{-1}) were maintained in each aquarium. The pH (pH 8.2, 7.8, 7.6, 7.4, 7.2) of each experimental aquarium was manipulated and maintained using CO_2 gas (as described in the seawater manipulation section). The shrimps were fed (10% of body weight) with commercial pellet feed (which was used during acclimatization) twice per day at 06:00 h and 18:00 h and the photoperiod was maintained as 12 h:12 h light and dark. The molts, unfed feeds, and wastes of shrimps grown were collected daily in each aquarium during the seven weeks experiment while renewing 40% of seawater.

2.4. Chemicals products

All of the chemicals used for this study were of high analytical reagents grade purity which was procured from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India.

2.5. Survival, growth, food indices and molting rate

The survival, growth [length gain (LG), weight gain (WG), and specific growth rate (SGR)], food indices [feed intake (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER)] and molting rate (MR) were computed by following the equations prescribed by earlier studies (Tekinay and Davies, 2001; Muralisankar et al., 2015)

$$\text{Survival (\%)} = \text{no. of live shrimp/no. of shrimps introduced} \times 100$$

$$\text{LG (cm)} = \text{final length (cm)} - \text{initial length (cm)}$$

$$\text{WG (g)} = \text{final weight (g)} - \text{initial weight (g)}$$

$$\text{SGR (\%day}^{-1}\text{)} = \log \text{ weight gain (g)/total days} \times 100$$

$$\text{FI (g day}^{-1}\text{)} = \text{feed eaten (g)/total days}$$

$$\text{FCR} = \text{feed intake (g)/weight gain (g)}$$

$$\text{PER (g)} = \text{weight gain (g)/protein intake (g)}$$

$$\text{MR (no. of molt day}^{-1}\text{)} = \text{total no. of molts/total days.}$$

2.6. Analysis of biochemical constituents

The biochemical constituents (total protein, amino acids, carbohydrate and lipid), ash, and moisture were analyzed in the muscle of shrimps according to the standard methods. Briefly, the total protein content was estimated as per the standard method of Lowry et al. (1951) using ethanolic precipitated samples. Bovine serum albumin was served as a standard. The total amino acid was determined by the procedure of Moore and Stein (1948) using sodium tungstate and sulfuric acid extracted samples with the amino acid leucine as standard. The level of carbohydrate content was estimated using trichloroacetic

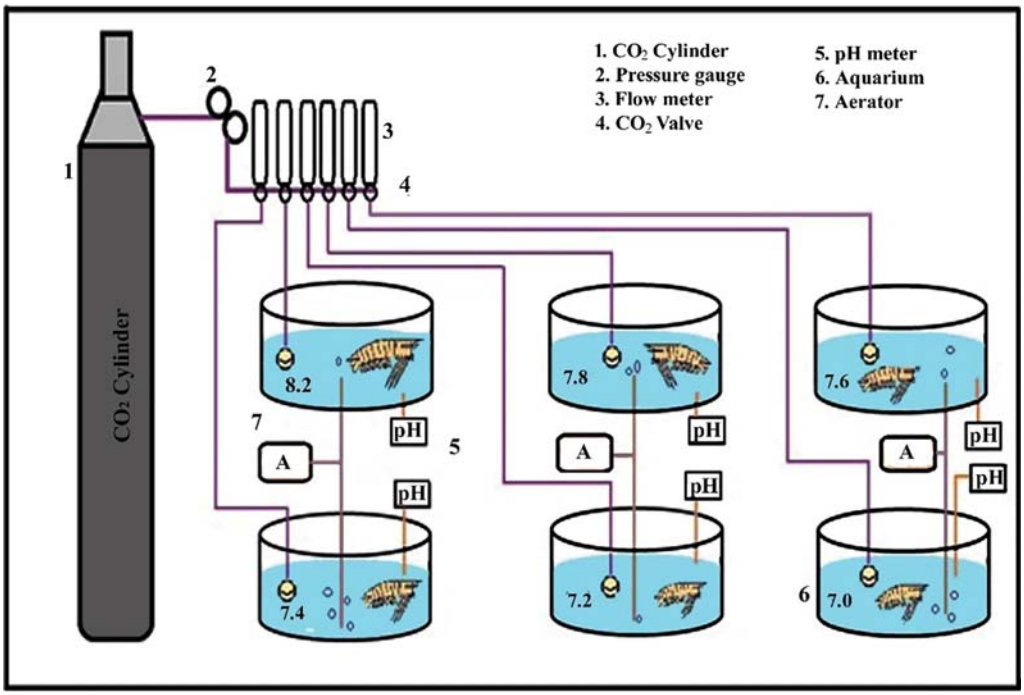

Fig. 1. Schematic representation of the acidification experiment.

acid extracted samples with glucose as standard (Roe, 1955). The lipid content was determined by the following methods of Barnes and Blackstock (1973) using chloroform-methanol extracted samples (Folch et al., 1957) with Olive oil as standard. The carcass ash and moisture contents of shrimps were analyzed by the standard procedures (AOAC, 1995).

2.7. Analysis of chitin content

The estimation of chitin content was performed according to Pinelli et al. (1998). Briefly, the shells of shrimp from each pH experiment were removed and thoroughly washed with distilled water and dried to remove moisture. Then the dried shells were boiled in NaOH solution (3%) for 15 min and cooled at room temperature. The contents were then centrifuged at 857 $\times g$ for 30 min and the supernatant was discarded to remove protein. This boiling process was repeated with residues and centrifuged to remove excess protein. The residue was washed with distilled water, suspended in acetone, and centrifuged at 857 $\times g$ for 15 min to remove the pigments, lipid fractions, and other acetone-soluble materials. The residues with sodium dilute hypochlorite solution, and then the contents were treated with 0.1% HCl at room temperature for 2 h, followed by centrifuging at 857 $\times$ g for 30 min. The residue was washed using double distilled water and dried at room temperature. The dry product was ground to yield powdered chitin and reported as mg/g.

2.8. Analysis of minerals levels

The minerals such as sodium (Na), potassium (K), and calcium (Ca) of shrimp from each experiment were analyzed using Flame Photometer (Labtronics LT- 671) according to the procedures of Jeffery et al. (1989). In short, 1 g of each shrimp muscle sample was digested in 10 ml of triacid (9:2:1 ratio of HNO_3, H_2SO_4, and $HClO_4$) until getting a clear solution. Then the digested sample was cooled and made up to

Table 1
Physicochemical parameters of acidified seawater at different pH.

Parameters	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Salinity (ppt)	34.0 ± 0.32^a	34.0 ± 0.25^a	34.0 ± 0.20^a	34.0 ± 0.30^a	34.0 ± 0.18^a	34.0 ± 0.21^a
Temperature (°C)	25.0 ± 0.11^a	25.0 ± 0.08^a	25.0 ± 0.12^a	25.0 ± 0.11^a	25.0 ± 0.09^a	25.0 ± 0.10^a
Ammonia (mg L^{-1})	0.20 ± 0.01^a	0.20 ± 0.08^a	0.20 ± 0.04^a	0.20 ± 0.02^a	0.20 ± 0.05^a	0.20 ± 0.01^a
Dissolved oxygen (mg L^{-1})	5.76 ± 0.40^a	5.76 ± 0.20^a	5.80 ± 0.70^a	5.80 ± 0.30^a	5.72 ± 0.50^a	5.76 ± 0.3^a
pH	8.22 ± 0.08^a	7.86 ± 0.06^b	7.64 ± 0.03^c	7.46 ± 0.06^d	7.28 ± 0.07^e	7.17 ± 0.20^f
Total alkalinity (μmol kg^{-1})	1998.2 ± 42.0^a	1998.2 ± 42.0^a	1998.2 ± 42.0^a	1998.2 ± 42^a	1998.2 ± 42.0^a	1998.2 ± 42.0^a
pCO_2 (μatm)	214.42 ± 4.80^f	658.89 ± 14.27^e	1104.10 ± 23.67^d	1815.37 ± 38.65^c	2946.67 ± 62.45^b	4742.43 ± 100.20^a
HCO_3^- (μmol kg^{-1})	1394.14 ± 31.27^a	1705.51 ± 36.95^b	1803.22 ± 38.66^c	1870.70 ± 39.83^d	1915.89 ± 40.60^e	1945.54 ± 41.11^f
CO_3^{2-} (μmol kg^{-1})	239.14 ± 5.36^a	116.46 ± 2.52^b	77.69 ± 1.66^c	50.85 ± 1.08^d	32.86 ± 0.69^e	21.05 ± 0.44^f
Ω Ca	5.80 ± 0.13^a	2.82 ± 0.06^b	1.88 ± 0.04^c	1.23 ± 0.02^d	0.79 ± 0.01^e	0.51 ± 0.01^f
Ω Ar	3.81 ± 0.08^a	1.85 ± 0.04^b	1.24 ± 0.02^c	0.81 ± 0.01^d	0.52 ± 0.01^e	0.33 ± 0.01^f
Total CO_2 (μmol kg^{-1})	1639.38 ± 36.77^a	1840.72 ± 39.88^b	1912.32 ± 41.00^c	1973.20 ± 42.01^d	2032.59 ± 43.08^e	2101.52 ± 44.40^f

n = 3; mean ± SD; values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with DMRT). pCO_2, partial pressure of CO_2; HCO_3^-, bicarbonate ions; CO_3^{2-}, carbonate ions; Ω Ca and Ω Ar, calcium carbonate saturation state for aragonite and calcite.

50 ml of double-distilled water for the analysis. After analysis, the concentrations of all studied minerals were converted to mg/kg based on the weight of the sample taken for digestion.

2.9. Assessment of antioxidants and lipid peroxidation

Muscles tissue of shrimps from each treatment were homogenized (10% w/v) individually in ice-cold Tris buffer (50 mM, pH 7.4), followed by centrifugation (9300 ×*g*) for 20 min at 4 °C and the supernatant was used as enzyme source for the assessment of antioxidant activities. Soluble protein concentration in the muscle of each tested shrimp was estimated as per the standard procedure of Lowry et al. (1951). Superoxide dismutase (SOD) activity was analyzed by autoxidation of pyrogallol (10 mM) in tris buffer (50 mM, pH 7.0) and the specific activity of SOD was expressed as U/mg protein (Marklund and Marklund, 1974). Catalase (CAT) activity was determined using the substrate hydrogen peroxide in phosphate buffer and the specific activity of CAT was reported as μmol of H_2O_2/min/mg protein (Sinha, 1972). Lipid peroxidation (LPO) in the shrimp's tissue homogenates was determined by measuring the thiobarbituric acid reactive substance (TBARS) formations and expressed in malondialdehyde (MDA)/mg protein (Ohkawa et al., 1979).

2.10. Metabolic enzymes activity analysis

Precisely, 0.1 g of shrimp muscle tissues were homogenized in sucrose solution (0.25 M), followed by centrifuging (3300 ×*g*) for 20 min at 4 °C and then the supernatant was separated and used as the source for the analysis of metabolic enzymes like glutamic oxaloacetate transaminase (GOT) and glutamic pyruvate transaminase (GPT). GOT and GPT were estimated according to the earlier standard methods (Reitman and Frankel, 1957). L-Aspartic acid was used as the substrate for GOT and L-alanine was used as the substrate for GPT assays with 2 mM sodium pyruvate as a calibrator for both assays. The activity of these enzymes was expressed in U/ml.

2.11. Enumeration of total hemocytes population

At the final day of the acidification experiment, 0.1 ml of fresh hemolymph was sampled from the ventral sinus of the first abdominal segment from each experimental shrimp using a sterile 26 gauge needle attached with 1 ml syringe containing 0.2 ml of freshly prepared anticoagulant (10 mM Tris-HCl, 250 Mm sucrose, 100 Mm sodium citrate, pH 7.6). The collected hemolymph was increased up to 1 ml using anticoagulant. From this 0.2 ml of anticoagulated hemolymph was fixed using 10% formalin (1:1 ratio) for 30 min, followed by diluting 0.1 ml of fixed hemolymph with ice-cold 20 mM phosphate buffer (pH 7.2) at 1:2 ratio. The diluted hemolymph was stained using 20 μl of Rose Bengal stain and incubated for 20 min. The total hemocytes population was determined by hemocytometer under the light microscope and calculated as per the earlier procedure of Muralisankar et al. (2014).

