3.1 Chemicals

Thiobarbituric acid (TBA), cumene hydroperoxide, sephadex G-25, BSA, glutathione reductase (GR), horse radish peroxidase (HRP), homovanilic acid (HVA), cumene hydroperoxide, dithiobis nitrobenzoic acid, sodium salts of glutamate and malate, pyruvate, triton-X100, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 1-chloro-2, 4 dinitrobenzene (CDNB), Hoechst dye number 33258, tert-butyl hydroperoxide, adenosine diphosphate (ADP), 2, 2- diphenyl-1-picryl hydrazyl (DPPH) and tris-Cl, catalase were purchased from Sigma Chemical Company, USA. Tert-butyl hydroperoxide was obtained from Merck-Schudant, Germany. Proteinase-K and RNase were obtained from Fermentas Life sciences, USA. Dithiothretol was supplied by Biogene, USA. Sucrose, phenolnaphtaline, phenol, EDTA, riboflavin, phenylmethyl sulphonyl fluoride (PMSF), hydroxylamine hydrochloride, L-methionine riboflavin, guanidine hydrochloride, ATP, disodium succinate hexahydrate, oxidized and reduced glutathione (GSH and GSSG), sodium azide, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), ascorbic acid, sodium dodecyl sulphate (SDS), acryl amide, bis-acrylamide, TEMED, ammonium persulphate (APS), nitrobluetetrazolium (NBT), KCN, phenolnaphtaline, manganous salt, chloroform and sodium molybdate were purchased from SISCO Research Laboratory, Mumbai, India. Silver nitrate (AgNO3) was purchased from Qualigens Fine Chemicals, Mumbai, India. Rubeanic acid was obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Potassium carbonate, hypochlorite was obtained from Fischer Inorganics & Aromatics Ltd, Chennai, India. H2O2 and isoamyl alcohol were obtained from SD Fine chemicals, Mumbai, India. All other chemicals used were of the analytical grade.

3.2 Animals

Mature male and female mud crabs (S. serrata) at inter molt stage were procured from Arakhakuda village site located on the bank of Chilika lagoon (19º 28’ and 19º 54’ N and 85º 05’ and 85º 38’ E, fig. 3.1) of Odisa, India for the present study.

3.2.1 Animals for seasonal studies

For seasonal studies, both male and female crabs were collected in winter (December-January, 2007), summer (April-June, 2008) and rainy (July-September, 2008) seasons. Body
weights of crabs were recorded at the time of collection and they were released immediately into a wooden box (10 m x 1 m x 1m) containing wet weeds and mud and transported to a temporary field station where crabs were sacrificed for tissue collection as described in section 3.6.

3.2.2 Animals for laboratory experiments

Adult male or female mud crabs were collected from Chilika lagoon as described in section 3.2.1 and transported to the laboratory in gunny bags. The crabs were disinfected by dipping them into 17 ppt saline water having 500 ppm (parts per million) potassium permanganate (KMnO₄) for 5-7 min and then were acclimated in 17 ppt saline water without KMnO₄ for 24 hours in the laboratory. During this acclimatization period different mortality rates (~13%) were observed when subjected to different salinity treatment experiments. Only active crabs were selected and used for experiments. After body weights of crabs were recorded, they were released into glass aquaria (75 cm x 30 cm x 30 cm) with artificially made saline water of required salinity. Artificial sea water of 35 ppt was made according to Robinson (1954) and diluted to 17 ppt or 10 ppt with tap water supplied by Bhubaneswar Municipality Corporation, Bhubaneswar, India where ever necessary. Aquaria had 5 cm high bed of sand which was pre-treated with KMnO₄ followed by extensive washing with tap water and was high enough to keep the crabs in merged condition. All aquaria were continuously aerated and covered with plastic opaque covers to make least disturbances to the animals. Saline water was changed daily at 18.00 hours and crabs were exposed to 12h:12h natural light and day periods throughout the experimentations. The crabs were fed once daily during night (at 19.00 hours) with fresh chick liver pieces.

3.3 Experimental designs

Following experiments were designed to find out the effects of salinity on the oxidative stress (OS) physiology of mud crabs. For each experiment, 9 to 10 animals were used.

- Experiment 1: Effects of different seasons on the OS physiology of crabs were investigated in both the sexes.

For the present experiment, three seasons (i.e. winter, summer and rainy) were selected. The salinity of the study site of Chilika lagoon was reported to be 10 ppt, 17 ppt and 35 ppt for rainy, winter and summer seasons, respectively (Mohapatra et al., 2007a;
Panigrahi et al., 2007). For seasonal studies, 10 each of adult male (67.333 ± 6.39 g) and female (70.83 ± 8.62 g) crabs at intermoult state were collected in winter (December-January, 2007), summer (April-June, 2008) and rainy (July-September, 2008) seasons. Body weights of crabs were recorded at the time of collection and they were released immediately into a wooden box (10 m x 1 m x 1m) containing wet weeds and mud and transported to a temporary field station where crabs were sacrificed for tissue collection. After sacrificing animals, muscle, gills and hepatopancreas (HP) tissues were collected as described above, packed separately in cryovials (5 ml) and were immediately frozen in liquid nitrogen canes. In the field, all the sacrificing was performed within 6 hrs to 11 hrs of the morning. The tissues were then transported to the laboratory keeping in the liquid nitrogen cane and stored at -80 ºC till further analysis.

Experiments 2 to 5 were conducted in the laboratory by acclimatizing either both sex or only male crabs to different salinities (i.e., 10 ppt, 17ppt and 35 ppt) that were prevalent in different seasons.

- **Experiment 2**: Effects of different salinities on O2 consumption, carbon dioxide release and ammonia excretion in crabs were investigated.

  Adult male (67.34 ± 8.53 g) and female (64.59 ± 6.99 g) crabs were collected from the site and transported to the laboratory where they were acclimatized in 17 ppt saline water for a day as described in section 3.2.2. Only healthy crabs (n=12, each for both male and female) were transferred to 10 ppt saline water and kept for 21 days. At the end of 21 days, the crabs were transferred to 17 ppt saline and kept for 21 days. Finally, the crabs were transferred to 35 ppt saline water for another 21 days. Before moving from one salinity grade to next, oxygen consumption, CO2 release, and ammonia excretion of each crab (n=10) was measured in triplicate as mentioned in section 3.5.

- **Experiment 3**: Effects of different salinities on OS physiology of gills, hepatopancreas and abdominal muscle of male crabs were investigated.

  Adult male crabs (65.95 ± 8.49 g) were collected from the site and transported to the laboratory. They were acclimatized for 24 hrs in the laboratory as described in section 3.2.2. After acclimatization only healthy crabs (n=12) were kept in 10 ppt, 17 ppt and 35 ppt saline water for 21 days. Animals (n=9) were sacrificed. Muscle, gills and HP tissues were collected as described in section 3.6. The tissues were stored at -80 ºC till further analysis.
• Experiment 4: Effects of different salinities on \( \text{O}_2 \) consumption by gill mitochondria with different substrates were investigated.

