1. Introduction

“Ocean acidification” refers to reducing the ocean pH from any starting point to any end point in the pH scale due to high level atmospheric CO2 mixing with seawater and subsequent change in the seawater carbonate system (IPCC, 2011). In pre-industrialisation time, the level of atmospheric CO2 was 280 ppm whereas the current level of atmospheric CO2 is >400 ppm; this has resulted in a reduction of ocean pH from 8.25 to 8.14 (Orr et al., 2005; Iglesias-Rodriguez et al., 2010). If the “business as usual scenario” continues, the excess CO2 emission will reduce pH up to 0.5 units by the end of this century (Hall-Spencer et al., 2008).

Marine calcifiers are sensitive to ocean acidification and are affected by two possible routes of action (Yang et al., 2016). One is the seawater acidiﬁcation makes corrosion or deformation of the animal shell due to prolonged exposure and another one is by the demand of carbonate ions in seawater which is the essential source for shell formation. The latter is due to hydrogen ions dissociation and its incorporation with existing carbonate ions followed by transformation into bicarbonate ions. As a result, abundant level of bicarbonate is deposited in seawater. Here, shelled animals can only imbibe carbonate ions from seawater to build its outer skeleton shell; the process called calcification or biomineralization and cannot uptake bicarbonate forms instead of carbonate. Thus, increasing level of CO2 lowers the calcium carbonate saturation level hence it is less favourable to the precipitation of calcium carbonate shells and more favourable for the dissolution of skeletons (Zeebe, 2012). Reduction of calcification rate has been observed in variety of taxonomic groups especially in corals and molluscs (Michaelidis et al., 2005; Kuffner et al., 2008). Calcification rate in shelled organisms is varied by the level of CaCO3 saturation states hence the construction of CaCO3 is to be proportional to their saturation level (Ilyina et al., 2009). In addition, the external acid-base inﬂuence of seawater causes the animals to alter the regular metabolism, mainly because of ionic fluctuations.

Ocean acidification has a profound impact on the size and weight of shells and skeletons of marine calcifiers (Spicer et al., 2007; Watson et al., 2012; Duquette et al., 2017; Qu et al., 2017; Coll-Llado 2018). Sea urchins are marine calcifiers which play an important ecological role as major grazers in the marine ecosystem, as well as being an experimental model organism for developmental biologists. They directly absorb most of the essential nutrients they require from the seawater in which they live (Vidavsky et al., 2014), hence it is believed that these organisms are...
the first responders to any chemical imbalance in the acidified waters. Sea urchin larvae exposed to low pH has negatively influenced the digestive efficiency of the organisms (Stumpf et al., 2012). High CO2 induced ocean acidification affect the sea urchin's fertilization, larval growth and physiology, juvenile and adult growth and reproduction (Emerson et al., 2017; Brothers et al., 2015; Bray et al., 2014; Ross et al., 2011). Sewell et al. (2014) reported that the fertilization success of sea urchin would significantly get affected as consequence of ocean acidification phenomenon for the next fifty years. In such case, ocean acidification phenomenon could lead to cascading effects in the complex marine food web.

Sea urchin \textit{Salmacis virgulata} belong to the phylum Echinodermata is the most common species distributed in the near shore marine ecosystem of the Gulf of Mannar waters, South East Coast of India where they play an important ecological role in shallow subtidal region as grazers of marine algae. The Gulf of Mannar is a large shallow bay forming part of the Laccadive Sea in the Indian Ocean. The Gulf is noted for religious tourism (Kathiravan et al., 2017), trawl net operations (AnalaShajeeva et al., 2017) and Tucitcin sea port traffic posing a major threat of anthropogenic CO2 emission and ocean acidification. The mean annual rainfall of the region ranged from 762 mm to 1270 mm (Neelakantan, 1998) and pH from 7.0 to 8.3 (Anandakumar and Thajuddin 2013) respectively. pH of Gulf of Mannar waters remains alkaline between 7.7 and 8.5 (Arumugam et al., 2013; Sivakumar, 2009) due to various factors like photosynthesis, respiration, decomposition and freshwater inflow (Rajasagar, 2003). In this scenario of high spatiotemporal variation of pH and the “business as usual” condition of anthropogenic source of CO2 input, there is no concrete data on the impact of ocean acidification on calcifiers of the Gulf of Mannar region.

The present study tests the hypothesis that the reduced ocean pH and CaCO3 availability will adversely affect the biomineralization process of shell formation by direct physiological stress. Ocean acidification can induce oxidative stress due to change in the acid-base regulation and process of reactive oxygen species. Hence, the increasing anthropogenic CO2 emission and OA is a testament to weaken the physiology of sea urchin. The profound impact of ocean acidification in the sea urchin, \textit{S. virgulata} was studied by choosing four different pH levels that depict the ambient 8.2 in Gulf of Mannar waters (Arumugam et al., 2013) and elevated pCO2 levels using CO2 gas bubbling method (Riebsell et al., 2010) in microcosm setup that represent future ocean acidification conditions such as pH 8.0, 7.8 and 7.6 (Clark et al., 2009; Martin et al., 2011). Here, the term microcosm refers to the setup of miniature environment in terms of glass bowls or part of ex situ environment for studying the effects on selected number of samples. So far, large number of studies have started to focus on the role of elevated pCO2 condition on biomineralization properties of marine calcifiers; however, the role of physiology especially biomarkers have been poorly studied hence we assessed the biomarker enzyme responses as well as the ovarian cell condition to ambient pH and reduced pH conditions.  