2.12. Statistical analysis

The data obtained were expressed as mean ± SD for all parameters. The data of each parameter were analyzed by one-way analysis of variance, followed by Duncan's multiple range test using SPSS (version 16.0) software to compare the significant differences among acidification treatments, and differences were considered significant when $p < 0.05$.

3. Results

3.1. Physicochemical parameters of acidified seawater at different pH

The physicochemical parameters, such as salinity, temperature, ammonia, dissolved oxygen, and total alkalinity were insignificantly ($p > 0.05$) varied all pH manipulated (pH 7.8 to 7.0) seawater compared to control (pH 8.2). Whereas, the pH, carbonates, calcium carbonate saturation state for aragonite and calcite were significantly ($p < 0.05$) decreased in the pH manipulated seawater from pH 8.2 to 7.0. In this context, pCO_2, bicarbonates, and total CO_2 levels were significantly ($p < 0.05$) higher in pH 7.8 to 7.0 when compared to the control pH 8.2 (Table 1).

3.2. Survival, growth, food indices and molting rate

The present study, the survival of *L. vannamei* was significantly ($p < 0.05$) reduced in pH 7.2 and pH 7.0 compared to control pH 8.2, whereas, an insignificant ($p > 0.05$) difference in survival was observed in shrimps exposed to pH 7.8 to pH 7.6 compared to control. The length and length gain were significantly ($p < 0.05$) decreased in *L. vannamei* exposed to pH 7.6 to 7.0 when compared to control, however, an insignificant ($p > 0.05$) variance was observed in these parameters between control pH 8.2 and pH 7.8. While, the weight and weight gain were found to be significantly ($p < 0.05$) declined in shrimps exposed to pH 7.8 to 7.0 compared to control, however, shrimps exposed to pH 7.8 and pH 7.6 showed an insignificant ($p > 0.05$) difference in these parameters. While, the specific growth rate was significantly ($p < 0.05$) decreased in *L. vannamei* exposed to pH 7.4 to 7.0 compared to control pH. In this context, an insignificant ($p > 0.05$) difference was noticed in the specific growth rate in the shrimps reared at pH 7.8 and 7.6 compared to control (Table 2).

The feed intake and protein efficiency ratio were decreased significantly ($p < 0.05$) in *L. vannamei* exposed to pH 7.4 to 7.0 compared to control, however, these parameters were statistically insignificant

Table 2
Survival, growth, and food indices of shrimp (*L. vannamei*) exposed to acidified seawater at different pH.

Parameters	pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Survival (%)	93.30 ± 1.50^{a}	92.20 ± 1.92^{ab}	90.0 ± 0.81^{b}	90.0 ± 1.20^{b}	86.60 ± 1.06^{d}	80.00 ± 1.40^{c}
Initial length (cm)*	2.81 ± 0.25	2.81 ± 0.25	2.81 ± 0.25	2.81 ± 0.25	2.81 ± 0.25	2.81 ± 0.25
Final length (cm)*	7.66 ± 0.47^{a}	6.96 ± 0.61^{ab}	6.73 ± 0.87^{b}	5.73 ± 0.87^{c}	4.93 ± 0.96^{d}	4.11 ± 0.87^{e}
Initial weight (g)*	0.107 ± 0.03	0.107 ± 0.04	0.107 ± 0.05	0.107 ± 0.06	0.107 ± 0.07	0.107 ± 0.08
Final weight (g)*	2.21 ± 0.39^{a}	1.63 ± 0.47^{b}	1.62 ± 0.63^{b}	0.95 ± 0.45^{c}	0.80 ± 0.48^{cd}	0.49 ± 0.23^{d}
Length gain (cm)*	4.85 ± 0.44^{a}	4.15 ± 0.58^{ab}	3.92 ± 0.83^{b}	2.92 ± 0.83^{c}	2.12 ± 0.92^{d}	1.30 ± 0.83^{e}
Weight gain (g)*	2.108 ± 0.39^{a}	1.532 ± 0.47^{b}	1.451 ± 0.46^{b}	0.851 ± 0.45^{c}	0.695 ± 0.48^{cd}	0.387 ± 0.23^{d}
Feed intake (g day^{-1})	0.368 ± 0.07^{a}	0.362 ± 0.02^{a}	0.364 ± 0.05^{a}	0.294 ± 0.04^{ab}	0.246 ± 0.08^{bc}	0.178 ± 0.06^{c}
SGR (% day^{-1})	2.65 ± 0.16^{a}	2.37 ± 0.25^{a}	2.33 ± 0.35^{a}	1.85 ± 0.39^{b}	1.61 ± 0.57^{bc}	1.28 ± 0.39^{c}
FCR	0.174 ± 0.03^{c}	0.236 ± 0.01^{bc}	0.250 ± 0.03^{bc}	0.345 ± 0.05^{ab}	0.353 ± 0.12^{ab}	0.459 ± 0.17^{a}
PER	0.016 ± 0.003^{a}	0.015 ± 0.004^{a}	0.018 ± 0.005^{a}	0.012 ± 0.006^{b}	0.013 ± 0.008^{b}	0.007 ± 0.004^{b}
Daily molt (no. of molt day^{-1})	1.13 ± 0.06^{a}	1.06 ± 0.02^{a}	1.10 ± 0.10^{a}	0.90 ± 0.10^{b}	0.86 ± 0.09^{b}	0.74 ± 0.12^{b}

n = 3; *, n = 5; mean ± SD; mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with DMRT). SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

($p > 0.05$) in shrimps reared in control, pH 7.8 and 7.6. In this context, the feed conversion ratio was significantly ($p < 0.05$) lower in *L. vannamei* exposed to control pH 8.2 compared to pH 7.4 to 7.0, while, an insignificant ($p > 0.05$) difference was recorded in the feed conversion ratio of shrimps reared in the pH 7.8 and 7.6 compared to control pH 8.2. Daily molt rate was significantly decreased in *L. vannamei* exposed to pH 7.4 to 7.0 when compared to control, while, the shrimps exposed to pH 8.2 to 7.6 showed an insignificant alteration in daily molt rate (Table 2).

3.3. Biochemical constituents

In the current study, the biochemical constituents, such as protein, amino acids, carbohydrate, and lipid were found to be significantly ($p < 0.05$) decreased in *L. vannamei* reared at pH 7.8 to 7.6 compared to control shrimps reared at pH 8.2. Among various pH exposures, the maximum level of decrease in biochemical constituents was noticed in shrimps exposed at pH 7.0 and 7.2. The ash content was decreased significantly ($p < 0.05$) in shrimps exposed to pH 7.4 to 7.0 compared to control and other pH experiments. While the significant ($p < 0.05$) elevation was recorded in the moisture content of shrimps exposed to pH 7.4 to 7.0 compared to control and other pH experiments (Table 3).

3.4. Chitin content and minerals

The chitin content was significantly ($p < 0.05$) decreased in shrimps exposed to pH 7.4 to 7.0 compared to control (pH 8.2), however, an insignificant ($p > 0.05$) difference was obtained in the chitin level of shrimps exposed to pH 7.4 to 7.0. Content of minerals, such as sodium, potassium, and calcium were significantly ($p < 0.05$) decreased in pH 7.0, followed by pH 7.2 and pH 7.4 when compared to control (pH 8.2). In this context, shrimps exposed to pH 7.8 and pH 7.6 showed an insignificant ($p > 0.05$) in these minerals contents compared to control pH 8.2 (Table 3).

3.5. Antioxidants, LPO, metabolic enzymes, and total hemocytes

The enzymatic antioxidants (SOD and CAT), LPO, and metabolic enzymes (GOT and GPT) were analyzed at the end of the acidification experiment. SOD, CAT, and LPO levels were significantly ($p < 0.05$) higher in *L. vannamei* exposed to pH 7.0, followed by pH 7.2, 7.4, and 7.8 compared to control (pH 8.2), while, the difference observed in the level of LPO in shrimps exposed to pH 7.4 to 7.0 was insignificant ($p > 0.05$). Further, the level of GOT and GPT were significantly ($p < 0.05$) elevated in shrimps exposed to pH 7.0 to 7.6 compared to control (pH 8.2). However, activities of GOT and GPT in the shrimps exposed to control (pH 8.2) and pH 7.8 was insignificant ($p > 0.05$). The total hemocyte was significantly ($p < 0.05$) lower in shrimps exposed to pH 7.0, followed by other pH exposures when compared to shrimps exposed to pH 8.2 (Figs. 2–4).

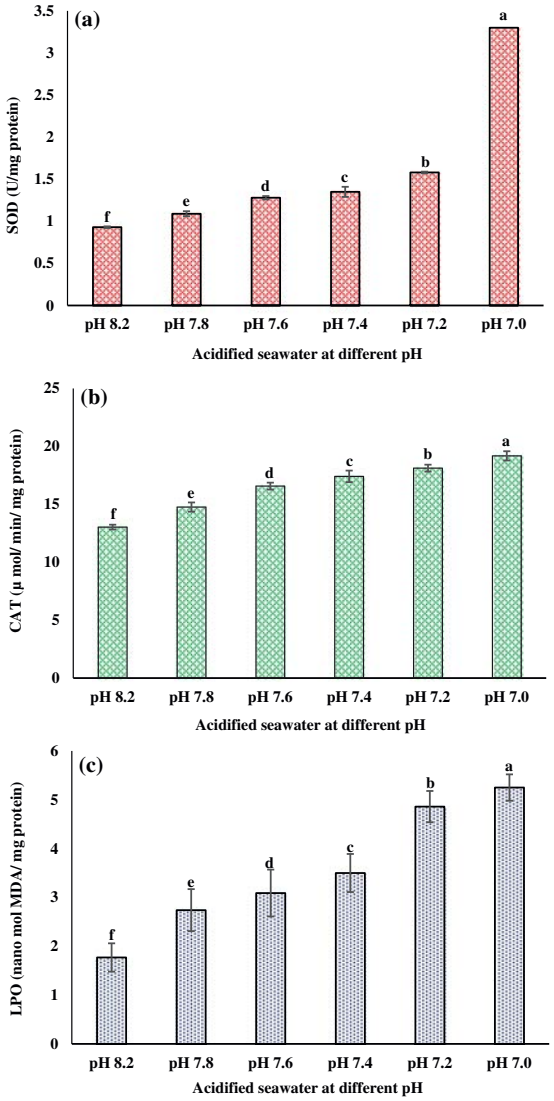

Fig. 2. Antioxidants and lipid peroxidation of *L. vannamei* exposed to CO_2 driven acidified seawater. n = 3 (three samples from each treatment), mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups. SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation.

Table 3
Muscle biochemical constituents, chitin and minerals level of shrimp (*L. vannamei*) exposed to acidified seawater at different pH.