Adult male crabs \((64.07 \pm 5.38 \text{ g})\) were collected from the site and transported to the laboratory. They were acclimatized for one day in the laboratory as described in section 3.2.2. After acclimatization, crabs \((n=12)\) were kept in 10 ppt, 17 ppt and 35 ppt saline water for 21 days. Animals \((n=9)\) were sacrificed. Gill tissues were dissected out as described elsewhere. Mitochondria were isolated from the fresh gill tissues as described in section 3.8. Oxygen consumption of mitochondria with different substrate and ADP was measured.

• Experiment 5: Effects of different salinities on oxidative stress parameters and on activities of respiratory enzyme complexes of gill mitochondria of male crabs were investigated.

Adult male crabs \((64.07 \pm 5.38 \text{ g})\) were collected from the site and transported to the laboratory. They were acclimatized for one day in the laboratory as described in section 3.2.2. After acclimatization crabs \((n=12)\) were kept in 10 ppt, 17 ppt and 35 ppt saline water for 21 days. Animals \((n=9)\) were sacrificed. Mitochondria from gill tissue were dissected out as described elsewhere. Oxidative stress parameters and activities of respiratory enzyme complexes of mitochondria were estimated.

In order to understand the effects of salinity on oxidative physiology of crabs, following biochemical parameters were measured:

• Oxidative stress parameters (LPx, PC and \( \text{H}_2\text{O}_2 \)),
• Total tissue antioxidant capacity,
• Small antioxidant molecules (ascorbic acid and non-protein -SH group) and
• Antioxidant enzymes (SOD, CAT, GPx and GR)

In addition to the above in some experiments, DNA unwinding assay was performed to assay genotoxicity of salinity on crabs.

3.4 Water quality measurement

Different hydrological parameters of water (aquaria or samples from sites) such as temperature, salinity, pH and DO were measured with the help of specific electrodes (\( \mu \text{P} \) based soil and water analysis kit, Esico. Co., New Delhi, India). Using the same electrodes, the above parameters were also measured at the laboratory in water samples collected form
the field for experiment 1. Temperature of the lagoon water was measured using a mini thermometer.

3.5 Measurement of O2 consumption, CO2 release and NH3 excretion

A respiratory chamber (RC) was designed with a cylindrical glass jar (Volume 3121 cm³) fitted with a hard plastic net at 7 cm height at its bottom. The RC was placed on a magnetic stirrer and magnetic bid was kept under the plastic net to circulate the water within the jar. The upper side of the RC was equipped with an O2 electrode connected with a monitor (μP based soil and water analysis kit, Esico. Co., New Delhi, India, fig. 3.2). One crab was introduced into the chamber filled with freshly O2 saturated artificial saline water of required salinity. Prior to release the crabs into the RC, each was acclimated in a similar chamber for 10 min with the water of same salinity. During experiments, the RC was sealed to prevent the diffusion of atmospheric O2 into the chamber. Fall of O2 concentration in the chamber was recorded at 15 min interval up to 60 min and the result was expressed as mg O2 consumed/100 g body weight/hr. At the end of one hour, 500 ml of water sample was withdrawn into an air tight dark bottle for CO2 measurement and 4 ml water sample into a microfuge tube for NH3 measurement. Initial concentrations of O2, CO2 and NH3 in water were determined before the experiment and the values were deducted from the respective values obtained after the end of the experiment. Free CO2 was measured by titrating 50 ml of water sample with 0.1 ml of phenolnaphtaline indicator against N/10 NaOH according to APHA (1985). The end point of titration was achieved when pink color was developed in the solution. The result was expressed as mg of CO2 released/100 g body weight/hr. NH3 in water sample was quantified according to Russell (1944). In brief, to 1.5 ml of water sample 0.1 ml of manganous salt was added followed by 1 ml of 25% alkaline phenol and 0.5 ml of hypochlorite solution. The mixture was gently rotated and boiled for 5 min, cooled and was centrifuged at 1,000 rpm for one mine at 25 ºC. Absorbance of the color of solution was measured at 625 nm. NH3 concentration was calculated from its standard curve and the results were expressed as mg of NH3 excreted/100 g body weight of crab/hr.

3.6 Tissue collection and sub cellular fractionations.

Animals were sacrificed by removing their carapace from their abdomen by a jerk and hepatopancreas, gills and abdominal muscle tissues were dissected out quickly (fig. 3.3).
Tissues were washed in ice cold normal saline (0.67\%, w/v), blotted, flash frozen in liquid nitrogen and stored at -80 °C for further analyses. Mitochondrial fraction was always isolated from fresh gill tissues as described later. In brief, homogenates of tissues (10\% (w/v)) were prepared at 4 °C in homogenizing buffer (50 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, 0.5 mM sucrose, 150 mM KCl and 1mM PMSF, pH 7.8) with the help of Potter-Elvejhem type, motor driven glass Teflon homogenizer at 250 rpm speed with 7-8 up and down strokes. On
the other hand, gill tissues were homogenized in pre-cooled mortar and pestle keeping on ice. The crude homogenates of tissues were centrifuged at 1, 000 x g for 10' at 4 °C in a cooling centrifuge (REMI, Model C-24) to sediment nuclei and tissue debris. The supernatant fractions (post-nuclear fraction: PNF) were centrifuged at 10,000 x g for 10' at 4 °C to obtain the clear supernatant which was referred as post mitochondrial fraction (PMF). The mitochondrial pellet (mitochondrial fraction: MF) obtained were washed and stored at -80 ºC for further analysis.

3.7 Biochemical determinations of protein and oxidative stress physiology parameters

3.7.1 Estimation of protein concentration

Protein concentration in tissue fractions was determined according to the method of Bradford (1976) by taking BSA as standard.

**Principle:** Coomassie brilliant blue (CBB) can remain in anionic, cationic or neutral form depending upon the pH. Under acidic condition (pH >1.3), the anionic form of CBB (G 250) binds with arginine and to a lesser degree with lysine and histidine and secondarily with the aromatic amino acids tryptophan, phenylalanine and tyrosine. Protein-CBB dye binding may be due to the development of hydrophobic interactions and van der Waals forces between proteins and the anionic species of the CBB dye (Georgiou et al., 2008). The final product absorbs light at 595 nm.

**Reagents:** 1) NaCl (0.15 N) solution in DW. 2) Bradford Reagent (CBP G-250, 0.01%, Ethanol, 4.74%, O-Phosphoric acid, 8.5%).

**Procedure:** In brief, 20 µl of suitably diluted samples or BSA (0.05 - 0.1 mg/ml in homogenizing buffer) was taken in 1.5 ml microfuge tubes containing 780 µl NaCl solution. Bradford reagent (200 µl) was added to it and mixed thoroughly. The absorbance of the sample was read at 595 nm against an appropriate blank. The protein concentration of the sample was determined by comparing the absorbance of the sample with the absorbance of BSA.