2. Materials and methods

2.1. Animal collection and acclimatization

Sea urchins (\textit{S. virgulata}) with a test diameter between 7.2 cm and 8.7 cm were collected from Keelakarai coastal waters (9°06’38.2”N 78°50’02.3’’E) of the Gulf of Mannar region, Southeast coast of India, in a depth of approximately five meters by SCUBA diving. From the collection site, the animals were transferred to Pudumadam Field Research lab using refrigerated van covering a distance of 35 km in one and half hours. The animals were transferred into seawater tanks (50 L) with an aerator, and acclimatized for seven days. Here Acclimation refers the animal accustomed to a new climate or to new conditions whereas acclimatization refers to the process in which an individual organism adjusts to a change in its environment, allowing it to maintain performance across a range of environmental conditions. Apart from control, all animals were acclimatized with the respective low pH seawater. Seawater was renewed twice per day. For first three days of acclimation, filtered seawater collected from sea urchin sampling site was used. For another four days of acclimatization, the seawater collected from near shore (approximately 300 m distance) to Marine field research lab at Pudumadam coast, Gulf of Mannar region was used for subsequent water renewal. Animals were fed with seaweeds (\textit{Sargassum} spp) during acclimatization.

2.2. Experimental setup

CO2 pertubated microcosm setup demonstrated by Riebsell et al. (2010) was modified and adapted for the laboratory experiment (Figure 1). Four different pH levels were selected based on the prediction represented by Gattuso and Lavigne (2009) and Wolf-Gladrow et al. (2007) using R software - Searcarb package that reflect an ambient level (pH 8.2) and three elevated pCO2 scenario levels of ocean acidification (pH 8.0, 7.8 and 7.6). For CO2 gas bubbling, the CO2 gas cylinder was connected to three reservoir tank containing filtered seawater each 10 L prepared from sand filtration then the CO2 was released slowly to the extent to reach the desired seawater pH (7.6, 7.8, and 8.0). CO2 perturbation was not carried out in fourth reservoir tank which was the control (pH 8.2). Continuously, from these reservoir tanks, CO2 pertubated seawater and ambient seawater was opened to four open cell glass bowls (each 7 L) and the same setup was duplicated. Three acclimatized healthy sea urchins (average test diameter size of 8.5 cm) were introduced into each experimental glass bowls and replicative glass bowls with CO2 treatments. Feeding was stopped on last day morning of acclimatization. After 24 h, the experiment was started. Regular pH monitoring using pH meter (accuracy ±0.001 pH) was done in each experimental chamber every 4 h in day and night. During slight deviation in pH, CO2 pertubated seawater was opened from reservoir tank to recompense for maintaining the desired pH. Seawater was siphoned and renewed twice per day. The experiment was run for 14 days.

2.3. Physicochemical parameters

For every 4 h, the pH, temperature, salinity, and total alkalinity of seawater were measured during the experiment. Among these, pH and temperature both was observed using pH ion meter (Eutech bench pH/Ion meter Model pH 2100). Salinity was checked using hand refractometer (Atago Master-S). Total alkalinity was measured by volumetric method using phenolphthalein and bromocresol green indicators and H2SO4 as acid titrant after poisoning with mercuric chloride (APHA, 2005). Among the observed parameters, pH, temperature, salinity and total alkalinity were given as input for CO2calc software (Version 1.0.3.) and the other associated seawater carbonate parameters such as ΩCa, ΩAr, HCO3, CO3, pCO2, and TCO2 were calculated. Two ml of hemolymph was extracted using syringe needle on the animal’s oral side and measured its pH on alternate days (once in 48 h) using the pH ion meter. Experiment was done at fourteenth day. Three sea urchins were randomly selected from each treatment and their shells, spines, digestive tissues and ovary were dissected out and processed for further analysis.

2.4. Biomineralization assays

The following assays were carried out to qualitatively analyze the calcium carbonate in \textit{S. virgulata} test contents to study the negative changes in response to different pH treatments. Shell powder samples of \textit{S. virgulata} were prepared by sunlight drying for 48 h then crushing and grinding of whole test using RIMi laboratory blender. Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out to get an infrared spectrum of \textit{S. virgulata} shell powder (control) at the wave number range between 400 and 4000 cm⁻¹ with the 2 cm resolution of wave number detection using FTIR 8400S Shimadzu with Germanium coated KBr Plate. Thermogravimetric Analysis (TGA) was carried out to analyse mass
properties of the sea urchin shell powder for all pH treatments at a temperature range between 21 °C and 99 °C (Exstar 6000). X-Ray Diffraction Analysis (XRD) was carried out to determine the phase crystalline nature of the sea urchin shell powder with different pH conditions at the copper 2θ range of 10°–80° in a fixed time mode at room temperature (X’Pert Pro). Basal spines of sea urchin were examined under Scanned Electron Microscopy (SEM) analysis (Model: TESCAN VEGA3 SBH) operated at 5 kV and its number per area calculation were analyzed using ImageJ software.