Composition		pH 8.2	pH 7.8	pH 7.6	pH 7.4	pH 7.2	pH 7.0
Protein (mg g^{-1})		217.75 ± 8.90^{a}	190.88 ± 6.40^{b}	172.49 ± 6.80^{c}	158.58 ± 9.20^{d}	150.23 ± 7.03^{de}	144.31 ± 3.40^{e}
Amino acid (mg g^{-1})		190.07 ± 4.90^{a}	139.8 ± 4.80^{b}	100.77 ± 2.30^{c}	100.76 ± 3.60^{c}	80.30 ± 5.20^{d}	76.22 ± 4.50^{d}
Carbohydrate (mg g^{-1})		168.86 ± 5.10^{a}	161.17 ± 5.00^{ab}	156.69 ± 5.40^{b}	153.52 ± 5.50^{b}	126.08 ± 4.00^{c}	96.67 ± 5.98^{d}
Lipid (mg g^{-1})		5.39 ± 0.22^{a}	4.70 ± 0.09^{b}	3.80 ± 0.20^{c}	3.62 ± 0.20^{cd}	3.39 ± 0.10^{de}	3.32 ± 0.10^{e}
Ash (%)		21.73 ± 0.44^{a}	20.0 ± 0.57^{ab}	21.0 ± 0.97^{ab}	19.66 ± 0.74^{b}	16.0 ± 0.65^{c}	16.0 ± 0.40^{c}
Moisture (%)		73.0 ± 0.81^{b}	76.0 ± 0.70^{ab}	75.0 ± 0.641^{ab}	77.0 ± 0.41^{a}	76.0 ± 0.52^{a}	76.0 ± 2.85^{a}
Chitin (mg g^{-1})		0.25 ± 0.10^{a}	0.25 ± 0.03^{a}	0.19 ± 0.02^{a}	0.09 ± 0.04^{b}	0.03 ± 0.014^{b}	0.02 ± 0.021^{b}
Minerals (mg kg^{-1})	Na	88.0 ± 1.54^{a}	76.16 ± 1.32^{b}	53.90 ± 1.20^{c}	53.6 ± 2.21^{c}	40.8 ± 1.10^{d}	34.20 ± 1.14^{e}
	K	70.30 ± 1.60^{b}	78.12 ± 1.14^{a}	76.50 ± 1.11^{a}	60.90 ± 1.21^{c}	51.40 ± 1.01^{d}	45.10 ± 1.12^{e}
	Ca	8.20 ± 1.00^{a}	5.60 ± 0.25^{b}	4.20 ± 0.12^{c}	3.20 ± 0.59^{d}	2.40 ± 0.35^{de}	1.70 ± 0.09^{e}

n = 3; mean ± SD; values within the same row sharing different alphabetical letter superscripts are statistically significant at $p < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with DMRT).

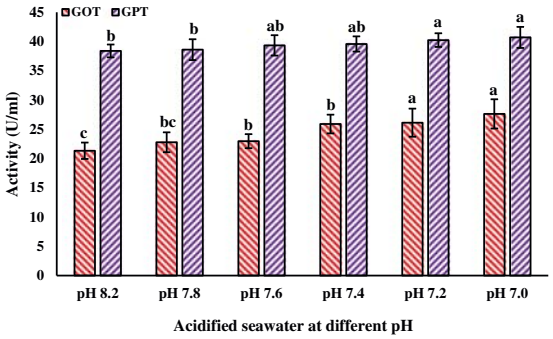

Fig. 3. Metabolic enzymes activity of *L. vannamei* exposed to CO_2 driven acidified seawater.
n = 3 (three samples from each treatment), mean ± SD; bars sharing different letters in each parameter are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups; GOT, glutamic oxaloacetate transaminase; GPT, glutamic pyruvate transaminase.

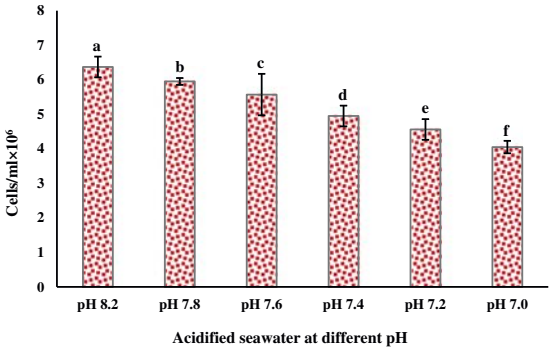

Fig. 4. THC of *L. vannamei* exposed to CO_2 driven acidified seawater.
n = 3 (three samples from each treatment), mean ± SD; bars sharing different letters are considered as significant at $p < 0.05$ while comparing to control (pH 8.2) and other pH treated groups. THC, total hemocytes count.

4. Discussion

The culture of crustaceans including shrimps, crabs, and lobsters has gained global attention in recent years for their significant role in seafood production. Crustaceans are also ecologically important organisms in marine environments. Marine pollution including changes in the carbon chemistry (acidification) of water can affect the physiological process of crustaceans which creates ecological consequences especially in the food chain (Whiteley, 2011). Crustaceans are moderately tolerant to changes in ocean carbon chemistry, such as pH regulation, regulation of the calcification process. Crustaceans including shrimps, crabs, and lobsters showed various responses including survival, feeding, molting, growth, calcification, etc., under the acute and chronic exposure in the acidified environment (Boßelmann et al., 2007; Small et al., 2010; Roleda et al., 2012; Keppel et al., 2012; Appelhans et al., 2012; Taylor et al., 2015; Mustafa et al., 2015; Furtado et al., 2017).

The physicochemical properties of seawater play a significant role in the physiological regulations of living organisms including crustaceans. Chemistry of seawater can be altered by the acidification processes which lead to physiological stress to the living organisms in the marine environment. In the present study, insignificant alterations in salinity, temperature, ammonia, dissolved oxygen, and total alkalinity of all CO_2 driven acidified seawater (pH 7.8 to 7.0) indicates that the acidification of seawater did not produce any significant alteration in these parameters during the seven weeks experiment. Further, the significant elevations in pCO_2, bicarbonates ions, and total CO_2, and significant decreases in pH, carbonates, calcium carbonate saturation state for aragonite and calcite in acidified (pH 7.8 to 7.0) seawater showed that the CO_2 driven acidification had produced key changes in the chemical properties of seawater. Similarly, CO_2 mediated drastic changes in chemistry (pCO_2, bicarbonates, ΩCa, and ΩAr) have been noticed by Arnold et al. (2009) and Pedersen et al. (2014). Further, Egilsdottir et al. (2009) observed that the significant changes in the seawater chemistry including bicarbonates, carbonates, ΩCa, and ΩAr due to CO_2 driven acidification. Also, the alterations in the chemical properties (pCO_2, bicarbonates, carbonates, and ΩAr) of seawater chemistry due to CO_2 driven acidification has been observed earlier (Mu et al., 2015).

The survival, growth, and utilization of feeds are pivotal parameters that directly affect the economy of edible organisms like fish and shellfishes. In the current study, the significant decrease in survival, daily molt, length gain, weight gain, and specific growth rate, feed intake and protein efficiency ratio of *L. vannamei* exposed to acidified seawater indicated that the CO_2 driven seawater acidification had shown an adverse effect on the shrimps. Among different acidification experiments, pH 7.6 to 7.0 produced maximum adverse effects on *L. vannamei*, it indicates that this range of pH could be served as toxic to shrimps. The decreased survival, growth, of shrimp, *Palaemon pacificus* exposed to acidified seawater has been reported earlier (Kurihara et al., 2008). The starfish, *Asterias rubens*, and crab, *Carcinus maenas* showed poor feeding and growth exposed to CO_2 driven acidified seawater has been reported (Appelhans et al., 2012). Vijayan and Diwan (1995) and Gireesh and Gopinathan (2004) reported that the variations in culture water pH had produced poor survival, molting, feed intake and growth in the shrimp, *Penaeus* (*Fenneropenaeus*) *indicus* and oyster, *Paphia malabarica*. Further, the poor survival, growth, hatching rate and metabolic stress in the crabs (*Cancer magister*, *Hyas araneus*, and *Petrolisthes cinctipes*) when reared at low pH have also been recorded earlier (Harms et al., 2014; Paganini et al., 2014; Miller et al., 2016). Also, changes in seawater pH led to decreases in hatching and survival of brine shrimps (*Artemia franciscana* and *Artemia parthenogenetica*) have been reported previously (Sui et al., 2014; Zheng et al., 2015).

The physiological condition of an organism can be determined by the biochemical composition of the edible organism which is directly correlated to the nutrients level, such as protein, essential amino acids, fatty acids, carbohydrate, and minerals. The present study, the significant decrease in biochemical elements (protein, amino acids, carbohydrates, and lipid) of *L. vannamei* reared in acidified (pH 7.8 to 7.0) seawater suggests that the CO_2 driven seawater acidification had produced an adverse impact on the synthesis and storage of these biochemical constituents in shrimps. Likewise, the impact of seawater acidification on the total protein content studied in the shrimp, *L. vannamei* has been reported earlier by Furtado et al. (2015). The significant reduction of amino acids content in the green crab, *Carcinus maenas* exposed to acidified seawater has been reported by Hammer et al. (2012). The decreased total protein content in neonates of shark, *Chiloscyllium plagiosum* exposed under CO_2 driven acidified seawater has also been reported previously (Lopes et al., 2018).

Chitin is an amino polysaccharide that is commonly found in all crustaceans which give strength to exoskeleton, shape, and protect the organisms from the predators. In the present investigation, the significant decrease in chitin content in *L. vannamei* inferred that the acidified seawater had produced an adverse effect on the synthesis of chitin. This result is agreed with previous studies of Mustafa et al. (2015) who reported that the reduced shell chitin content in the same shrimp species (*L. vannamei*) exposed to acidified seawater. Minerals play an essential role in several biological functions including the formation of skeletal, colloidal systems maintenance, acid-base equilibrium regulation, etc., of living organisms (Lall, 2002). The decreased level of minerals, such as Na, K, and Ca in *L. vannamei* indicates that the

acidified seawater can prevent the intake of minerals from water and food. Further, the decreased level of Ca in the carcass of shrimps exposed to acidified seawater suggest that the acidic condition of surrounding water led to decreased calcification rate in the experimental shrimps. Earlier studies reported that the crabs (*Paralithodes camtschaticus* and *Chionoecetes bairdi*) produced a significant decrease in calcification when exposed to CO_2 driven acidified seawater (Long et al., 2013; Swiney et al., 2016). Also, the shrimp *Lysmata californica* exposed to acidified seawater showed significant alterations in the mineralization of exoskeleton (Taylor et al., 2015).

Antioxidants are collectively fighting against free radicals to resist the damaging of cells and tissues. Among antioxidants, SOD and CAT are considered as the primary defense antioxidants which are essential for the entire defense strategy against superoxide anion radical (O_2^-) during the tissue injury following the oxidative process and phagocytosis and normal body metabolism (Fang et al., 2002; Ighodaro and Akinloye, 2017). The process of LPO is free radicals mediated chain reactions which resulted in oxidative degradation of lipids, especially polyunsaturated fats. The main targets parts of biological systems are cell membranes. The biotic and abiotic toxic substances are responsible to initiate the LPO reactions. In animals, the significant fluctuations in metabolic enzymes indicate the injury of normal liver function. In this study, the significant increment of antioxidants such as SOD and CAT in *L. vannamei* reared at CO_2 mediated low pH compared to control pH suggesting the high contribution of these antioxidants to remove the reactive oxygen species which formed by acidic stress. The significant increase in LPO of *L. vannamei* exposed to CO_2 driven low pH compared to control showed that the acidic stress can produce the membrane damage in shrimps. The metabolic enzymes in *L. vannamei* exposed to acidified seawater indicated that the CO_2 driven acidified seawater had produced metabolic stress to the shrimps. Therefore, it suggests that alterations in the carbon chemistry of seawater may toxic to shrimps. Earlier studies also noted that the elevated level of SOD, CAT, and glutathione transferase (GST) in *L. vannamei* and *Artemia sinica* exposed to acidified seawater (Furtado et al., 2015; Zheng et al., 2015). The increased activities of antioxidants, such as SOD, CAT, glutathione peroxidase, glutathione, acid phosphatase and alkaline phosphatase, and the metabolic enzyme GPT in the mussel, *Mytilus coruscus* has also been reported by Hu et al. (2015). Further, the alterations in antioxidants, such as GST, SOD, and CAT, and LPO in sand smelt larvae (*Atherina presbyter*) and flatfish larvae (*Solea senegalensis*) when reared at acidified seawater have also been investigated previously (Pimentel et al., 2015; Silva et al., 2016).