3.7.2 Determination of oxidative stress parameters

3.7.2.1 Measurement of lipid peroxidation

Level of lipid peroxides (LPx) in samples was determined by monitoring formation of thiobarbituric acid reactive substances (TBARS) based on the method of Ohkawa et. al. (1979).
Principle: PUFA are subjected to oxidation by free radicals and the product is known as LPx. Malondialdehyde (MDA) is the most abundant aldehyde resulting from lipid peroxidation and usually determined based on its reaction with thiobarbituric acid (TBA) in an acidic medium. The final product has absorbance maxima at 532 nm. Several other aldehydes can form chromogens with TBA other than MDA; therefore, the final product in this assay is expressed in terms of TBARS (thiobarbituric acid reactive substances). To prevent auto-peroxidation of samples during heating, butylated hydroxytoluene was used in the assay mixture.

Reagents: 1) Potassium chloride (KCl) solution (1.15%; w/v) in DW. 2) Acetic acid (CH₃COOH, 20%, v/v) in distilled water, pH adjusted to 3.5 with NaOH. 3) Sodium dodecyl sulfate solution (SDS, 8.1%, w/v) in DW. 4) Aqueous solution of thiobarbituric acid (TBA, 0.8%, w/v) prepared fresh in DW prior to assay. 5) Butylated hydroxytoluene solution (BHT, 0.76%, w/v) in alcohol, prepared fresh prior to assay. Cocktail was prepared fresh every time before use.

Composition of the cocktail:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per tube (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, pH 3.5</td>
<td>0.75</td>
</tr>
<tr>
<td>SDS, 8.1%</td>
<td>0.1</td>
</tr>
<tr>
<td>TBA, 0.8%</td>
<td>0.75</td>
</tr>
<tr>
<td>BHT, 0.76%</td>
<td>0.05</td>
</tr>
<tr>
<td>Total</td>
<td>1.65 ml</td>
</tr>
</tbody>
</table>

Procedure: Sample was suitably diluted with cold KCl (1.15%; w/v) solution so that the final protein concentration of the sample was 1.5 to 2.0 mg/ml. Sample (0.35 ml) was added to the tube containing TBA reagent (1.65 ml) and kept at 95 °C for 1 hr. Tubes were centrifuged at 1, 000 x g for 10' after they were cooled to room temperature. Supernatants were carefully decanted into other tubes and absorbance of reddish-pink chromogen was read at 532 nm against suitable blank in a UV-VIS spectrophotometer. Results were calculated from the molar extinction coefficient of TBARS as 1.56 x 10⁵ M⁻¹cm⁻¹ (Wills, 1969) and were expressed as nmoles of TBARS formed per mg protein.

3.7.2.2 Measurement of protein carbonylation

Protein carbonyl content of samples was determined according to the method of Levine et al. (1994 and 1990).
**Principle:** Membrane bound and other proteins are subjected to oxidation by free radicals. It resulted into production of protein carbonyls (PC). PC is usually determined based on its reaction with dinitrophenylhydrazine (DNPH). The final product is formed as protein hydrazones that have absorbance maxima at 355-390 nm. BSA is used as standard to calculate the protein content of the sample.

**Reagents:**
1) PDEP buffer: Potassium phosphate buffer, pH 7.4 (50 mM) containing digitonin (0.2%), EDTA (2 mM) and phenylmethysulfonyl fluoride, PMSF (40 μg/ml). PMSF stock solution was prepared in methanol.
2) Streptomycin sulfate (10%, w/v) in potassium phosphate buffer, pH 7.4 (50 mM).
3) Hydrochloric acid, HCl (2.5 M).
4) 2, 4-dinitrophenylhydrazine, DNPH (10 mM) in 2.5 M HCl.
5) Trichoroacetic acid, TCA (20%, v/v).
6) Absolute ethanol/ethylacetate solution in the ratio 1:1 (v/v).
7) Guanidine hydrochloride (6 M) in K₂HPO₄ (20 mM), pH adjusted to 2.3 with concentrated HCl.

**Procedure:** Sample was suitably diluted with potassium phosphate buffer (50 mM, pH 7.4) so that protein concentration of sample was approximately 1.0 -1.5 mg protein per ml. 0.45 ml of sample was pipetted into a microfuge tube. To this, PDEP buffer (0.45 ml) and streptomycin sulfate (10%, 0.1 ml) were added. Contents were thoroughly mixed and allowed to stand for 10'. Tubes were centrifuged at 1, 000 x g for 10'. Supernatant was pipetted into two new centrifuge tubes having 0.4 ml in each. Equal volume of DNPH or 2.5 M HCl were added separately into the tubes, vortexed and kept in dark for 1 h. For each sample tube with DNPH, the blank tube was run with HCl (2.5 M). During incubation, tubes were vortexed with every 15' interval. Tubes were then transferred to ice box and 20% ice-cold TCA (0.8 ml) was added to each tube to stop the reaction. Tubes were kept in ice for 10' and centrifuged at 1, 000 x g for 10'. Supernatants were discarded. Protein pellet of each tube was carefully washed by dissolving the pellet in absolute ethanol: ethylacetate solution (ratio 1:1, 2 ml) and centrifuging at 1, 000 x g for 5'. After three washings, the final pellet was dissolved in 1 ml of guanidine hydrochloride (6 M) and absorbance was recorded at 366 nm in a UV-VIS spectrophotometer (Cary 100, Varian). For each sample, absorbance at 366 nm was determined as DNPH reading minus HCl blank reading. Protein recovery was estimated for each sample at 280 nm using a standard curve of BSA in guanidine hydrochloride. Carbonyl content was calculated using molar extinction coefficient of aliphatic hydrazones i.e., 22 x 10³ M⁻¹ cm⁻¹ and expressed as nmol carbonyl/mg protein.
3.7.2.3 Measurement of hydrogen peroxide

Hydrogen peroxide (H$_2$O$_2$) content in tissue sample was measured spectrofluorimetrically according to Anguelov and Chichovska (2004) with a little modification.

**Principle:** HRP mediated oxidation of homovanillic acid (HVA) by H$_2$O$_2$ forms a HVA dimer that has specific excitation and emission wave lengths at 312 nm and 420 nm, respectively. The reaction takes place as follows:

\[
\text{HRP} \rightarrow \text{HRP-H}_2\text{O}_2 \text{ (Compound I)}
\]

\[
\text{HRP-H}_2\text{O}_2 + 2\text{HVA} \rightarrow \text{HRP} + 2 \text{H}_2\text{O} + \text{HVA-HVA dimer}
\]

**Reagents:** 1) Phosphate EDTA buffer (PBE): 2 mM EDTA in 50 mM PB pH 7.6. 2) Glycine-NaOH (0.1 M) buffer, pH 12. 3) Homovanillic acid, (HVA, 1.25 mM in DW). 4) Horseradish peroxidase (HRP, 10 units/ml potassium phosphate buffer, pH 7.6, 50 mM). 5) Trichloroacetic acid (TCA, 50 % in DW).