2.5. Biomarker assays

Biomarkers have the important role in indication of physiological stress to the pH treated animals. After completion of 14th day experiment, *S. virgulata* gut tissues from different pH treatments were immediately ground with phosphate buffer solutions and these were centrifuged to obtain enzyme sources. These enzymes were used for biomarker assays namely Glutathione-S-Transferase (GST), Catalase (CAT), Acetylcholine Esterase (AChE), Lipid Peroxidase (LPx) and Reduced Glutathione (GSH) assays using centrifugation followed by spectrophotometer. Two samples from each treatment were assayed. These enzymes indicate the physiology related response against the oxidative stress due to changes in acid-base regulation. AChE activity was measured using the substrate analogue namely acetylthiocholine iodide which converted to thiocholine. Chemical reaction of thiocholine with dithionitrobenzoic acid (DTNB) - the chromogenic substrate makes the formation of nitrobenzoic acid - yellow anion that absorbs at 412 nm (Ellman et al., 1961). CAT activity was determined using Sinha (1972) method by observing decrease of H2O2 in absorbance at 570 nm and enzyme activity was expressed as H2O2 consumed/min/mg protein. GST activity was tested by spectrophotometer at 340nm by enumeration of CDNB (1-chloro-2, 4-dinitro-benzene) conjugated with reduced glutathione as a function of duration (Habig et al., 1974). LPx level was assayed by quantification of malondialdehyde (MDA), a decomposed product of PUFA (polyunsaturated fatty acids) and hydro peroxides were determined using thiobarbituric acid reaction method described by Ohkawa et al. (1979). MDA’s absorbance was read at 532 nm following the removal of fluctuated materials using centrifugation and it was expressed in nano moles malondialdehyde (MDA)/mg protein. GSH level was quantified according to Moron et al. (1979) method by measuring the reaction with 5, 5’dithiobis (2 nitro benzoic acid) DTNB to give the absorbance at 412 nm and the level was measured as μM of GSH mg−1 protein.

2.6. Histology sectioning

Female gonad tissues of *S. virgulata* (three numbers) from each treatment were dissected out for histology sectioning. Automated tissue processor was used for this test (Lang et al., 2006). The gonads were fixed in Bouin’s solution (a histological fixative – prepared using 15:5:1 ratio of saturated picric acid in 95% ethanol: formaldehyde: glacial acetic acid) for 24 h. Later, the gonads were processed by dehydration, cleared in xylene and finally embedded in paraffin wax. The block has been sectioned at 5 μm using a rotary microtome followed by deparaffination and washing. The samples were stained using haematoxylin and eosin. Five sections were selected and one and the one which looked clear tissue damage was used. Finally, the prepared slides were examined under the light microscope which can able to detect colour i.e., RGB images at resolution of 40X magnification.

2.7. Data analysis

The results of FTIR, XRD and TGA graphs were plotted with origin pro-8.1. Descriptive statistics was used for physicochemical parameters and One-way ANOVA was used for biomarker enzyme responses using Microsoft Excel 2007.
3. Results

3.1. Physicochemical parameters

The mean pH, temperature, salinity, total alkalinity in four different pH treated seawaters and the respective carbonate parameters such as $\Omega_{\text{Ca}}$, $\Omega_{\text{HCO}_3}$, $\text{CO}_3$, $\text{pCO}_2$, and $\text{TCO}_2$ were given in Table 1. These results represent the mean SD ($N = 80$ data collected for 14 days $\times$ 6 times per day) of microcosm observation experiment. There were no mortalities observed during the experiment. The real time extraction and observation of sea urchin haemolymph pH was 7.24 $\pm$ 0.08, 7.68 $\pm$ 0.05, 7.66 $\pm$ 0.07 and 7.94 $\pm$ 0.04 (mean with standard deviation) during all pH treatments.