The physiological and immunological status of an organism can be determined by hematological parameters like total blood cells count. The determination of hemocytes in crustaceans including shrimps is an essential tool for the detection of the health status. In the current study, the significant decrease in total hemocytes count indicated that the shrimp exposed to CO_2 driven acidified seawater had impaired the production of hemocytes. This result is agreed with a previous study in *L. vannamei* which shown the decreased level of hemocyte populations when shrimps reared in CO_2 driven acidified seawater (Huang et al., 2018). The impact of seawater acidification on hemocyte physiology in the tanner crab (*Chionoecetes bairdi*) has been studied by Meseck et al. (2016). Also, the diminished level of hemocyte population has been noted in Norway lobster (*Nephrops norvegicus*) exposed to acidified seawater environment (Hernroth et al., 2012).

In conclusion, the present study revealed that survival, growth, feed utilization, biochemical constituents, chitin and minerals, and hemocytes populations of shrimps were found to be significantly decreased in CO_2 driven acidified seawater which indicates the adverse effects of acidified seawater in *L. vannamei* with the sign of elevated antioxidants, lipid peroxidations, and metabolic enzymes which shows that the shrimps were under free radical stress and metabolic stress. Therefore, the present study concluded that the elevated level of CO_2 driven seawater acidification can produce harmful effects on *L. vannamei* postlarvae which indicates potential threats of ocean acidification on shrimps.

CRediT authorship contribution statement

T. Muralisankar: Conceptualization, Funding acquisition, Investigation, Project administration & Writing original draft. P. Kalaivani: Software & Formal analysis. S.H. Thangal: Formal analysis & Validation. P. Santhanam: Writing - review & editing.

Declaration of competing interest

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

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Occurrence of Microplastics in the Gastrointestinal Tracts of Edible Fishes from South Indian Rivers

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Abstract
Microplastics (MPs) in the gastrointestinal (GI) tracts of the five fish species from the Kollidam and Vellar rivers of Tamil Nadu, Southern India were evaluated. A total of 315 MPs were isolated from GI tracts of 23 fishes (Chanos chanos, Chanda nama, Chelon macrolepis, Carangoides malabaricus and Gerrus filamentosus) sampled from both rivers. MPs ranged from 109 to 129 µm (119 ± 79.7) and 181 to 284 µm (122 ± 92.6) in size, with fibres (85.7%) and fragments (14.3%) being the most common ones in the fishes from Kollidam and Vellar river, respectively. The colour pattern of ingested MPs was dominated by blue, transparent, red, yellow and black in collected fishes from both rivers. In this study, MPs were higher in fishes with omnivore feeding habits due to their broad diet habits. Moreover, urban wastes, fishing and agricultural activities are the possible primary sources of MPs in both rivers.

Keywords Abundance · Fibre · Fragment · Kollidam river · Vellar river

Introduction

Plastic pollution has become a severe threat to the environment around the globe due to mismanagement and poor recycling rates of plastics. India lifted to the 15th position among the plastics polluting country in the list of nations with lower recycling rates in plastic waste treatment (Geyer et al. 2017). Microplastics (MPs) are particles < 5 mm and widespread in all global water bodies. Secondary MPs originated from environmental processes such as photo-oxidation, thermal stress and tidal actions (de Sá et al. 2018). Fishing practices and terrestrial activities contribute nearly 20% and 80% of MPs, respectively (Cole et al. 2011). MPs enter the rivers and disturb the river, estuarine, and marine ecosystems (Dris et al. 2015). MPs have been found in zooplankton, amphipods, polychaete, echinoderms, crustaceans, fishes, birds and mammals (Desforges et al. 2015; Long et al. 2015; Rummel et al. 2016; Herzke et al. 2016; Hurley et al. 2017; Fossi et al. 2018).

A wide range of organisms is ingesting the MPs, which adversely affects the biology and physiology of organisms. MPs showed detrimental effects on feeding, intestinal tract blockage, false sensation of satiation, reproduction, and energy metabolism in aquatic animals (Cole et al. 2013; Xu et al. 2018; Jiang et al. 2020). Earlier studies reported the accumulation of MPs in marine organisms, including fish and shellfishes (Jonathan et al. 2021; Selvam et al. 2021). Nevertheless, the information on the accumulation of MPs in freshwater organisms is limited, including in India. Hence, the present study aimed to evaluate MPs pollution status (abundance, size, shape, colour, and surface topography) in the Kollidam and Vellar rivers of South India with reference to edible fishes. The selected rivers for the study serve as habitats for the various faunal diversity and livelihood for the local fishermen communities until they end up in the Bay of Bengal. Kollidam and Vellar rivers flow in subparallel patterns to each other. Both rivers are geographically located in the Cuddalore district along the southeast coast of Tamil Nadu, India. Kollidam River is the Northern distributary of the Cauvery River splits from Cauvery near Srirangam of Tiruchirappalli district. It flows about 150 km among six significant districts such as Tiruchirappalli,

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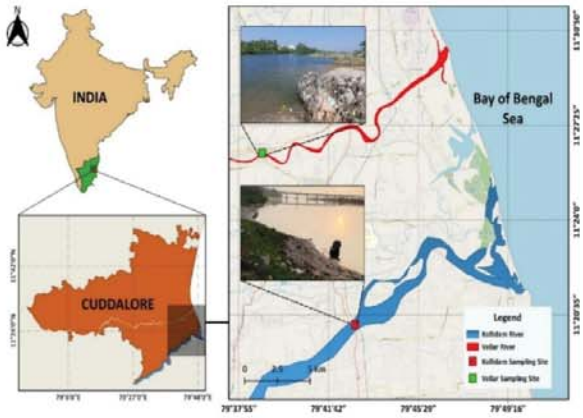

Fig. 1 Geographical data of the selected study sites of both Vellar and Kollidam Rivers

Tanjore, Ariyalur, Mayiladuthurai, and Cuddalore and ends up in the Bay of Bengal near Sirkali in Nagapattinam district. Vellar river originates in the Chittori hills of the Eastern Ghats in the Salem district of Tamil Nadu at an elevation of 900 m it has a total length of 210 km and consists of 22 urban parts of eight districts, its watershed covers 2.14 lakh hectares (NWM 2017). It runs through Salem and Cuddalore districts in Tamil Nadu and confluences with the Bay of Bengal near Parangipettai in the Chidambaram district. Urban, fishing and agricultural activities are the primary sources of MPs in both rivers.

Materials and Methods

Fishes were collected from the Vellar river at Bhuvanagiri, Chidambaram district (11°26′23.00″ to 11°26′27.15″ N, 79°33′53.35″ to 79°33′57.05″ E) and Kollidam river at Kollidam, Mayiladuthurai district (11°19′58.30″ to 11°20′23.19″ N, 79°42′42.82″ to 79°43.07′71″ E) with the help of local fishermen from February to May 2020 (Fig. 1). Both sampled sites are freshwater environments and directly connected with the urbanised zones and regular fishing activities. The collected fishes were anaesthetised, stored in a sterile sample container, and then transported to the laboratory for further process. Fishes were cleaned with de-ionised water to remove the debris and minimise external MPs contamination. The lengths and weights of sampled fish ranged between 11.7 to 27.8 cm and 21.77 to 217.72 g, respectively (Table S1 supplementary material). Collected fishes were identified based on their morphological features using standard manuals and the data obtained from the Fish Base website. Identification keys of length, shape, depth, mouth, distribution, nature of fish spines, and scales were used to identify sampled fishes (Jayaram 1999; Froese and Pauly 2021). Twenty-three fishes were examined for taxonomical classification from Kollidam (n = 13) and Vellar (n = 10) rivers. From these five fishes such as *Chanos chanos* (n = 5), *Chelon macrolepis* (n = 9), *Chanda nama*, (n = 3), *Gerrus filamentosus* (n = 4), and *Carangoides malabaricus* (n = 2) were taxonomically identified.

Each fish's gastrointestinal tract (GI) was dissected in plastic-free containment and rinsed using ultrapure water to reduce the chances of plastic contamination. Dissected guts were transferred into sterile glass containers for digestion. Digestion of fish guts and extraction of MPs was done using the alkaline digestion method of Karami et al. (2017). Extracted MPs were viewed under the Magnus stereomicroscope with 20–40× magnification and treated with a hot needle test to observe the melting points of the particles to confirm the MPs (de Witte et al. 2014). Particles were imaged using an Ultrascope 9.0v camera, and size was measured with ImageJ software v.1.50i (http://imagej.nih.gov). The morphological characterisation of MPs was done using the distinguishing method criteria like size, shape, and colour (Hidalgo-Ruz et al. 2012; Li et al. 2016). Extracted MPs from both rivers were quantified and picked up using 0.3 × 0.15 mm tip-sized micro forceps and segregated for surface morphological analysis using a field emission scanning electron microscope (FE-SEM) (FEI Quanta 250 FEG).

For quality control, the sampled fishes were rinsed with filtered de-ionised water, and stainless-steel containers with icepacks were used to transfer the fishes to the laboratory. The laminar chamber stage was cleaned before the dissection of fishes to avoid cross-contamination between each fish. Natural cotton lab coats were used inside the laboratory to maintain quality control. Glassware was rinsed using filtered de-ionised water before the dissection of GI tract samples. Samples were not exposed to any open environmental conditions. The incubator, ultra-probe sonicator, centrifuge, orbital shaker, vacuum filtration unit with pump, laminar airflow, and stereo zoom microscope were cleaned with 90% ethanol and kept sterile before use. Pre-filtered de-ionised water was used to prepare the chemicals for sample processing. Before chemical preparation, the filtered de-ionised water was allowed for MPs examination and confirmed as no evidence for MPs contamination. Moreover, procedural and laboratory environmental blank samples were prepared using the same concentration of chemicals without the GI tract of fish, followed by examining and confirming the absence of MPs. The filters consisting of MPs were only exposed to air during microscopic examination inside the laminar chamber. The data obtained from all the parameters were expressed in mean ± SD. Significance variations in properties of MPs were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at $p < 0.05$ using SPSS (16.0) software.

Table 1 Abundance and Length of MPs in the GI tracts of fishes collected from the Kollidam and Vellar river

Site	Species	No. of MPs	MPs/ Indiv.	Length of MPs (μm)			Level of Significance < 0.05
				Min.	Max.	Mean length	
Kollidam	*C. chanos*	50	10.0 ± 5.5^{a}	18.4	333.9	121 ± 72.6^{a}	0.473
	C. macrolepis	39	7.8 ± 3.1^{a}	11.6	273.4	109 ± 74.1^{a}	
	C. nama	39	$13 \pm 14^{a*}$	32.1	267.1	$129 \pm 75^{a*}$	
	∑	128					
Vellar	*C. macrolepis*	59	$14.8 \pm 5.6^{a*}$	13.0	506.2	$202 \pm 97.8^{b*}$	0.004
	G. filamentosus	91	$22.8 \pm 4.9^{a*}$	19.4	377.5	$284 \pm 83.3^{a*}$	
	C.malabaricus	37	$18.5 \pm 7.8^{a*}$	19.6	487.6	$181 \pm 95.4^{b*}$	
	∑	187					
∑ Kollidam + Vellar		315	Overall				0.056

Mean values within each site sharing the same alphabetical letter superscripts are not statistically significant at $P < 0.05$; *denotes the significant difference of MPs between all fish species from both locations (one-way ANOVA and subsequent post hoc multiple comparisons with DMRT)