**Procedure:** Tissue sample was homogenized in 50 mM phosphate buffer containing 2 mM EDTA, pH 7.6 (PBE) at 4 ºC. The crude homogenate (10%, w/v) was centrifuged at 1000 x g for 10 min at 4 ºC to obtain PNF. The PNF was pre-incubated for one hour at 25 ºC in the assay mixture (0.6 ml) containing 2 Unit/ml horse radish peroxidase (HRP) and, 200 µM homovanillic acid (HVA) in PBE. After incubation, 0.9 ml of PBE and 0.375 ml of glycine-NaOH (0.1 M) buffer, pH 12 were added to the tube. HRP mediated dimer product of HVA by H$_2$O$_2$ was measured with fluorescence spectrophotometer (Hitachi, Japan, Model F 2500). Instrument specifications during measurement were 5 nm of both extinction and emission slit widths, 400 PMT voltages and 312 nm and 420 nm of extinction and emission wave lengths, respectively. Pure H$_2$O$_2$ was taken as standard and the results were expressed as µg of H$_2$O$_2$ per g of wet tissue. To calculate internal loss of H$_2$O$_2$ during sample preparation and assay, muscle, gill and hepatopancreas of a group of five crabs were homogenized separately as described above and precipitated immediately with 5% trichloroacetic acid and centrifuged at 1, 000 rpm for 5 min to collect the supernatant. Samples were then neutralized with tris base and H$_2$O$_2$ was measured as described above. After H$_2$O$_2$ content was determined in both the precipitated sample and their respective control sample (non-precipitated sample) as described above, a loss of $2.12 \pm 0.11$, $1.51 \pm 0.07$ and $1.56 \pm 0.12$ fold of higher H$_2$O$_2$ content was observed in muscle, hepatopancreas and gills, respectively, in the precipitated samples when compared form the respective control samples. The observed data obtained for non precipitated samples in the experiment 3 were multiplied.
with the corresponding factors for calculation of actual H₂O₂ content in the respective tissues. But in experiment 1, the tissue homogenates were directly precipitated and H₂O₂ content was determined as described above. In the experiment 1, 3 M K₂HPO₄ was used to neutralize the samples instead of tris base.

3.7.3 Genotoxic stress parameter: Alkaline DNA unwinding test

Alkaline DNA unwinding assay was performed fluorimetrically according to the method of Shugart (1988a and 1988b).

Principle: Hoechst dye is a fluorescent compound that binds to A-T rich region of minor groove of double stranded DNA when it is in super coiled structure (Pjuara et al., 1987, Quintana et al., 1991). The dye does not bind the DNA when it is in unwound form. Therefore, a higher fluorescence value is obtained due to the binding of the dye with unwound DNA than intact DNA when it is in super coiled from. The reaction product has fluorescent properties of 360 nm and 450 nm excitation and emission wave lengths, respectively.

Reagents: 1) Lysis buffer (50 mM tris, 100 mM EDTA, 0.5% SDS, pH 8). 2) RNAse (10 µg/ml) and proteinase-K (100 µg/ml). 3) Phenol: chloroform: isoamyl alcohol (in 25:24:1 ratio). 4) Sodium acetate (0.3 M). 5) Tris (10 mM) EDTA (1 mM) buffer, pH 8. 6) 0.05 N NaOH and 0.05 N HCl solutions. 7) SDS (0.2%, w/v) containing 2 mM EDTA. 8) Hoechst dye no.- 33258 (1mg/ml stock).

Procedure: DNA was isolated from tissue samples using standard protocol. In brief, a 10% (w/v) homogenate (50 mg tissue) was made in lysis buffer with the help of a hand homogenizer in 1.5 ml microfuge tubes at room temperature. Proteinase-K (100 µg/ml) was added to it and was incubated for one hour followed by treatment of RNAse (10 µg/ml) for another one hour. The samples were then mixed with equal volume of phenol and centrifuged at 12,000 x g for 10 min at 4 ºC. The aqueous phase was transferred to a new tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to it and again centrifuged at 12,000 x g at 4 ºC for 10 min. The aqueous phase was extracted with equal volume of chloroform followed by centrifugation at 12,000 x g for 10 min at 4 ºC. The aqueous phase was again transferred to another tube and added with double volume of absolute alcohol and sodium acetate (0.3 M) and kept at -40 ºC for 30 min. The sample was then centrifuged at 12,000 x g for 10 min at 4 ºC to obtained DNA pellet. The pellet was
washed with 75% ethanol at 12,000 x g at 4 ºC and dissolved in 20 µl of Tris (10 mM) EDTA (1 mM) buffer, pH 8. DNA was quantified by taking absorbance at 260 nm at proper dilution with double distilled water. Around 5 µg of DNA was mixed with 1µg of Hoechst dye, number 33258 for DNA unwinding assay. Briefly, for each sample, three sets of incubation were performed at 4 ºC, 38 ºC and 80 ºC with 50 µl of 0.05 N NaOH solution for 15 min. The mixture was then neutralized with 50 µl of 0.05 N HCl solution. Then 5 µl of SDS (0.2%,w/v) containing 2 mM EDTA was added, mixed and followed by 100 µl of Hoechst dye (1µg) and kept for 15 min at room temperature. The volume of reaction mixture was adjusted to 3 ml with 0.2 M PB, pH 6.9. Fluorescence of the sample were measured at 360 nm excitation and 450 nm emission wave length and 5 nm for both the slit widths in a fluorescence spectrophotometer (Hitachi, Japan, Model F-2500). The results were calculated from the fluorescence value of three incubations of each sample and expressed as F-value, where F-value = (F 38 ºC - F 80 ºC) / (F 4 ºC-F 80 ºC) and F indicated fluorescence of sample at the respective temperatures. F value closer to 1.0 or less than 1.0 indicates more intact DNA or more unwound DNA, respectively.

3.7.4 Determination of antioxidant enzyme activities

SOD, GPx and GR activities were measured in sephadex G-25 column elutes of PMF of tissue samples. Sephadex G-25 column was equilibrated with homogenizing buffer before use. The column was prepared in a 1 ml syringe. The syringe was put in a cleaned cool centrifuge tube and was subjected to centrifugation at 2,000 rpm for two min at 4 ºC to get rid of the buffer before loading the sample. The eluted buffer was discarded. After loading 200 µl of PMF sample to the column, it was again centrifuged at 2,000 rpm for two min at 4 ºC and enzyme activities were assayed in the elute. Elute was immediately stored in ice box. Activities of all enzymes were measured at 25 ºC.

3.7.4.1 Superoxide dismutase

SOD activity was measured according to Das et al. (2000).