3.2. Biomineralization

3.2.1. FTIR

The representative FTIR spectral analysis from chosen replicates studied with the wide range from 400 to 4000 cm$^{-1}$ of the S. virgulata shell powder exposed in three experimental pH and control showed distinct peak values (Figure 2). The peak at 713 cm$^{-1}$ represents the double degenerate planar bending (V4) in calcite phase of CaCO$_3$ in S. virgulata exposed to all four pH treatments. This is due to asymmetric bending mode of carbonate ions. The out of plane bending (V2) was displayed by a peak at 875 cm$^{-1}$ bending mode of carbonate ions. The peak at 1417 cm$^{-1}$ was found in pH 7.8 and 7.6. These characteristics could be derived from the symmetric bending mode of carbonate ions. The peak at 1417 cm$^{-1}$ showed the doubly degenerate planar bending (V3) in calcite structure for CaCO$_3$ in S. virgulata shell powder exposed to pH 8.2 (control) and pH 8.0 treatments whereas a similar V2 peak at 873 cm$^{-1}$ was found in pH 7.8 and 7.6. These characteristics could be derived from the symmetric bending mode of carbonate ions. The peak at 1417 cm$^{-1}$ showed the doubly degenerate planar bending (V3) in calcite structure for CaCO$_3$ in S. virgulata shell powder exposed to pH 8.2 (control) and pH 8.0 treatments whereas a similar V3 peak at 1415 and 1411 cm$^{-1}$ was found in pH 7.8 and 7.6 respectively. It represents the presence of aromatic groups C=C in fingerprint region. The peak 2521 cm$^{-1}$ indicated the N-H bonds represent ammonium ions. The peak range between 2926 and 2928 cm$^{-1}$ signs the change in methylene group with compare to control. The structure of bonds elucidated for the peaks between 3414 cm$^{-1}$ from pH 7.6 and 3421 cm$^{-1}$ from pH 8.2 enunciated the change in O-H stretching mode in S. virgulata test skeleton.

3.2.2. TGA

Thermal analysis of S. virgulata shell powder revealed a rapid calcium carbonate weight loss in pH 7.6 and 7.8 compared to the pH 8.0 and 8.2 (Figure 3). The initial weight loss occurred when the temperature was below 100 °C which denotes removal of moisture content. Further increase in temperature revealed subsequent weight loss of subsequent organic molecules. The weight loss of amorphous calcium carbonate in the test shell powder was significant between high and low CO$_2$ conditions. A narrow range of temperature difference was observed for I, II, III and IV weight loss for pH 7.6 at 235 °C, 250 °C, 598 °C and 601 °C and for pH 7.8 at 272 °C, 286 °C, 609 °C and 616 °C respectively. Similarly, for pH 8.0 I, II, III and IV weight loss were recorded at 417 °C, 435 °C, 668 °C and 674 °C whereas for pH 8.2 at 477 °C, 495 °C, 686 °C and 698 °C.

Table 1. Physicochemical parameters of seawater during the experiment.

| Parameters                  | pH 7.6     | pH 7.8     | pH 8       | Control (pH 8.2) |
|-----------------------------|------------|------------|------------|-----------------|
| pH                          | 7.63 ± 0.02| 7.81 ± 0.03| 8.00 ± 0.01| 8.26 ± 0.04     |
| Temp (°C)                   | 28.5 ± 0.54| 28.46 ± 0.54| 28.49 ± 0.55| 28.54 ± 0.54    |
| Sal                         | 30.69 ± 1.18| 30.32 ± 1.18| 30.23 ± 1.11| 30.28 ± 0.95    |
| TA (μmol kg$^{-1}$)         | 1942 ± 43  | 1978 ± 23  | 2066 ± 11  | 2148 ± 7.2      |
| pCO$_2$ (μatm)              | 2282.35 ± 92.99| 692.20 ± 70.25| 197.90 ± 11.00| 89.45 ± 18.33  |
| TCO$_2$ (μmol kg$^{-1}$)    | 1010.53 ± 22.52| 929.48 ± 3.30| 869.70 ± 8.57| 765.70 ± 32.11  |
| HCO$_3$ (μmol kg$^{-1}$)    | 940.06 ± 25.00| 879.13 ± 0.46| 777.78 ± 14.82| 634.29 ± 46.38  |
| CO$_3$ (μmol kg$^{-1}$)     | 11.47 ± 1.89| 31.88 ± 5.19| 86.64 ± 6.68| 129.029 ± 14.81 |
| $\Omega_{\text{Ca}}$        | 0.28 ± 0.04| 0.80 ± 0.02| 2.18 ± 0.14| 3.25 ± 0.34     |
| $\Omega_{\text{Ar}}$        | 0.19 ± 0.03| 0.52 ± 0.08| 1.43 ± 0.10| 2.14 ± 0.24     |
3.2.3. XRD

The XRD patterns of powdered *S. virgulata* shell under different pH exposures such as 7.6, 7.8, 8.0 and 8.2 (control) represented the structural properties of biomineral phases at 20 values from 10° to 80° (Figure 4). In all pH treatments, the 20 position showed peak 29.8° which is also known as the position of reflection d (104) which confirms the structure of calcite in all samples. The other peak positions also represented the calcite type of calcium carbonate. In low pH exposed shells the reduced calcite blooming which denotes the predominance of calcium carbonate, calcite and amorphous calcium carbonate (ACC). The calcite crystals exposed to all pH treatments. The given XRD patterns were assigned for calcite and amorphous calcium carbonate (ACC). The calcite crystals were available for 20 value at 23.1, 29.6°, 36.2°, and 39.8° and the ACC crystal were available at 28.0°, 31.7°, 46.4°, 57.0° and 71.1°. Compared to pH treated shell powders the control was predominantly composed of calcite type of calcium carbonate. In low pH exposed shells the reduced calcite peak was seen whereas in control (8.2) the peak distinctly showed the calcite blooming which denotes the predominance of calcium carbonate content.