Results and Discussion

In the present study, 315 MPs were isolated from the GI tracts of fishes sampled from both river sites. A total of 128 and 187 MPs were isolated from GI tracts of fishes from the Kollidam and Vellar rivers, respectively. Among fishes from the Kollidam river, the maximum number of MPs were isolated from *C. chanos*, followed by *C. macrolepis* and *C. nama*. In fishes sampled from the Vellar river, a higher abundance of MPs was found in *G. filamentosus*, followed by *C. macrolepis* and *C. malabaricus* (Table 1). The MPs level in fish/individuals did not show any significant ($p > 0.05$) difference in-between the species collected from each sampling location. However, the total number of MPs per individual was significantly ($p < 0.05$) higher in the fishes collected from the Vellar river than that of the Kollidam river, which denotes the higher amount of MPs pollution Vellar compared to the Kollidam river. This might be due to the discharge of MPs from the urbanised areas near the Vellar river basin than the Kollidam river (Table 1). The feeding habits of fishes, trophic levels and the availability of MPs in the ecosystem can affect the ingestion of MPs. In this study, all examined fishes, such as *G. filamentosus, C. malabaricus* and *C. macrolepis*, from the Vellar river showed a high level of MPs per individual, suggesting that these species possess omnivore feeding habits that facilitate feeding on wide verities of diet. However, the carnivore fish *C. nama* from the Kollidam river showed higher MPs per individual than omnivore fishes (*C. macrolepis* and *C. chanos*), suggesting that this fish feeding might be associated with MPs ingested prey. Similarly, MPs are in fishes with the same feeding habits, such as *Cyprinus carpio, Carassius auratus, Hypophthalmichthys molitrix, Pseudorasbora parva, Megalobrama amblycephala*, and *Hemiculter bleekeri* sampled from Taihu Lake, China has been observed (Jabeen et al. 2017). Also, 10–13 MPs per individual in the fishes such as *Catostomus commersonii*, *Pimephales promelas*, *Carpoides cyprinus*, *Notropis stramineus*, *Notropis hudsonius*, *Fundulus diaphanus*, *Micropterus sp.*, *Notropis atherinoides*, *Neogobius melanostomus*, and *Cyprinella spiloptera* has been noticed from the major tributaries of Lake Michigan, the USA (McNeish et al. 2018) which is similar to the outcome of the present study.

The mean length of 128 MPs isolated from the fishes *C. nama, C. chanos* and *C. macrolepis* from the Kollidam river was recorded from 32.14 to 267.08 μm, 11.60 to 273.38 μm, and 18.40 to 333.91 μm, respectively. MPs in *C. macrolepis, G. filamentosus*, and *C. malabaricus* sampled from the Vellar river showed lengths between 13.01 and 506.24 μm, 19.40 to 377.53 μm, and 19.63 to 487.611 μm respectively. The mean length of MPs isolated from fishes of the Kollidam river showed an insignificant ($p > 0.05$) difference compared to each other. The mean length of MPs isolated from *G. filamentosus* showed a significant ($p < 0.05$) increase compared to MPs isolated from other fishes from the Vellar river. However, the mean length of MPs isolated from *C. macrolepis* and *C. malabaricus* showed an insignificant ($p > 0.05$) difference sampled from the Vellar river (Table 1). Moreover, the mean length of MPs was significantly ($p < 0.05$) higher in the fishes collected from the Vellar river than that of the Kollidam river, which indicates the ingestion of an increased range of MPs by fishes in the Vellar river compared to the Kollidam river (Table 1). The high abundance of MPs of different sizes might be caused by indirect and false ingestion of fishes in the Vellar river rather than the Kollidam river. Food selection with suitable sized particle feeding of fishes can cause the difference in MPs ingestion (Cole et al. 2013). Also, the much deviation in the MPs isolated from the GI tract of fishes indicated that the high rate of fragmentation led to ingestion by fishes as false feeding in the sampled environments. Similar to our study, 0.3 to 0.6 mm size MPs have been observed in *C. Carpio, Carassius cuvieri, Lepomis macrochirus, Micropterus salmoides, Silurus asotus*, and *Channa argus* from Han River, South Korea (Park et al.

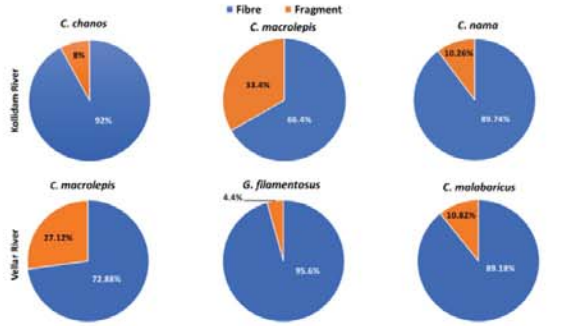

Fig. 2 Shape distribution of MPs in the fish species collected from the Kollidam and Vellar Rivers

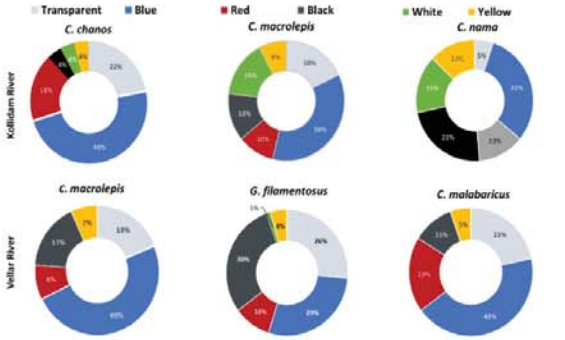

Fig. 3 Colour distribution of MPs in the fish species collected from the Kollidam and Vellar Rivers

2020). A South-West Nigerian Eleyele Lake report revealed that the fishes (*Coptodon zillii* and *Oreochromis niloticus*) ingested 124 and 126 µm sized MPs (Adeogun et al. 2020). In the present study, the size of MPs is much smaller than in the above-cited previous studies. Moreover, the smaller plastic particles can serve as vectors for carrying metals and organic compounds like polychlorinated biphenyls (PCBs), organochlorines, dioxins, etc., to the organisms (Wang et al. 2020; Hildebrandt et al. 2021).

The morphological distribution of MPs in this study indicated that the fibres were the dominant in the GI tract of fishes sampled from both rivers, constituting nearly 85.71% of the total 315 MPs, followed by fragments with fragments of 14.29%. MPs isolated from fishes belonging to the Kollidam river were categorised as fibres and fragments with 83.59% and 16.41%, respectively. Among the fish species from the Kollidam river, *C. chanos* had the maximum number of fibres, followed by *C. nama* and *C. macrolepis*, whereas the fragments were found in the order of *C. macrolepis* > *C. chanos* > *C. nama* (Fig. 2; Table S2 and Fig. S1-S3 supplementary material). In the Vellar river, the GI tract of *G. filamentosus* showed a maximum level of fibres, followed by *C. malabaricus* and *C. macrolepis*. Meanwhile, the fragments were foremost in the GI tract of *C. macrolepis* compared to other fishes (Fig. 2; Table S2 and Fig. S4-S6 supplementary material). In the present study, a maximum level of fibres suggests that the mismanaged fishing gears and textile wastes are the primary sources of the MPs invasion in fish guts. Likewise, the alien fish *Piaractus brachypomus* sampled from Ramsar wetland Vembanad Lake, Kerala, India, accounted for 50% of fibres in their GI tracts (Devi et al. 2020). Similarly, fish *Gambusia holbrooki* from Melbourne, Australia, in more numbers of fibres than other shaped MPs (Su et al. 2019). Defragmentation of plastic wastes into secondary MPs is the major contributor to plastic litter in the rivers. In the present study, fibre-shaped MPs were predominantly distributed in all sampled fishes. This might be due to discharges of plastic debris originating from the leftovers and fragmented fishing gears, agricultural and urban discharges to the rivers.

The colour-wise distribution of MPs in fishes guts showed significant differences in their accumulation pattern sampled from Kollidam and Vellar rivers. The blue-coloured MPs, followed by transparent were dominant in the GI tract of fishes sampled from both rivers (Fig. 3; Table S3 and Fig. S1-S6 supplementary material). MPs in the GI tract of fishes sampled from the Kollidam river showed the colours in the order of blue > transparent > red > white > yellow, whereas the river Vellar showed in the order of blue > transparent > red > yellow > white (Fig. 3; Table S3 supplementary material). In this study, blue-coloured MPs were dominant in the GI tract of fishes, these coloured fibres and fragments can be derived from textile wastes and fishing gears (Browne et al. 2008; Li et al. 2016). These particles might be similar to the feeds in aquatic conditions, which led to false feeding by fishes (Ory et al. 2018). The remaining-coloured MPs might be direct false feeding or transferred from other prey. Similarly, the *Dicentrarchus labrax, Diplodus vulgaris*, and *Platichthys flesus* from Mondego estuary western coast of Portugal showed dominant blue, transparent and black coloured MPs particles (Bessa et al. 2018). Further, different proportions of colours on ingested MPs from the GI tracts were observed on fish species of *O. niloticus* and *Cirrhinus molitorella* from the Rivers of Guangdong province, south China (Sun et al. 2021). MPs' colour distribution can significantly affect fishes' ingestion rate due to the difficulty in differentiating the feeds. Moreover, transparent MPs are non-identical in the aquatic systems, blue is used in fishing nets and textile waste, and their different colours and buoyance resemble the planktonic feeds.

The surface morphological characteristics of MPs were carried out with a cluster of MPs picked up from the filter obtained from the extraction step. The complex surface topography of the extracted MPs indicates the linear strings

due to the heavy accumulation of fibres in the GI tracts of fishes collected from both sites. The surface of the MPs strings was convex, rough non-porous and with many folds (Fig. S7 supplementary material). These strings appeared irregular and had a brittle body with sharp and blunt edges. These damages may be caused by environmental factors like continuous mechanical disturbances caused by water current flow in the river and photo-oxidative weathering of MPs caused by UV radiation (Kalogerakis et al. 2017; Ding et al. 2019). The damage and peeled spots were observed on the MPs strings' surface (Fig. S7).

Conclusions

MPs in the GI tract of fishes sampled from the Vellar and Kollidam rivers indicate MPs pollution in both rivers. Further, the fibre content was higher in the GI tract of fishes sampled from both rivers, suggesting that the mismanaged fishing gears, textiles, and urban waste materials might be the primary source of fibres in the fishes. The overall occurrence of MPs elevated in the fish *C. macrolepis* sampled from the Vellar river compared to the same species from the Kollidam river, indicating a higher MPs pollution in the Vellar river. Therefore, these findings suggest that the MPs were added to fishes' diets, which may carry them to the next level of the ecosystem's food chain with potential risks. Moreover, both Kollidam and Vellar rivers end up in the Bay of Bengal and can serve as a carrier of MPs to the marine environment.

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REGULAR ARTICLES

Utilization of marine fisheries wastes for the production of the freshwater fish *Cyprinus carpio*

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Abstract
The present study was aimed to assess the effects of complete replacement of fish meal with fisheries waste meals on survival, growth performance, digestive enzyme activities, and muscle compositions of the freshwater fish *Cyprinus carpio*. The proximate composition and mineral contents of three different fisheries wastes, such as *Rastrelliger kanagurta*, *Sphyraena barracuda*, and *Fenneropenaeus indicus* were analyzed. Based on the nutrient content of these fisheries waste, one control fish meal diet and three different complete fish meal replacement diets (diet 1, diet 2, and diet 3 formulated with *R. kanagurta*, *S. barracuda*, and *F. indicus* waste meals, respectively) were formulated. Fingerlings *C. carpio* were fed with these diets for a period of 6 weeks. Results from feeding experiments showed insignificant ($p > 0.05$) differences in survival, growth, and feed intake of *C. carpio* fed with control and three different fisheries waste diets. However, the digestive enzyme activity and muscle biochemical compositions were significantly ($p < 0.05$) altered in *F. indicus* waste meal fed *C. carpio* compared to other fisheries waste meal and control diets fed fish groups. Therefore, the present study suggests that *R. kanagurta*, *S. barracuda*, and *F. indicus* waste meals can be considered as alternative feed ingredients for fish meal to formulate low-cost feeds for *C. carpio* culture.