**Principle:** SOD (EC 1.15.1.1) dismutates O$_2^••$ to H$_2$O$_2$ and O$_2$. The present assay is based on an indirect method involving photo reduction of riboflavin which generates O$_2^••$. It is allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite reacts with sulfanilic acid to produce a diazonium compound that subsequently reacts with naphthylamine forming a red azo compound which absorbs light at 543 nm. When SOD
present in the assay mixture, nitrite formation is inversely proportional to the amount of SOD as it scavenges $O_2^{•−}$ competitively with nitrite. One unit of enzyme activity was defined as amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions.

**Reagents:** 1) Tris buffer, pH 8 in DW (100 mM). 2) L-methionine (20 mM) prepared in 100 mM tris buffer, pH 8. 3) Triton-X-100 (1 %, v/v) in 100 mM tris buffer, pH 8. 4) Hydroxylamine hydrochloride (10 mM) prepared 100 mM tris buffer, pH 8. 5) Ethylenediamine tetraacetic acid (EDTA, 100 µM) prepared in 100 mM tris buffer, pH 8. 6) Riboflavin (50 µM) prepared in 100 mM tris buffer, pH 8. 7) Orthophosphoric acid (5%, v/v) in distilled water. 8) Sulfanilamide (1%, w/v) in orthophosphoric acid (5%, v/v). 9) N- (1-naphthyl) ethylenediamine (0.1%, w/v) in distilled water. 10) Greiss reagent: Freshly prepared mixture of sulfanilamide (1%) and N- (1-naphthyl) ethylenediamine (0.1%) in the ratio 1:1 (v/v). 11) Cocktail: Freshly prepared mixture of the reagents 1-5 in specified proportions as mentioned below.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume per tube (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer, 100 mM pH 8</td>
<td>1.110</td>
</tr>
<tr>
<td>L-methionine (20 mM)</td>
<td>0.075</td>
</tr>
<tr>
<td>Triton-X-100 (1%)</td>
<td>0.040</td>
</tr>
<tr>
<td>Hydroxylamine hydrochloride (10 mM)</td>
<td>0.075</td>
</tr>
<tr>
<td>EDTA (100 µM)</td>
<td>0.100</td>
</tr>
<tr>
<td>Total</td>
<td>1.400 ml</td>
</tr>
</tbody>
</table>

**Procedure:** The assay was performed in dark since the reaction itself is light dependent. Blank tubes, internal control and sample tubes were assigned in duplicates as follows:

Blank------------------cocktail (1.40 ml) + buffer (0.18 ml)

Internal control----cocktail (1.40 ml) + buffer (0.1 ml) + riboflavin (0.08 ml)

Sample---------------cocktail (1.40 ml) + sample (0.1 ml) + riboflavin (0.08 ml)

The assay mixture (1.58 ml) contained hydroxylamine hydrochloride (0.47 mM), L-methionine (0.9 mM), EDTA (6.33 mM), triton-X 100 (0.026%), in 100 mM tris buffer, pH 8 and/or sample (0.1 ml containing 50 -150 µg protein) and riboflavin (2.5 µM). The tubes were vortexed and the reaction was started by exposing it to two 20 W fluorescent lamps (separated by 30 cm, fitted parallel to each other inside an aluminum coated wooden chamber) for 10 mins at 25 ºC. After 10' exposure to light, Greiss reagent (1 ml) was added
to all tubes and mixed thoroughly. Absorbance of internal control and various sample tubes were read against blank at 540 nm in a UV-VIS spectrophotometer (Cary 100, Varian). One unit of enzyme activity was calculated from the value \( \frac{V_o}{V} - 1 \) (\( V_o \) = absorbance of internal control, \( V \) = absorbance of sample). Final SOD activity was calculated by subtracting the value obtained for boiled samples (95 °C for 30 min) from the corresponding unboiled samples. Enzyme activity was expressed as Units/mg protein.

### 3.7.4.2 Catalase

Catalase enzyme activity was measured in the samples according to the method of Aebi (1974).

**Principle:** Catalase (CAT, EC 1.11.1.6) breakdown of H\(_2\)O\(_2\) to water and oxygen. The enzyme activity was measured in the samples by monitoring the decrease in absorbance of H\(_2\)O\(_2\) at 240 nm.

**Reagents:**
1) Potassium phosphate buffer, pH 7.0 (50 mM).
2) Absolute ethanol Triton-X-100 (10%, v/v).
3) Hydrogen peroxide (H\(_2\)O\(_2\), 25 mM) prepared in potassium phosphate buffer, pH 7.0 (50 mM).

**Procedure:** Prior to the assay, to 0.5 ml of PMF, 5 µl ethanol (1%) was added to prevent formation of the inactive complex of CAT (complex-II) by reacting with H\(_2\)O\(_2\). This was kept in ice for 30'. After incubation, 50 µl triton-X 100 (1%, v/v) was added and incubated for 15' to increase the observable CAT activity by releasing it from peroxisomes (Cohen *et al.*, 1970). Freshly prepared H\(_2\)O\(_2\) (25 mM) in 2.9 ml PB (50 mM, pH 7) was pipetted into glass test tubes kept in water bath at 25 °C. The above treated PMF sample (0.1 ml) appropriately diluted so as to contain 50-200 µg protein was then added to the tube. The solution was quickly transferred to quartz cuvette for measurement. Enzyme activity was calculated from the extinction coefficient of H\(_2\)O\(_2\) as 43.6 M\(^{-1}\)cm\(^{-1}\) and was expressed as nKat per mg protein. (One Kat is defined as one mole of H\(_2\)O\(_2\) consumed per second per mg protein).

### 3.7.4.3 Glutathione peroxidase

GPx assay was performed according to Paglia and Valentine (1967).

**Principle:** GPx (EC 1.11.1.9) catalyses detoxification of H\(_2\)O\(_2\) and other organic peroxides by oxidizing reduced glutathione (GSH). The assay measures rate of GSH oxidation by hydroperoxides as catalysed by GPx present in the sample. Rather than measuring progressive loss of GSH, this substrate is maintained at a constant concentration
by adding exogenous GR and NADPH, which immediately convert any GSSG produced to its reduced form. Rate of GSH oxidation can then be indirectly determined by following decrease in absorbance of reaction mixture at 340 nm as NADPH is converted to NADP⁺. Assay was conducted in presence of KCN and sodium azide to inhibit inherent catalase activity of sample. Total GPx activity was measured using cumene hydroperoxide as substrate.

**Reagents:** 1) PB pH 7.6 (50 mM). 2) Reduced glutathione (GSH, 30 mM) prepared in PB pH 7.6 (50 mM). 3) Potassium cyanide (KCN, 4.5 mM) prepared in PB pH 7.6 (50 mM). 4) Sodium azide (NaN₃, 30 mM) prepared in PB pH 7.6 (50 mM). 5) Nicotinamide adenine dinucleotide phosphate reduced (NADPH, 4.5 mM) prepared in PB pH 7.6 (50 mM). 6) Glutathione reductase enzyme (GR, 10 units/ml) diluted in PB pH 7.6 (50 mM). 7) Cumene hydroperoxide (7.5 mM), diluted in PB pH 7.6 (50 mM). 8) tert-butyl hydroperoxide (tert-BuOOH, 7.5 mM) diluted in PB pH 7.6 (50 mM). 9) Reaction cocktail: Freshly prepared mixture of potassium phosphate buffer: GSH: KCN: NaN₃ in the ratio 23:1:1:1.