3.3. SEM imaging

The SEM imaging of the basal spine region of *S. virgulata* exposed to all pH treatments were given in Figure 5 a-e. The basal spine surface found corroded with cracks and breaks in pH 7.6 treatments whereas no crack found in pH 8.2 and 8.0. The basal spine surface has different numbers of pores in each pH treatments and number per area has been calculated. Large number of pores found in pH 7.6 was 29 × 10^−2 /μm^2 whereas 19 × 10^−2 /μm^2 found in pH 7.8. Average number of pores found in pH 8.0 was 17 × 10^−2 /μm^2 whereas 13 × 10^−2 /μm^2 found in pH 8.2. These attributed that the calcite in *S. virgulata* spines exposed to low pH treatments lead to demineralization, dissolution and corrosion.

3.4. Biomarker assay

The enzymatic response of *S. virgulata* in control and pH treated conditions varied highly significant (p < 0.01) and their varying patterns ascertainment that the animals were being stressed. The ANOVA for each enzymatic response were given in Table 2. AChE and CAT (Figure 6 a and b) levels were significantly decreased in pH 8, 7.8 and 7.6 when compared with control. GST, LPx and reduced glutathione (Figure 6 c, d and e) levels were significantly increased in pH 8, 7.8 and 7.6 when compared to pH 8.2. AChE activity in hypercapnia condition with maximum decrease was observed at 0.043 μM ACTI/min 1/mg 1 protein at pH 7.6 compare to remaining pH treatments. The CAT activity also reduced at 0.032 mM H2O2 consumed/min 1/mg 1 protein in pH 7.6 whereas maximum activity observed at 0.061 mM H2O2 consumed/ min 1/mg 1 protein in pH 8.2. GST had maximum activity at 0.078 μM GSH & CDNB conjugate formed min 1/mg 1 protein for pH 7.6 and 8.2 respectively. LPx showed higher response at 0.903 and lower response at 0.125 μM of GSH/min 1/mg 1 protein for pH 7.6 and 8.2 respectively. The R^2 value of AChE, CAT, GST, LPx and GSH were 0.416, 0.2652, 0.0061, 0.1044 and 0.4521 respectively. The regression/trend line of AChE, CAT, GST, LPx and GSH were polynomial, power, polynomial, exponential and logarithmic respectively.

3.5. Histopathology

The gonad sections of *S. virgulata* exposed to different pH exposures consist of ovaries with mature oocytes. The control animal possessed mature ovaries with vitellogenic oocytes and the treated animal of pH 8.0, 7.8, 7.6 showed lesions in oocytes (Figure 7 a-d). In addition, the animals from the pH 8.0 showed slight disruption in the density of egg whereas the pH of 7.8 and 7.6 showed the destruction of cell envelope and more disruption in the density of egg cell and globules of accessory cells intruding an oocyte. Particularly these cell envelopes are jelly coat of ovaries, which has polysaccharides with unique structures. These polysaccharides are very crucial for stimulating the acrosome reaction in species-specific manner. This event is commonly occurred during pre-fertilization stages in terms of binding of sperm and fusion with the egg. Cell disruption and rupture was visible in gonads exposed to pH 7.6 and pH 7.8. Gonad lesions were not found in ovarian cells exposed to control pH 8.2 whereas slight gonad lesions and damage in egg jelly were found in *S. virgulata* exposed to near future acidification pH 8.0.

4. Discussion

Marine calcifiers produce skeletal structures of carbonate in the form of aragonite and calcite (Kawahata et al., 2019). In Echinoderms, two forms of CaCO3 are available i.e., Mg-Calcite and Amorphous Calcium Carbonate (ACC) (Addadi et al., 2003). Amorphous calcium carbonate has important function in CaCO3 formation process as a response of transient precursor phase of calcite or aragonite. Six different forms of CaCO3 exist in nature namely ACC, calcite, aragonite, vaterite, mono-hydrocalcite and Ikaite. Interplay of one form of CaCO3 to other form reported in many animals. For instance, nacre shells purely made up of aragonite crystal and they can transform to hydroxyapatite by hydrothermal transformation in high pressure (Agagouliari, 2012). Sea urchins have complex skeletal structure; though inter-relation among amorphous calcium carbonate, calcite and intra-crystalline organics is not clear; the biomineralization process in sea urchin is well documented where the transient form of hydrated ACC dehydrates to form ACC and subsequently transformed to calcite (Wolpert and Gustafson 1961; Brecvice and Nielsen 1989; Clarkson et al., 1992; Beniash et al., 1997; Decker et al., 1987; Yutao Gong et al., 2012). Sea urchin test and spicules consist of calcite and magnesium (Drzozdov et al., 2016) that grow from a single calcite crystal seed by transformation of a transient ACC phase (Beniash et al., 1999). The possible influence of intra-crystalline organics in sea urchin on size and orientation of calcite crystals is evident from the studies conducted by Berman et al. (1988; 1990 and 1993) where the extract of the skeletal parts from *Paracentrotus lividus* revealed a conchoidal fractured surface for the calcite crystals grown in the presence of 4.