Keywords Fish meal · *Cyprinus carpio* · Proximate composition · Feed · Fisheries waste · Digestive enzymes

Abbreviations

FWM	Fisheries waste(s) meals(s)
WM	Waste(s) meal(s)
WMD	Waste(s) meal(s) diet (s)

Introduction

Fish and fish products have presently emerged as the largest group in agricultural exports in the world. According to the FAO (2016), India has produced 4.9 million metric tons of aquaculture products during 2014. Among the different aquaculture practices, the culture of freshwater organisms including grass carp (*Ctenopharyngodon idellus*), common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*), catla (*Catla catla*), Scampi (*Macrobrachium rosenbergii*), and monsoon river prawn (*Macrobrachium malcolmsonii*) has been playing significant role in global food production. Among these freshwater organisms, *C. carpio* is considered as a very important cultivable species in Asian and European countries due to the presence of essential nutrients like protein, essential amino acids, fatty acids, lipid, vitamins, and minerals for mankind. Next to grass carp and silver carp, *C. carpio* ranked as the third position in world total finfish production which contributed about 9% of the global finfish production (FAO 2012).

Artificial feeds play a significant role in aquaculture operations which contains all essential nutrients like protein, amino acids, carbohydrate, lipid, fatty acids, vitamins, and minerals for farming species. However, the fish feed is one of the major cost components in aquaculture operations which reaches about 50–60% of total production cost (Adikari et al. 2017). Among the different types of feed ingredients, fish meal is a primary feed ingredient in commercial feed formulations which contains a chief source of protein, essential amino acids, lipids, vitamins, and minerals. However, the

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cost of fish meal is increasing continuously due to over-exploitations and unpredictable availability in nature. Hence, it is a necessity to find out alternate feed ingredient for replacing fish meal with low-cost animal protein sources (Forster et al. 2003; Lunger et al. 2007; FAO 2010).

Waste from fish-processing industries is an important environmental contamination source. Fish heads, viscera (gut) gills, skin, and fins are discarded as fish waste. Dumping of this waste creates both disposal and pollution problems to the environment (Norziah et al. 2009). About 50% of waste materials from the total fish capture is not used as a food source and involves almost 32 million tonnes of waste per year in the world (Kristinsson and Rasco 2000). India alone generates greater than 2 million metric tonnes of fisheries by-products per year without recovery of any useful product (Nurdiyana and Mazlina 2009). This processing discards the major valuable components like nutrients, pigments, and enzymes (Sachindra et al. 2006). The utilization of fisheries wastes for the production of various value-added products, such as proteins, fatty acids, fish oils, amino acids, vitamins, minerals, enzymes, bioactive peptides, etc., is becoming a significant industry process which leads to reducing the waste and help to eliminate harmful effects on the environment (Fuchise et al. 2009; Gumisiriza et al. 2009; Esposito et al. 2010; Mathew 2010; Ghaly et al. 2013). Hence, the present study was aimed to assess the proximate composition and minerals including trace elements of marine fish waste meals, such as *R. kanagurta*, *S. barracuda*, and *F. indicus*, followed by evaluating the effect of complete replacement of fish meal with these fisheries waste meals on the survival, growth, digestive enzyme activities, such as protease, amylase and lipase, and muscle biochemical constituents, such as protein, amino acids, carbohydrate, and lipid of the freshwater fish *C. carpio* fingerlings as alternative feed ingredient for fish meal to formulate for low-cost fish feed.

Materials and methods

Collection and processing of fisheries waste

The wastes including head, skin, viscera, fin, and tail of fish *Rastrelliger kanagurta* (Indian mackerel) and *Sphyraena barracuda* (Great barracuda), and head, legs and shells of shrimp, *Fenneropenaeus indicus* (Indian white shrimp) were collected from Ukkadam fish market in Coimbatore, Tamil Nadu, India. The waste materials of each species packed in separate polythene bags and transported to the laboratory. The collected fisheries wastes were sun-dried at 28.64 ± 1.77 °C for 6 h per day for 2 weeks. During the drying process, fisheries wastes were covered with mosquito nets to protect from flies, insects, birds etc. Further, these dried waste materials were powered finely using mixer grinder and stored individually in airtight plastic containers at − 20 °C until further analysis.

Analysis of proximate composition of fisheries waste

The proximate composition of each fisheries waste meal (FWM) including crude protein, lipid, fiber, nitrogen-free extracts (NFE), ash, moisture, and energy levels was estimated according to standard procedures (Table 1). The amounts of crude protein, lipid, fiber, moisture, and ash contents were analyzed according to standard procedures of AOAC (1995). The content of nitrogen-free extracts (NFE) was calculated using the formula [NFE (%) = 100 − (moisture + crude protein + crude lipid + total ash + crude fiber)] prescribed by Natarajan (2006). The level of gross energy was calculated by multiplying generalized physiological energy values of proteins (19 kJ), NFE (15 kJ), and lipids (36 kJ) with the energy levels contributed by the crude protein, NFE, and crude lipid fractions of FWM [energy contributed by crude protein (kJ/g) = protein content of feed (%) × 19 = x; energy contributed by NFE (kJ/g) = NFE content of feed (%) × 15 = z; energy contributed by crude lipid (kJ/g) = lipid content of feed (%) × 36 = y; gross energy (GE kJ/g) = (x + y + z)/100] (Natarajan 2006).

Analysis of minerals and trace elements

Content of trace elements (Zn, Cu, Cd, and Pb) and other mineral salts (Na, K, and Mg) of FWM was analyzed according to AOAC (1995) standard procedures (Table 2). Briefly, 1 g of each FWM was digested separately using 10-mL 9:2:1 ratio of HNO_3, H_2SO_4, and $HClO_4$ in a hot plate at 80 °C. Further, the volume was made up to 25 mL, followed by filtered using Whatman filter paper No. 45. The trace elements and other minerals were analyzed using atomic absorption spectrometry (Perkin-Elmer; Model 2380) and results were expressed as mg/kg.

Table 1 Proximate composition of collected fisheries waste

Composition (%)	*R. kanagurta*	*S. barracuda*	*F. indicus*
Protein	38.00 ± 1.20^{b}	37.52 ± 1.41^{b}	41.56 ± 1.84^{a}
Carbohydrate	15.67 ± 1.0^{a}	3.59 ± 0.20^{c}	9.02 ± 1.05^{b}
Fiber	1.0 ± 0.21^{b}	1.0 ± 0.20^{b}	11.37 ± 1.21^{a}
Lipid	8.17 ± 0.84^{b}	18.86 ± 1.42^{a}	4.55 ± 0.71^{c}
Ash	31.29 ± 1.17^{b}	33.83 ± 1.71^{a}	25.19 ± 1.54^{c}
Moisture	5.88 ± 0.57^{b}	5.20 ± 0.49^{b}	8.87 ± 0.72^{a}
Gross energy (kJ/g)	12.69 ± 1.40^{b}	16.81 ± 1.42^{a}	12.27 ± 1.51^{b}

$n = 3$ (three samples from each treatment); mean ± SD; mean values within the same row sharing the same superscript are not significantly different ($p > 0.05$)

Table 2 Contents of mineral salts and trace elements in the collected fisheries waste

Minerals/elements (mg/kg)	*R. kanagurta*	*S. barracuda*	*F. indicus*
Na	93.94 ± 1.24[a]	76.17 ± 1.58[b]	76.74 ± 1.45[b]
K	17.50 ± 1.18[a]	16.88 ± 1.50[b]	16.08 ± 1.47[b]
Mg	10.08 ± 1.20[a]	10.04 ± 1.10[b]	10.04 ± 1.01[b]
Zn	50.0 ± 1.0[a]	49.62 ± 0.64[a]	43.66 ± 0.95[b]
Cu	4.64 ± 0.1[b]	5.00 ± 0.18[b]	27.99 ± 1.0[a]
Cd	0.97 ± 0.01[a]	0.28 ± 0.01[b]	0.25 ± 0.01[b]
Pb	1.39 ± 0.09[a]	1.28 ± 0.47[a]	1.45 ± 0.70[a]

$n = 3$ (three samples from each treatment); mean ± SD; mean values within the same row sharing the same superscript are not significantly different ($p > 0.05$)

Preparation of fishmeal replacement diets

Experimental feeds were prepared using commonly available feed ingredients (fish meal, soybean meal, groundnut oil cake, wheat bran, and tapioca flour) in the local markets. Briefly, fish meal, soybean meal, and groundnut oil cake were separately grounded using a mixer grinder and sieved through a 60-μm mesh. Each ingredient was weighed at desired concentrations (Table 3) to formulate 37% protein diets which is the optimum requirement for culturing of *C. carpio*. These weighed feed ingredients were thoroughly mixed at different ratios for preparing four different diets (one control fish meal diet and three 100% fish meal replacement diets (diet 1, diet 2, and diet 3 fish meal replaced with *R. kanagurta*, *S. barracuda*, and *F. indicus* waste meal, respectively). These blends were cooked in a closed aluminum container at 105 °C for 15 min, followed by cooling at room temperature. Further, Cod liver oil, vitamins, and egg albumin were added and thoroughly mixed for obtaining a stiff dough. The dough was pelletized by an indigenous hand pelletizer with the mesh size of 0.1 mm diameter (Pigeon manufactures, Kolkata, India) and was cut into 3.0 ± 0.18-mm-sized pieces. The pellets were dried at room temperature (27 °C) until constant weight was reached. The prepared feeds was stored individually in airtight plastic containers at − 20 °C until to use the feeding trials. The proximate composition of the prepared feeds was analyzed according to the standard methods (AOAC 1995; Natarajan 2006) (Table 4).

Collection and acclimatization of experimental fishes

Fingerlings *C. carpio* (1.12 ± 0.2 cm and 0.30 ± 0.01 g length and weight, respectively) were collected from the Bhavanisagar dam in Erode District, Tamil Nadu, India. Fingerlings were safely transported to the laboratory using plastic polythene bags half filled with oxygenated dam water and acclimatized to ambient laboratory conditions for 1 week in plastic tubs (50 L) with tap water. The acclimatized water was analyzed for temperature (27 ± 1.04 °C), dissolved oxygen (7.50 ± 0.20 mg/L), pH (7.43 ± 0.21), total dissolved solids (0.61 ± 0.05 g/L), biological oxygen demand (18.40 ± 2.05 mg/L), chemical oxygen demand (67.0 ± 2.70 mg/L), and NH_3 (0.017 ± 0.001 mg/L) according to the standard methods of APHA (1995). During acclimatization period, adequate aeration was given to the fingerlings and they were fed with control feed prepared with basal feed ingredients twice (at 06:00 h and 18:00 h, respectively) per day, and about 60% of rearing tap water was renewed daily in order to maintain a healthy environment.

Feeding experiments

For this study, four groups of *C. carpio* fingerlings (1.88 ± 0.27 cm length and 0.34 ± 0.11 g weight) were assigned for this experiment in triplicate (three independent groups per diet) for a period of 6 weeks. One group was served as a control and fed the control feed (formulated with fish meal). The remaining three groups were fed with feeds formulated with respective FWM diets (diet 1, diet 2, and diet 3). The fish groups were separately maintained in a 20–L plastic aquarium with a stocking density of one fish/L. During the experimental period, about 60% of aquarium water was renewed every day by siphoning method with minimum disturbance to the fishes and the water quality parameters of each aquarium were maintained as same to acclimatization condition. Fish fingerlings were fed these experimental feeds at 5% of body weight (body weight was measured every 10-day interval for adjusting the feeding level) twice per day (at 06:00 h and 18:00 h). Fishes were maintained on a 12-h light/12 h dark photoperiod during the experimental period. The unfed feed and feces were removed by siphoning filtration during the feeding trial while renewing the rearing water.