**Procedure:** In a quartz cuvette, freshly prepared cocktail (2.6 ml) was carefully pipetted. To this, 0.1 ml each of NADPH, GR and sample (50-150 µg protein) were added at interval of 1' each and allowed to stabilize. Addition of hydroperoxide substrate started the reaction. Utilization of NADPH by glutathione system was recorded at 340 nm at 2' interval for 6' in a UV-VIS spectrophotometer (Cary 100, Varian). A sample blank was also run in parallel. The enzyme activity was calculated from the extinction co-efficient of NADPH as 6.22 mM⁻¹cm⁻¹ and expressed as nmol of NADPH oxidized per min per mg protein.

### 3.7.4.4 Glutathione reductase

GR was assayed following the method of Massey and Williams (1965).

**Principle:** GR (EC 1.6.4.2) catalyses reduction of oxidized glutathione (GSSG) to its reduced form (GSH). This reaction is simultaneously accompanied by oxidation of NADPH to NADP⁺. Enzyme activity of tissue sample can be assayed by monitoring the decrease in absorbance of the reaction mixture at 340 nm as NADPH is progressively converted to NADP⁺.

**Reagents:** 1) Potassium phosphate buffer (PB), pH 7.6 (50 mM). 2) Oxidized glutathione (GSSG, 120 mM) prepared in 50 mM PB, pH 7.6. 3) Nicotinamide adenine dinucleotide phosphate reduced (NADPH, 4.5 mM) prepared in 50 mM PB, pH 7.6.
**Procedure:** 2.7 ml of 50 mM PB, pH 7.6 was carefully pipetted into quartz cuvette. To this, 0.1 ml each of NADPH and GSSG were added at interval of 1’ each and allowed to stabilize. Suitably diluted sample (0.1 ml) containing 100-200 μg protein was added to start the reaction. Decrease in absorbance at 340 nm was recorded at interval of 2' each for 6' in a UV-VIS spectrophotometer (Cary 100, Varian). A sample blank was also run in parallel. Enzyme activity was calculated using molar extinction coefficient 6.22 x 10³ M⁻¹cm⁻¹ and expressed as nmol NADPH oxidized/min/mg protein.

3.7.5 Measurement of small antioxidant molecules and total antioxidant capacity

3.7.5.1 Ascorbic acid

Ascorbic acid content in samples was measured by the method of Mitsui and Ohta (1961) with a little modification.

**Principle:** Phosphomolybdate is stoichiometrically reduced by ascorbic acid in the presence of inorganic phosphorous to give a characteristic molybdenum blue colour which absorb light at 660 nm.

**Reagents:** All reagents were prepared in DW. 1) Sodium molybdate (3.33 %). 2) H₂SO₄ (0.25 N). 3) disodium monophosphate (0.25 mM).

**Procedure:** 1.0 ml of the PMF was precipitated with ice-cold TCA (5 %, w/v) immediately after fractionation. The precipitated samples were centrifuged at 10,000 x g for 10' at 4 °C to obtain the supernatant. Briefly, 1 ml of assay mixture contained 0.5 ml sample, 0.2 ml sodium molybdate (0.66 %), 0.2 ml H₂SO₄ (0.05 N) and 0.1 sodium phosphate (0.025 mM) and was kept at 60 °C water bath for 40 mins. It was then cooled under running water and centrifuged. Absorbance of the clear supernatant was taken at 660 nm. AA was taken as standard and the results were expressed in ng of ascorbic acid per g wet tissue.

3.7.5.2 Non protein -SH group

Non protein -SH content was determined according to the method of Sedlak and Lindsay (1968).

**Principle:** The -SH groups can reduce of 5, 5′-dithiobis-2-nitrobenzoic acid into 2-nitro, 5-thiobenzoic acid whose absorbance maxima is 412 nm.

**Reagents:** 1) Trichloroacetic acid, TCA (50%, v/v). 2) Tris base (saturated solution at 25 °C). 3) 5, 5′-dithiobis-2-nitrobenzoic acid, DTNB (0.01 M) in methanol. 4) Standard reduced glutathione, GSH (0.25 mM).

**Procedure:** 1.0 ml of the PMF was precipitated with ice-cold TCA (5 %, w/v) immediately after fractionation and centrifuged at 10,000 x g for 10 mins at 4 °C. To 0.5 ml
of supernatant, 80 µl of saturated Tris base was added to make the pH of the solution between 8-9. To it, 50 µl of 0.01 M dithiobis nitrobenzoic acid (DTNB) was added and incubated for 10 min. The color product was measured at 412 nm in a UV-VIS spectrophotometer (Cary 100, Varian) against a suitable blank. The values of -SH content of the sample were calculated from the standard curve of GSH which was linear in the concentration range of 0.1-0.4 mM. Results were expressed as nmol of -SH content per g of wet tissue.

3.7.6 Determination of total antioxidant capacity

DPPH scavenging assay was performed according to Marxen et al. (2007) in aqueous phase of PNF.

**Principle:** DPPH is a stable free radical which can bind basically with small molecules having antioxidant activity. After binding it absorbs light at 515 nm. Since, the reaction is mediated by many small antioxidant molecules, therefore, more often the assay is considered to measure the total antioxidant capacity of the tissue.

**Reagents:** 1) Phosphate buffer 50 mM containing 2 mM EDTA, pH 7.6 (PBE). 2) DPPH (2, 2- Diphenyl-1-picryl hydrazyl) solution (0.5 mg/ml in methanol).

**Procedure:** Tissue was homogenized in PBE as described in the section 3.6. The crude homogenate (10%, w/v) was centrifuged at 1,000 x g for 10 min to obtain PNF. To 1.4 ml of methanol, 100 µl of DPPH solution was added followed by 100 µl of sample. Absorbance of the reaction mixture was read at 515 nm. Results were expressed as % of decrease in absorbance by 100 µl of PNF from control set (without sample). The calculation was performed as % of inhibition = (Abs control – Abs of sample)/Abs control x 100 (Singh et al., 2002).