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Figure 4. X-ray diffractograms of different pH (8.0, 7.8 and 7.6) treated and control (8.2) *S. virgulata* shell powders. Calcite peak is shown with distinct symbols and the longest peak in pH 8.2 indicated the calcite blooming.
of acidic glycoprotein which is remarkably different from the exceptionally smooth cleavage surfaces of abiotic calcite.

FTIR vibrational bands of the present study confirmed the presence of CaCO₃ with sharp peak of in-plane carbonate bending (V₄) at 713 cm⁻¹ and out-of-plane bending (V₂) between 875 cm⁻¹ and 873 cm⁻¹ for calcite in all the treatment pH and control. The previous studies revealed comparable V₄ splitting characteristic of the CaCO₃ and their spectrum compared to control of the present study was in good agreement (Balmain et al., 1999; Lee et al., 2011; Weir and Lipponcott, 1961; Kamba et al., 2013). Further vibrational bands of 3429–3414 cm⁻¹ for V O–H (H₂O) and 1413–1411 cm⁻¹ asymmetric stretching (ν₃) of CO₃²⁻ trace the group change for calcite crystalline process (Lee et al., 2005; Gebauer et al., 2010). The low intense and broad absorption peak between 3425 and 3414 cm⁻¹ (O–H stretching) ascribe the presence of structural water within calcite samples. Similar absorption peak 3637–3000 cm⁻¹ was observed for crystallization of ACC (Cheng et al., 2019) and biogenic calcite spines 3400 cm⁻¹ (Radha et al., 2010). Published reports prove that ACC act as precursor for calcite and aragonite skeletal structures of marine calcifiers (Addadi et al., 2003; Weiner et al., 2003; Weiss et al., 2002; Beniash et al., 1997; Politi et al., 2004, 2006, 2008; Aizenberg et al., 1996, 2002; Killian et al., 2009). Even though all the recognizable spectra for calcite crystalline process is similar in control and pH

Figure 5. a) SEM image of basal surface of the spine of S. virgulata (arrow indicates the basal surface region) b) control (pH 8.2), c) pH 8.0, d) pH 7.8 and e) pH 7.6. The surface of spine looks weaker in low pH treated samples.

M. Anand et al. Heliyon 7 (2021) e05970
treatments; spectral band for O–H stretching in pH 8.0, 7.8 and 7.6 were typically broaden compared to control suggest a relative pH change influence on the crystal lattice of test shell.

Compared to pH 8.2 (control) and 8.0 (1413 cm⁻¹) the peak shift of C–O bond in 7.8 (1415 cm⁻¹) and 7.6 (1411 cm⁻¹) denote the disordered crystal lattice, similarly the shift in C–H bond vibration from 2928 cm⁻¹ (control) to 2926 cm⁻¹ (other pH treatments) confirm significant change in organic substances suggesting a variation in the crystal growth mechanism between animals exposed to high and low CO₂ conditions; in particular to ACC dehydration from ACC-H₂O to ACC and Calcite in a biologically controlled mechanism (Politi et al., 2008; Gong et al., 2012).

Similar to FTIR peak shift, the results of thermogravimetric curve of the test samples too revealed significant weight loss impression between the test shell exposed to high (pH 8.2 and 8.0) and low CO₂ conditions (pH 7.8 and 7.6). Observable variation in FTIR spectral vibration for O–H structural water in four different pH exposures is well in agreement with the initial water loss with endothermic event at below 100 °C and further second and third weight loss for subsequent organic molecules in the thermogravimetric analysis. In TGA, the weight loss impression might be by phase transformation of CaCO₃ form of calcite to unstable CaCO₃ form (Lingaraju et al., 2002). Since both the control and treated samples remain unchanged when the temperature reached 750 °C, the result is considered as the total decomposition of calcium carbonate leaving alone the ash (Kamba et al., 2013; Mohamed et al., 2012). The rapid weight loss in this study reveals the endothermic behaviour of calcium carbonate. All of the thermogram values in present study showed four step weight loss

Table 2. One-way ANOVA for the result of biomarker enzymes with different treated pH.