Survival, growth, and food index analysis

The survival, growth performance, such as length gain (LG), weight gain (WG), and specific growth rate (SGR), and food index parameters, such as feed intake (FI) and feed conversion ratio (FCR), were calculated by the following equations:

$$\text{Survival}\ (\%) = \text{no.of live fish/no.of fish introduced} \times 100.$$

$$\text{LG (cm)} = \text{final length (cm)–initial length (cm)}.$$

$$\text{WG (g)} = \text{final weight (g)–initial weight (g)}.$$

$$\text{SGR}\ (\%/\text{day}) = {}_{log}\ \text{final weight (g)} - {}_{log}\ \text{initial weight (g)/total number of days} \times 100.$$

$$\text{FI (g/day)} = \text{feed eaten (g)/total number of days}.$$

$$\text{FCR} = \text{feed intake (g)/weight gain (g)}.$$

Estimation of digestive enzymes activity

Activities of digestive enzymes such as protease, amylase, and lipase were assayed in the fishes at the end of the experimental period. The whole intestine of fish from each feeding experiment was carefully dissected out and homogenized in ice-cold distilled water and centrifuged at 9300*g* under 4 °C for 20 min. The supernatant was used as a crude enzyme source. The activity of protease was analyzed by the casein-hydrolysis method of Furne et al. (2005). One unit of enzyme activity represents the amount of enzyme contributed to liberate 1 μg of tyrosine per min. Casein and L-tyrosine were used as substrate and standard, respectively. Amylase activity was determined by the starch-hydrolysis method (Bernfeld 1955). One unit of amylase activity was defined as the amount of enzyme that produced 1 μg of maltose per min. Starch and maltose were used as substrate and standard, respectively. Lipase activity was estimated by titration method (Furne et al. 2005). The activity of one unit of lipase was calculated as the volume of free fatty acids released from triacylglycerol per unit of time estimated by the amount of NaOH required for maintaining the constant pH and represented as milli equivalents of alkali consumed. Virgin Olive oil and lipase were used as substrate and standard, respectively. All these enzymes are expressed as specific activity (U/mg protein = enzyme activity/soluble protein (mg/mL). The protein content of crude enzyme extracts was analyzed according to the standard method (Lowry et al. 1951).

Table 3 Ingredients and proximate composition of experimental diets

Ingredients (g/100 g)	Control	Diet 1	Diet 2	Diet 3
Fish meal	25	0	0	0
Soybean meal	25	32	32	32
Groundnut oil cake	25	20	20	20
Wheat bran	10	8	8	8
Tapioca flour	5	5	5	5
Egg albumin	7	7	7	7
Sunflower oil	2	2	2	2
Vitamin mix[a]	1	1	1	1
Fisheries waste	0	25	25	25
Proximate composition (%)				
Protein	44.43	38.64	38.54	39.54
Carbohydrate	20.92	27.58	24.21	24.87
Fiber	7.24	7.84	7.84	10.43
Lipid	5.57	5.52	8.20	4.62
Ash	12.22	11.42	12.01	11.14
Moisture	9.62	9.0	9.20	9.40
Gross energy (kJ/g)	13.58	13.46	13.90	12.90

Control: formulated with fish meal; diet 1: fish meal replaced by *R. kanagurta* waste meal; diet 2: fish meal replaced by *S. barracuda* waste meal; diet 3: fish meal replaced by *F. indicus* waste meal

[a] Becosules capsules (manufactured by Pfizer), each capsule contains Thiamine Mononitrate IP 10 mg; Riboflavin IP 10 mg; Pyridoxine Hydrochloride IP 3 mg; Vitamin B12 (as tablets 1:100) IP 15 mcg Niacinamide IP 100 mg; Calcium pantothenate IP 50 mg; Folic acid IP 1.5 mg; Biotin USP 100 mcg; Ascorbic acid IP 150 m

Proximate composition analysis of fish

Concentrations of muscle total protein, total amino acid, and total carbohydrate contents of fish were analyzed according to the standard methods of Lowry et al. (1951), Moore and Stein (1948), and Roe (1955), respectively. The content of total lipid was extracted (Folch et al. 1957) and estimated according to the method of Barnes and Blackstock (1973). Ash and moisture contents of fish were determined by the standard procedures of AOAC (1995).

Statistical analysis

All the data were expressed as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) using SPSS (16.0) software to compare the significant ($p < 0.05$) differences among treatments. The data of survival, specific growth rate, ash, and moisture were arcsine transformed prior to one-way analysis.

Results

Proximate composition of collected fisheries waste

In the present study, protein and fiber contents were significantly ($p < 0.05$) higher in *F. indicus* waste meal (WM) when compared to *R. kanagurta* and *S. barracuda* WM. Whereas the NFE content was significantly ($p < 0.05$) higher in the *R. kanagurta* waste meal, followed by *F. indicus* and *S. barracuda* WM. In this context, the lipid content was significantly ($p < 0.05$) higher in *S. barracuda* WM when compared to the other WM. The ash content was significantly ($p < 0.05$) higher in *R. kanagurta* and *S. barracuda* WM rather than in *F. indicus* WM, however, an insignificant ($p > 0.05$) difference was noted in ash content between *R. kanagurta* and *S. barracuda* WM. The moisture content was significantly ($p < 0.05$) higher in *F. indicus* WM than in *R. kanagurta* and *S. barracuda*. Meanwhile, the gross energy level was significantly ($p < 0.05$) higher in *S. barracuda* and *F. indicus* WM when compared to the *R. kanagurta* WM, however, there was no significant ($p > 0.05$) difference noted in the energy level in *S. barracuda* and *F. indicus* WM (Table 1).

Table 4 Survival, growth, and food index of *C. carpio* fed with fish meal replacement feeds

Parameters		Control	Diet 1	Diet 2	Diet 3
Survival (%)		90.0 ± 5.00[a]	85.0 ± 5.00[a]	86.0 ± 5.70[a]	88.0 ± 2.88[a]
Feed intake (g/day)		0.36 ± 0.010[a]	0.35 ± 0.011[a]	0.33 ± 0.012[a]	0.34 ± 0.014[a]
Feed conversion ratio		1.84 ± 0.31[a]	1.48 ± 0.10[b]	1.52 ± 0.11[b]	1.45 ± 0.10[b]
Length (cm)[1]	Initial	1.88 ± 0.27	1.88 ± 0.27	1.88 ± 0.27	1.88 ± 0.27
	Final	3.56 ± 0.14[a]	3.62 ± 0.11[a]	3.82 ± 0.27[a]	3.60 ± 0.14[a]
Weight (g)[1]	Initial	0.34 ± 0.11	0.34 ± 0.11	0.34 ± 0.11	0.34 ± 0.11
	Final	0.80 ± 0.065[a]	0.90 ± 0.125[a]	0.91 ± 0.173[a]	0.90 ± 0.036[a]
Length gain (cm)[1]		1.68 ± 0.16[a]	1.74 ± 0.13[a]	1.94 ± 0.30[a]	1.72 ± 1.58[a]
Weight gain (g)[1]		0.46 ± 0.07[a]	0.57 ± 0.14[a]	0.53 ± 0.19[a]	0.56 ± 0.04[a]
Specific growth rate (% /day)		0.57 ± 0.03[a]	0.68 ± 0.04[a]	0.64 ± 0.02[a]	0.68 ± 0.03[a]

$n = 3$ (three samples from each treatment); mean ± SD; mean values within the same row sharing the same superscript are not significantly different ($p > 0.05$)

[1] $n = 5$ (five samples from each treatment)

Control: formulated with fish meal; diet 1: fish meal replaced by *R. kanagurta* waste meal; diet 2: fish meal replaced by *S. barracuda* waste meal; diet 3: fish meal replaced by *F. indicus* waste meal

Contents of mineral salts and trace elements

In the current study, the mineral salts, such as Na, K, and Mg, and trace elements, such as Zn and Cd contents, were significantly ($p < 0.05$) higher in *R. kanagurta* WM compared to *S. barracuda* and *F. indicus* WM, however, an insignificant ($p > 0.05$) difference was noted in these elements between *S. barracuda* and *F. indicus* WM. In this context, the content of Cu was significantly ($p < 0.05$) preeminent in *F. indicus* WM when compared to *R. kanagurta* and *S. barracuda* WM, whereas the difference between *R. kanagurta*, *S. barracuda*, and *F. indicus* WM was insignificant ($p > 0.05$) in the case of Pb content (Table 2).

Survival, growth, and food index

In this study, survival, length, weight, length gain, weight gain, feed intake, and specific growth rate were insignificantly ($p > 0.05$) increased in *C. carpio* fed with different types fish meal replacement diets (diet 1, diet 2, and diet 3) when compared to control fish meal diet, whereas the feed conservation ratio was significantly ($p < 0.05$) higher in the control diet-fed fish group when compared to *S. barracuda* and *F. indicus* waste meal diets (WMD) fed fish groups. However, an insignificant ($p > 0.05$) difference was noted in feed conversion ratio between control fish meal diet and *R. kanagurta* WMD fed fish groups (Table 4).

Activities of digestive enzymes

The activities of digestive enzymes, such as protease, amylase, and lipase, were significantly ($p < 0.05$) improved in fish fed with *F. indicus* WMD when compared to control and other fisheries WMD fed fishes, whereas an insignificant ($p > 0.05$) elevation was noted in protease and lipase activities among control, *R. kanagurta* and *S. barracuda* WMD fed fish groups. In context, the insignificant ($p > 0.05$) variation was seen in amylase activity between control and *R. kanagurta* WMD fed fish groups (Fig. 1).

Proximate composition of *C. carpio*

In the present study, the protein content was significantly ($p < 0.05$) higher in *C. carpio* fed with *F. indicus* waste meal diet, followed by *S. barracuda* WMD, control fish meal diet, and *R. kanagurta* WMD, while the amino acid and lipid contents were significantly ($p < 0.05$) increased in *C. carpio* fed on *F. indicus* WMD when compared to control fish meal diet and other fish meal replacement diets fed fishes, however, level of these biochemical constituents between control and *S. barracuda* WMD fed fishes was statistically insignificant ($p > 0.05$), while the carbohydrate content was significantly ($p < 0.05$) higher in *F. indicus* and *S. barracuda* WMD fed fishes when compared to control and *R. kanagurta* WMD fed fish groups. The level of ash and moisture were insignificantly ($p > 0.05$) differed in control and fisheries WMD fed fish groups (Table 5).

Discussion

Aquaculture has been identified as one of the fastest food production sectors which are responsible for the supply of fish protein for human consumption, and currently, 50% of world's

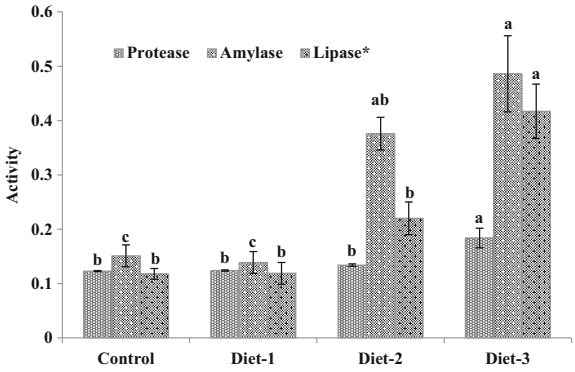

Fig. 1 Digestive enzymes activity (U/ mg protein) of *C. carpio* fed with control and fish meal replacement feeds. $n = 3$ (three samplings from each treatment); mean ± SD; Bars sharing the same alphabets are not significantly different ($p > 0.05$); *, $\times 10^2$. Control: formulated with fish meal; Diet-1: fish meal replaced by *R. kanagurta* waste meal; Diet-2: fish meal replaced by *S. barracuda* waste meal; Diet-3: fish meal replaced by *F. indicus* waste meal

fishes products are used as food (Ryder 2018). However, aquaculture industries have been facing serious issue due to the high operation cost. Fish feed is one of the major components in aquaculture operation which contributes about 60% of the total production cost (Adikari et al. 2017). Among the feed ingredients, fish meal plays a major role due to the presence of highly digestible protein, essential amino acids, fatty acids, vitamin, and minerals (Tacon and Akiyama 1997; Gatlin et al. 2007; Tacon and Metian 2008; Naylor et al. 2009). However, fish meal is expensive due to low or unpredictable availability (Maliwat et al. 2017). According to Amaya et al. (2007), fish meal is the primary and most expensive ingredient in commercial feed formulations which contributes about 50% of total feed ingredients. Hence, it needs to search for alternative protein sources to replace fish meal in aquafeed formulations. In regard to this, several studies have been reported earlier with various alternative feed ingredients (Ali et al. 1994; Ai et al. 2006; Cavalheiro et al. 2007; Lazzarotto et al. 2015; Lazzarotto et al. 2018; Kriton et al. 2018).