3.8 Isolation of mitochondria and measurement of respiration

3.8.1 Homogenization

Mud crabs (*S. serrata, 80.83 ± 10.21 g*) were collected from Arakhakuda region of Chilika lagoon. They were acclimatized in laboratory at 17 ppt artificially made saline water as described in section 3.2.2. Gill tissues were removed immediately after sacrificing the animals. The tissues were washed in ice cold PB (50 mM), pH 7.6. A 10% (w/v) homogenate of finely minced gill tissue was prepared in ice-cold mitochondria isolation buffer (MIB) comprising of sucrose (400 mM), KCl (100 mM), EGTA (6mM), EDTA (3 mM), HEPES (70 mM), BSA (1%) and PMSF (1 mM) (each time freshly prepared required concentration...
of PMSF solution was added to the buffer prior to use) pH 7.6, with a Potter-Elvejhem type motor-driven glass Teflon homogenizer with three up and down strokes at 250 rpm speed at 4 °C. The homogenate was filtered through four layers of sterilized cheesecloth. The filtrate was then subjected to repetition with the above procedure and the second homogenates was filtered again with the four layer of sterilized cheesecloth. Both the filtered liquid pooled together and subjected for centrifugation. Apart from sacrificing the animals, all steps were carried out at 4 °C or in ice and utmost care was taken to prevent foam formation in the homogenate.

3.8.2 Differential centrifugation

The homogenate was subjected to differential centrifugation at 4 °C in a cooling centrifuge (REMI C 24, Mumbai, India). Firstly, it was centrifuged at 1,000 x g for 10' to precipitate nuclei and cell debris. Pellet was discarded and the supernatant was re-centrifuged at 1,000 x g for 5 min to confirm the removal any trace of nuclei and unbroken cells. The supernatant was centrifuged at 8,000 x g for 15' to sediment mitochondria. Mitochondrial pellet was washed thrice to remove cytosolic contaminations and remaining BSA protein. In each washing step, mitochondria were gently suspended in MIB without BSA and centrifuged at 6,000 x g for 5'. Removal of BSA form the mitochondrial pellet was ensured by checking the concentration of the protein content of the supernatants at each washing step. The final pellet was divided into three parts. Two parts were dissolved separately in chilled mitochondria isolation buffer (MIB) without BSA. Out of these two parts, one part was used to determine protein concentration, H₂O₂, LPx, PC and -SH content and another part was stored at -80 °C for assay of respiratory chain enzymes. The third part was dissolved in MRB and was used to carry out respiratory experiments. MRB was comprised of sucrose 560 mM, KCl 100 mM, HEPES 70 mM, BSA (0.5%) and K₂HPO₄ 10 mM, pH 7.6.

3.8.3 Measurement of mitochondrial respiration.

Complex I and II mediated respirations of isolated mitochondria were measured in 1 ml of chamber volume of a water jacketed Clark type electrode (Hansatech, London) at 25 °C with sodium salts of glutamate, malate, pyruvate and succinate (final concentration 5 mM each) as substrates, respectively. Until unless mentioned in each experiment about 1 mg mitochondrial protein was used. In brief, required amount (to make the final chamber volume 1 ml) of MRB was added to the chamber using a pipette and left for 1 min for stabilization.
Then mitochondria suspension was added and the stopper was placed over it. After 30 sec, the fall of oxygen content in the chamber was recorded for 10-20 mins till state IV respiration was achieved using glutamate, and pyruvate or malate (as complex I substrates) and succinate (as complex II substrate) and ADP. Substrate (10 µl) was added into the chamber through a Hamilton’s syringe via the stopper after 1 to 2 min interval after the graph gets stabilized to a steady state. Mitochondria in the chamber were left for the respiration for 2 min with the substrate. ADP (5 µl) was added (final concentration 62 nmol to 125 nmol per ml) to initiate state III respiration. Addition of ADP into the chamber facilitates the completion of the electron transport chain by transferring the electrons to O₂. The different phases of mitochondrial respiration were referred as state I, II, III and IV respiration. State I, II and III respirations were recorded with mitochondria (M), (M + substrate) and (M + substrate + ADP), respectively. State IV respiration was achieved after completion of state III respiration. The rate of O₂ consumption at each step was measured using the scale given in the oxygraph. Respiratory Control Index (RCI) was calculated by dividing state III respiration with state IV respiration. ADP molecules utilized to oxygen uptake (P/O) ratio were calculated manually from the oxygraph by dividing the ADP molecules utilized with the number of atomic oxygen consumed in state III respiration.

3.9 Assay of electron transport chain enzymes

3.9.1 Sub-mitochondrial fractionation

Sub-mitochondrial fractionation was done following the protocol mentioned by Lotscher et al. (1980) with a little modification.

3.9.2 Sonication

Mitochondrial sample was placed in an ice bath in aliquots and sonicated at a protein concentration of ~10 mg/ml. Sonication was done by giving four bursts for 15'' with each burst being followed by interval of 30'' in a B. Braun sonicator.

3.9.3 Removal of unbroken mitochondria

Sonicated sample was centrifuged at 10,000 x g for 10' at 4 °C to separate unbroken mitochondria. Supernatant thus obtained was subjected to protein estimation and used for the assay of respiratory chain enzymes.
3.9.4 Complex enzyme assay

3.9.4.1 NADH: Duroquinone oxidoreductase assay

This was assayed following the method of Gassner et. al. (1997).

**Principle:** NADH: DQ oxidoreductase (complex I, EC 1.6.5.3) transfers electrons from NADH to quinone in electron transport chain. As a result NADH is reduced and the rate of reduction can be measured at 340 nm.

**Reagents:** 1) Potassium phosphate buffer, PB pH 7.6 (20 mM). 2) Potassium cyanide (KCN, 200 mM) prepared in 20 mM PB pH 7.6. 3) Ethylenediamine tetraacetic acid (EDTA, 10 mM) prepared in 20 mM PB pH 7.6. 4) Duroquinone (DQ, 30 mM). It was first dissolved in ethanol to prepare a 60 mM stock solution which was diluted to 30 mM in 20 mM PB pH 7.6. 5) Nicotinamide adenine dinucleotide reduced salt (NADH, 13 mM) prepared in 20 mM PB pH 7.6.

**Procedure:** 845 μl of PB (20 mM, pH 7.6) was carefully pipetted into quartz cuvette. To this, 10 μl each of KCN, EDTA and DQ were added and allowed to stabilize. Appropriately diluted sample (100 μl) having 50-150 μg protein was added to this. Reaction was started by adding 25 μl of NADH and decrease in absorbance was followed at 340 nm at intervals of 2' for 6' in a UV-VIS spectrophotometer (Cary 100, Varian). A sample blank and reagent blank were also run. Enzyme activity was calculated using molar extinction coefficient of NADH as 6.22 x 10³ M⁻¹cm⁻¹ and results were expressed as nmol NADH oxidized/min/mg protein.

3.9.4.2 Succinate: 2, 6-dichlorophenolindophenol oxidoreductase (SDH) assay

SDH was assayed according to the method of Lambowitz (1979).

**Principle:** Succinate: DCPIP oxidoreductase (complex II, EC 1.3.5.1) transfers electrons from succinate to quinone, reducing the former in the process. It is the second point for entry of electrons into the ETC besides complex I. The assay utilizes an artificial electron acceptor DCPIP. The use of KCN inhibits the terminal complex of ETC. Thus, all the electrons from succinate are taken up by DCPIP. Reduction of DCPIP causes it to decolorize and decrease in absorbance is followed at 600 nm.