| Biomarker Enzymes       | Source of variation | SS     | Df | MS     | F      | P     |
|-------------------------|---------------------|--------|----|--------|--------|-------|
| Acetylcholine Esterase  | Between Groups      | 0.002598 | 3  | 0.000866 | 209.9697 | p < 0.001 |
|                         | Within Groups       | 1.65E-05 | 4  | 4.13E-06 |        |       |
| Catalase                | Between Groups      | 0.000805 | 3  | 0.000268 | 306.8095 | p < 0.001 |
|                         | Within Groups       | 3.5E-06  | 4  | 8.75E-07 |        |       |
| Glutathione S-Transferase| Between Groups     | 0.000913 | 3  | 0.000304 | 243.4667 | p < 0.001 |
|                         | Within Groups       | 5E-06   | 4  | 1.25E-06 |        |       |
| Lipid Peroxidase       | Between Groups      | 0.136762 | 3  | 0.045587 | 729.3973 | p < 0.001 |
|                         | Within Groups       | 0.00025 | 4  | 6.25E-05 |        |       |
| Reduced Glutathione    | Between Groups      | 0.047788 | 3  | 0.015929 | 6707.14  | p < 0.001 |
|                         | Within Groups       | 9.5E-06  | 4  | 2.38E-06 |        |       |

Figure 6. Physiological responses in gut tissues of S. virgulata at different pH exposure; a) AChE activity b) CAT activity c) GST activity d) LPx activity and e) GSH activity.
nature, water loss (may be either loose or bound) from the membrane stage, bulk or micro calcium carbonate, the phase transformation of stable calcite to unstable CaCO$_3$ form after the water molecules from the carbonate lattice and complete volatilization. Eventually pH 7.6 shows a higher range of weight loss compare to the other pH levels (pH, 7.8, 8.0 and 8.2 (control)), Ash was the final content of all samples. Such high sensitivity of weight loss may be connected to the weak O–H structural vibrations in the samples. Hence, TGA and FTIR results in this study enunciated the weakening of molecules in the dense CaCO$_3$ shell and their response to low pH (7.6 and 7.8) exposure.

XRD patterns of the present study reveal that the prevalence of calcite blooming in control samples compared to pH 7.6 and 7.8 (Wang et al., 2015). According to Goodwin (1969) and Amarowicz et al. (2012), sea urchin shells are mainly composed of calcium carbonate with the organic matrix. The 2θ value of 28.2°/C14 was not shown in pH 7.6 and 7.8 due to the characteristic changes in crystallinity of calcium carbonate shells. Calcium carbonate content had decreased in all low pH treatments, from pH 8.0 to 7.8 and 7.6. The combined results of FTIR, TGA and XRD revealed that a relative proportion of calcite in the shell was affected minimally to the near future CO$_2$ level scenario but mostly affected to high CO$_2$ level which substantially supports the theory that the reduced ocean pH and CaCO$_3$ availability will adversely affect the biomineralization process of sea urchin shell.

Morphology of sea urchin basal spine had clear and visible pores with corrosive surface. Similar results found on the portion of Aristotle’s lantern of sea urchin which showed the large porous structure and irregularly shaped holes as a prevailing sign of corrosion and structural breaks due to increasing pCO$_2$ up to 1000 μatm and feeding rate of calcifying and non-calcifying algae (Asnaghi et al., 2013). Same kind of shell damages found in the degradation of calcium carbonate skeleton by less uniform size and increased inner matrix pores of ossicle pores in A. liozula under high magnification (Bray et al., 2014). Previous study of the dried shell mass of juvenile oyster was 39% lower in hypercapnic condition in comparison to normocapnic condition (Beniash et al., 2010). Low pH treatment made the negative attribution in size and stability of shell due to low calcium carbonate saturation in seawater (Watson et al., 2012). However, this analysis clearly indicated that the strength of calcium carbonate of $S$. virgulata lost its intensity in low pH (pH 7.6 and 7.8) treatments. The present study confirms the negative impact of ocean acidification on biomineralization process of sea urchins by affecting highly soluble crystal forming calcified tissues is in consistent with observed results of other marine calcifiers nevertheless the degree of impact varies from species to species (Dupont et al., 2010; Gazeau et al., 2013; Yao and Somero, 2014; Stillman and Paganini, 2015).

Results of all the five biomarkers were similar to the mussels integrated biomarker response demonstrated by Damiens et al. (2007) and were highly significant (p < 0.01) with one-way ANOVA. The present study denotes a moderate elicitation level of decreasing AChE for the near future level of CO$_2$ (pH 8) whereas a significant high elicitation level of decreasing AChE in pH 7.6 & 7.8. Acetylcholine esterase is a glyco-protein (Godoy-Reyes et al., 2019) found in variety of chemical structure but their functions in all species are similar (Hodges et al., 2018). AChE breaks down ester bond of acetylcholine and segregate into acetate and choline. AChE plays an important role in perception of prey and to sense deterioration condition in natural environment (Dos Santos Miron et al., 2005). Reduction of AChE indicates abnormal neurotransmission activity it could affect the whole nervous system. Several studies on choline esterase activity were important biomarker for assessing neurotoxicity impact on invertebrates and vertebrates (Jebali et al., 2011). Reduced AChE activity was recorded when the mussels transplanted from the unpolluted reference site to contaminated site (Damiens et al., 2007). AChE activity of the present study is similar to the findings of Priya et al.
urchin (Moulin et al., 2011) in terms of reducing size and calcium carbonate skeletongenesis disruption (Kurihara, 2004, 2008).