In the present study, a significant level of protein and fiber in the *F. indicus* WM indicates that the shrimp waste contains better source of protein and fiber rather than *R. kanagurta* and *S. barracuda* WM. The insignificant differences in the protein and fiber content between *R. kanagurta* and *S. barracuda* WM suggests that these two waste meals had about a similar level of protein and fiber contents. The NFE (carbohydrate) and lipid contents were significantly increased in *R. kanagurta* and *S. barracuda* WM, respectively; it indicates that these meals are good source of carbohydrate and lipids. The significant level of ash and energy contents in the selected WM indicated that these meals are the rich source of total mineral and energy levels. Similarly, significant amount of crude protein, lipid, carbohydrates, fiber, ash, and energy contents in the shrimp (*Penaeus* spp., *Pandalus borealis*, *Trachypenaeus curvirostris*, *Penaeus notialis*, *Penaeus duorarum*, *Parapenaeus longirostris*, and *Penaeus kerathurus*) WM and fish (*Ethmalosa fimbriata*, *Sardinella* sp., *Upeneus* sp., *Rastrelliger brachisoma*, *Terapon jarbua*, *Liza macrolepis, Siganus javus*, and *Leiognathus* sp.) WM have been reported (Ibrahim et al. 1999; Fanimo et al. 2000; Heu et al. 2003; Nwanna 2003; Sotolu 2009; Obasa et al. 2011; Esteban et al. 2007; Ramalingam et al. 2014). The significant decreases in the moisture content in *R. kanagurta* and *S. barracuda* suggest that these WM are rich source for nutrients.

In the present study, the significant elevations in minerals and trace elements, such as Na, K, Mg, and Zn in *R. kanagurta* WM, followed by *S. barracuda* and *F. indicus* WM indicate that these fisheries waste are good source essential minerals. Previously, the essential minerals, such as Ca, P, Na, K, Mg, Fe, Mn, Zn, and Cu contents in the shrimp *P. borealis* and *T. curvirostris* waste meal, and fish *E. fimbriata* and *Sardinella* sp., WM have been reported (Ibrahim et al. 1999; Esteban et al. 2007). The significant level of Ca and P contents has also been reported in shrimps (*P. notialis*, *P. duorarum*, *P. longirostris*, and *P. kerathurus*) head silage meal (Nwanna 2003). In this study, the detection of trace level of heavy

Table 5 Proximate composition of *C. carpio* fed with fish meal replacement feeds

Parameters	Control	Diet 1	Diet 2	Diet 3
Protein (mg/g)	164.14 ± 0.86^{c}	108.08 ± 2.88^{d}	178.68 ± 2.27^{b}	182.62 ± 0.76^{a}
Amino acid (mg/g)	112.87 ± 1.84^{b}	86.752 ± 2.25^{c}	116.32 ± 2.25^{b}	123.72 ± 2.25^{a}
Carbohydrate (mg/g)	68.46 ± 0.80^{c}	68.05 ± 1.38^{c}	76.94 ± 3.61^{b}	96.42 ± 2.97^{a}
Lipid (mg/g)	35.15 ± 1.03^{b}	22.47 ± 0.90^{c}	35.34 ± 0.51^{b}	37.43 ± 0.76^{a}
Ash (%)	8.69 ± 1.20^{a}	8.54 ± 1.45^{a}	8.34 ± 1.81^{a}	9.52 ± 1.31^{a}
Moisture (%)	77.0 ± 2.0^{a}	77.0 ± 1.9^{a}	78.0 ± 2.412^{a}	79.0 ± 2.04^{a}

$n = 3$ (three samples from each treatment); mean ± SD; mean values within the same row sharing the same superscript are not significantly different ($p > 0.05$)

Control: formulated with fish meal; diet 1: fish meal replaced by *R. kanagurta* waste meal, diet 2: fish meal replaced by *S. barracuda* waste meal; diet 3: fish meal replaced by *F. indicus* waste meal

metals, such as Cd and Pb, indicated that the selected FM are nontoxic in the heavy metal view.

In aquaculture industries, assessment of survival, growth, and quality of feeds are considered as an important parameter which affects the economy of the cultivable organisms like fish and crustaceans. In the current study, the insignificant elevations in survival, length, length gain, weight, weight gain, feed intake, and specific growth rate of *C. carpio* fed with control (fish meal diet) and all three FWM (*R. kanagurta*, *S. barracuda*, and *F. indicus*) suggested that these selected FW performed on par to fish meal diet. The significant decreases in feed conversion ratio in *S. barracuda* and *F. indicus* WM fed fish group suggest the better quality of these feeds. The insignificant elevation in survival, weight gain, feed intake, specific growth rate, and feed conversion ratio has been reported in the catfish, *Clarias gariepinus* fed to fish meal and FWM (*E. fimbriata* and *Sardinella* sp.) diets (Sotolu 2009; Obasa et al. 2011). Lu and Ku (2013) reported that partial replacement of fish meal by shrimp WM showed an insignificant difference in survival and significant elevation in weight gain, feed efficiency, and protein efficiency ratio in juvenile cobia, *Rachycentron canadum*. Effects of replacement of fish meal by fish silage meal on the growth and nutrient efficiency of red tilapia *Oreochromis mossambicus*, *Oreochromis niloticus*, and *Oreochromis aureus* have also been reported earlier (Madage et al. 2015).

In this present investigation, significant elevations in the activity of digestive enzymes, such as protease, amylase, and lipase of *C. carpio* fed with *F. indicus* WMD, indicate that this shrimp meal has the ability to promote digestive enzyme secretion in the experimental fishes which led to better feed intake, survival, and growth performance. In context, an insignificant difference in the digestive enzymes activity among control (fish meal) diet, *R. kanagurta* and *S. barracuda* WMD fed fish groups indicated that these selected FWM performed on par to each other. Previously, the impacts of dietary fish silage on digestive enzymes (protease, amylase, and lipase) activity of *Labeo rohita* has been reported (Haider et al. 2017). The digestive tracts of *L. rohita* fingerlings produced a significant level of digestive enzyme activities, such as protease, amylase, lipase, cellulase, maltase, and invertase when fed on prawn head meal diet has also been reported (Sethuramalingam and Haniffa 2002).

Body chemical composition is a good indicator of the physiological status of an organism. The nutrients in edible organisms depend upon their proximate composition, such as protein, amino acid, carbohydrate, lipid, ash, and moisture levels (Vijayavel and Balasubramanian 2006). In this study, the significant improvement in muscle protein, amino acids, and carbohydrate contents of *C. carpio* fed with *F. indicus* and *S. barracuda* WMD showed that these waste meals had potent to improve the protein content in the experimental fishes due to the presence of considerable level of crude protein and carbohydrate in their by-products. Also, the insignificant variations in the muscle lipid content of *C. carpio* fed with the control diet, *F. indicus* and *S. barracuda* suggested that these FW influenced the lipid utilization in experimental fishes due to the presence of adequate amount of crude lipid in their meal. Further, in this study, the insignificant elevations of ash and moisture content in the fishes fed with control fish meal diet and all three fisheries WMD indicated that all these diets had better level of total minerals and other nutrients content. Similarly, *C. gariepinus* fed with FWM (*E. fimbriata* and *Sardinella* sp.) diets showed a significant elevation in protein, lipid, and ash content have been reported (Sotolu 2009; Obasa et al. 2011). The insignificant difference in protein, lipid, and ash contents of the fish, *C. carpio*, *R. canadum*, and *Heterobranchus longifilis* fed on fish meal, shrimp and crab WMD has also been reported earlier (Lu and Ku 2013; Keremah 2013; Ramasubburayan et al. 2013).

Results from the present study indicated that the FWM, such as *R. kanagurta*, *S. barracuda*, and *F. indicus*, are the good source of proximate composition and mineral contents. Feeding experiment revealed insignificant differences in survival, growth, feed intake, specific growth rate biochemical constituents and activity of digestive enzymes of *C. carpio* fed with control fish meal diet and fisheries WMD. Therefore, the present study suggests that the *R. kanagurta*, *S. barracuda*, and *F. indicus* WM can be considered as alternative feed ingredients for fish meal to produce cost-effective feeds for the culture of freshwater fish *C. carpio*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The manuscript does not contain clinical studies or patient data.

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(Sponsored by RUSA 2.0 Scheme, Alagappa University, Karaikudi)

24th & 25th July, 2019

Venue : 4th Floor, Conference Hall, Science Campus

Certificate

This is to certify that

Prof./Dr./Mr./Mrs./Ms Rasid Hamid Thangal, Research Scholar (Oral Presentation)
Dept of Zoology, Bharathiar University

has successfully participated as Delegate / Delivered an Invited Lecture / Presented a Poster in the 2nd International Conference on **"Molecular Physiology, Therapeutics and Experimental Medicine (MP-TEM 2019)"** *organized by the* **Department of Animal Health and Management, Alagappa University, Karaikudi, India** *during 24th and 25th July, 2019.*

Dr. JONG MING THONG
Universiti Sains Malaysia
Malaysia

Dr. P. SRINIVASAN
Organizing Secretary
MP-TEM 2019

Dr. B. VASEEHARAN
Convener
MP-TEM 2019

National Conference on Biology and Medicine (NCBM'19)

07th - 08th February, 2019

Jointly Organized by

Department of Zoology
&
Human Genetics and Molecular Biology
Bharathiar University
Coimbatore - 641 046, Tamil Nadu, India.

NCBM'19

Certificate of Appreciation

This is to certify that S. H. Thangal, Ph. D Research Scholar Dept. of Zoology, Bharathiar University, has participated / presented (Oral / Poster) / served as Chair Person/ Co-Chair/ Judge in the **National Conference on Biology and Medicine (NCBM'19)** held during 07th - 08th February 2019 organized jointly by the Department of Zoology & Human Genetics and Molecular Biology, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India.

Dr. P. Vinayaga Moorthi	Dr. T. Muralisankar	Dr. M. Ramesh	Dr. A. Vijaya Anand	Dr. B. Vanitha	Dr. N. Jeyakumar
Organizing Secretary	Organizing Secretary	Convener	Convener	Registrar i/c	Member, VC Committee

DEPARTMENT OF ZOOLOGY
BHARATHIAR UNIVERSITY, COIMBATORE – 641046

International Conference on

RECENT BIOTECHNOLOGICAL INNOVATION IN AQUACULTURE (RBIA)

(LIVE AQUA 2020)

Certificate

This is to certify that ~~Dr.~~ / Mr. / ~~Mrs.~~ / ~~Ms.~~ P. SAID HAMID THANGAL, RESEARCH SCHOLAR, DEPT. OF ZOOLOGY, BHARATHIAR UNIVERSITY has participated / presented a paper in the International Conference on "Recent Biotechnological Innovation in Aquaculture" organized by Department of Zoology, Bharathiar University, Coimbatore in association with ICAR - National Bureau of Fish Genetic Resources Lucknow, held during 27th - 28th February, 2020.

Dr. V. Ramasubramanian Organizing Secretary Department of Zoology	Dr. K. Murugan Registrar I/c Bharathiar University	Dr. T. T. Ajith Kumar Organizing Secretary ICAR - NBFGR	Dr. Kuldeep K. Lal Director ICAR - NBFGR	Prof. Dr. P. Kaliraj Vice - Chancellor Bharathiar University