According to Lu et al. (2013) AChE and CAT had similar direction for decreasing activity when exposed to polybrominated diphenyl ethers. CAT is an antioxidant enzyme which have protective mechanism against H2O2 so that it is considered as one of the powerful biomarker tools. In the present study, the CAT activity showed decreasing sign in low pH exposures revealed the hydrogen peroxide stress. Similar result was recorded by Seguin et al. (2017) where CAT, GST & LPx revealed significant effects in transplanted mussels at contaminated sites. GST, LPx and GSH levels were increased in the present study. In particular, GST the member of the glutathione families that act against oxidative stress; showed increasing activity (Pereira et al., 2010). The main role of GST is considered as a fast recovery biomarker and the main role is to detoxify the cellular toxicity. GSH and LPx is said to be a precise signal for oxidative stress parameters and undergo slow recovery for bringing normal condition of the organism therefore it plays a significant role in pollutant assessment. LPx is assumed that they can propagate cytotoxic products and leads to DNA damage. Generally, the increased LPx enzyme is considered as the response of more contaminant. According to Herdege et al. (2011), pH induced changes intricate and affects conformational changes in chemoreceptor function and it leads to behavioural changes like olfactory function in invertebrates and vertebrates. Thus, biomarker enzymes in the present study showed negative physiological response to the animals exposed to low pH treatment compared to control. Since these assays are important in environmental health monitoring, possible impacts of hypercapnia induced physiological effect can be identified (Lam, 2009).

Gonad development is the process which comprises the continuous progression for each stage to become intact and sexually competent. In general, the impact on genital organs has more possible to deteriorate their reproduction and development (Havenhand et al., 2008). The gonads of sea urchin in different pH treatments of the present study revealed that pH 7.6 and 7.8 exposure had more impact on genital organs and particularly, ovarian cells become abruptly damaged when compare to control. Similar gonad lesions were found at Schafer and Kohler (2009) on female sea urchin after polycyclic aromatic hydrocarbon (PAH) where the results agree well in regard to gonad lesion in which, the high amount of degenerating previtellogenic oocytes exhibiting when the animal was treated with phenanthrene. Therefore, it seems the possible consequence that it will interrupt the successful fertilization in sea urchin, if even fertilized, the detrimental morphology of larval sea urchin will interrupt the successful fertilization in sea urchin, if even fertilized, the detrimental morphology of larval sea urchin, if even fertilized, the detrimental morphology of larval sea urchin (Moulin et al., 2011) in terms of reducing size and calcium carbonate skeletonogenesis disruption (Kurihara, 2004, 2008).

Apart from the fertilization, lesions of sea urchin in many organs including gonads were characterized by Gilles and Pearse (1986), in which, close contact of diseased sea urchins with healthy individual led to infection. Consequently, the lesions progressed within 2 days for diseased urchin whereas in control sea urchin the tissue was regenerated in lesion area. Physiological response in the present study revealed a highly negative impact that could be the link between gonad lesion and fertilization deterioration. Bromhead et al. (2015) reported that the early stages of yellowfin tuna under extreme pCO2 levels (≥8800 μatm) showed significant reduction of larval survival.

The result of present study was in agreement to Frommel et al. (2016) report where the increasing damage in organs of yellowfin tuna showed decreased growth and endurance with high correlation. Giansuza et al. (2014) documented that the temperature plays the significant role in fertilization success, in addition, the development rate of pH effect was dependent upon the temperature and eventually the development was slower at higher temperature. The results of FTIR TGA XRD and SEM imaging substantiate the negative impact of high CO2 level exposures to sea urchin biomineralization process and the biomarker and histopathology findings bear strong evidence for the physiological stress experienced by S. virgulata was in accord to the findings on T. gratilla where the due impacts of low pH exposures alter the metabolism and energy demand of the animals in order to maintain the body acid-base balance (Stumpp et al., 2012; Collard et al., 2013; Dubois 2014).

5. Conclusion

The perspective of shell deformation, physiological response and gonad tissue damage in our study revealed ascertained manifestation when compared between control and treated animals. The IPCC predictions and on-going anthropogenic activity for CO2 emissions are alarming for the year 2100. But the threats and effects of ocean acidification to marine flora and fauna are subtle which need to be studied in detail by micro and mesocosm experiments especially for marine calcifiers. The present study has proved that the reduced ocean pH negatively impacts the biochemical pathway of biomineralization process of shell formation by direct physiological stress on energy and ion supplies.

Declarations

Author contribution statement

M. Anand: Conceived and designed the experiments; Analyzed and interpreted the data.
K. Rangesh: Performed the experiments; Contributed reagents, materials, analysis tools or data.
G. Jayanthi and B. Rajeswari: Performed the experiments; Contributed reagents, materials, analysis tools or data.
R.J. Priya: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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