**Reagents:** 1) Potassium phosphate buffer PB, pH 7.6 (50 mM). 2) Sodium succinate (1.2 M) prepared in 50 mM PB, pH 7.4. 3) Potassium cyanide (KCN, 60 mM) prepared in 50
mM PB, pH 7.4. 4) 2, 6-dichlorophenolindophenol (DCPIP, 1.125 mM) prepared in 50 mM PB, pH 7.4.

**Procedure:** A reaction cocktail was freshly prepared with 50 mM PB, pH 7.4. (2.75 ml), succinate (0.05 ml), KCN (0.05 ml) and DCPIP (0.05 ml) per tube. Tubes were covered with glass balls and equilibrated at 37 °C. Absorbance of cocktail was noted at 600 nm. Sample (0.1 ml) suitably diluted as to contain 50-150 μg protein was added to tube and properly mixed. Its absorbance was recorded at 0, 3 and 15 mins intervals. Turbidity factor was obtained as difference between absorbance of sample at 0 min and that of cocktail alone at 0 min and was deducted from actual absorbance values. Activity was expressed as units/min/mg protein. Results were calculated by using the following formula.

\[
\text{Activity} = \frac{\ln \left( \frac{A \text{ at 3 min}}{A \text{ at 15 min}} \right) \times 1000}{12 \times \text{protein (mg)}} / 0.1 \text{ Units/min/mg protein}
\]

**3.9.4.3 Succinate: cytochrome c oxidoreductase assay**

Enzyme activity was assayed following the method of Tisdale (1967).

**Principle:** Succinate: cytochrome c oxidoreductase (Complex II-III) catalyses transfer of electrons from succinate to oxidized cytochrome c, through quinone intermediate. Sodium azide is used to inhibit the action of terminal complex IV of the electron transport chain. Thus, the electrons from succinate are taken up by complex II and then passed on to complex III, which reduces cytochrome c. The process can be followed from changes in absorbance at 550 nm.

**Reagents:** 1) Potassium phosphate buffer (PB), pH 7.6 (10 mM). 2) Sodium succinate (780 mM) prepared in 10 mM PB, pH 7.6. 3) Ethylenediamine tetraacetic acid (EDTA, 16 mM) prepared in 10 mM PB, pH 7.6. 4) Sodium azide (780 mM) prepared in 10 mM PB, pH 7.6. 5) Cytochrome c (1%) prepared in 10 mM PB, pH 7.6.

**Procedure:** 870 μl of 10 mM PB, pH 7.6 was carefully pipetted into cuvette. 10 μl each of succinate, EDTA and azide were added and allowed to stabilize. Sample (100 μl) appropriately diluted so as to contain 25-50 μg protein was added to this. Reaction mixture (1 ml) was then incubated at 37 °C for 2 min. Reaction was initiated by addition of 25 μl of 1% oxidized cytochrome c and increase in absorbance was followed at 550 nm at intervals of 1
min for 6 min in a UV-VIS spectrophotometer (Cary 100, Varian). A sample blank and reagent blank were also run. Enzyme activity was calculated using molar extinction coefficient for cytochrome c (21 x 10³ M⁻¹cm⁻¹), and expressed as nmol cyt c reduced/min/mg protein.

3.9.4.4 ATPase assay

ATPase activity was assayed following the protocols mentioned by Cormier et al. (2001) and Chen et al. (1956).

**Principle:** ATPase (complex V, EC 3.6.1.34) catalyses the oxidative and iP phosphorylation of ADP into ATP *in vivo* and is a measure of ATP synthesis in the mitochondria. *In vitro*, F1 part of the isolated enzyme have ATPase action to hydrolyze ATP to ADP and inorganic phosphate (Pi). The Pi is then estimated using a color reagent as described (Chen et al., 1956).

**Reagents:** 1) ATPase buffer (TMB, 50 mM Tris, 5 mM MgCl₂, pH 7.6). 2) Adenosine 5'-triphosphate (ATP, 50 mM) prepared in ATPase buffer. 3) Trichloro acetic acid, TCA (50%, v/v). 4) Standard KH₂PO₄ (8 mg/ml in DW). 5) Sulphuric acid, H₂SO₄ (6N, v/v). 6) Ammonium molybdate (2.5%, w/v) prepared in DW. 7) Ascorbic acid (10%, w/v) prepared in DW. 8) Color reagent: Freshly prepared mixture of distilled water, 6N H₂SO₄, 2.5% ammonium molybdate and 10% ascorbic acid (2:1:1:1, v/v).

**Procedure:** Firstly, for incubation with ATP at 37 °C, following sets of tubes were prepared.

<table>
<thead>
<tr>
<th>Sample blank</th>
<th>ATP blank</th>
<th>ATP + sample tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (TMB): 800 µl</td>
<td>800 µl</td>
<td>700 µl</td>
</tr>
<tr>
<td>ATP: 100 µl</td>
<td>--</td>
<td>100 µl</td>
</tr>
<tr>
<td>Pre-incubation at 37 °C for 10 min. Sample (10-20 µg protein) was added.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample: --</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Incubation at 37 °C for 30 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCA: 100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Total</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The above reaction mixtures (1 ml volume each) were centrifuged at 5,000 rpm for 10'. Supernatants thus obtained were used for estimation of Pi with color reagent. Supernatant (0.1 ml) was mixed with distilled water (1.9 ml) and color reagent (2 ml).
Reaction mixture (4 ml) was vortexed and incubated at 37 °C for 2 h. Absorbance of the color product was finally read at 820 nm and KH₂PO₄ was taken as standard. Final sample absorbance values were obtained as: Abs of ATP + sample tube – (Abs of ATP blank + Abs of sample blank). The enzyme activity was expressed as μmol iP formed/h/mg protein.

3.10 OS and AOD parameters

In the mitochondrial fraction, LPx, PC and H₂O₂ were measured as OS parameters and non-protein -SH as small antioxidant as described in the respective sub-sections of section 3.7.

3.11 Statistical analysis

Until unless described, all results were expressed as mean ± standard deviations (SD). Difference among the means was considered significance at P < 0.05 levels. Separate statistics were used when ever required as described bellow.

3.11.1 OS physiology parameters

For seasonal study (experiment 1), data were compared and analyzed by two way ANOVA analysis followed by Duncan’s new multiple range test. Correlation coefficient was determined between oxidative stress parameters and physico-chemical parameters of the sampling site of the lagoon. Additionally, discrimination function analysis was performed to evaluate the contribution of the variables of oxidative stress parameters, antioxidant enzymes and small antioxidant molecules on the groups. The protocol for the discrimination function analysis was performed after Jennrich (1977) and Garson (2008).

For salinity treatment experiments (experiment 3, 4 and 5), data were compared and analyzed by one way ANOVA analysis followed by Duncan’s new multiple range test.

3.11.2 O₂ consumption, CO₂ and NH₃ excretion

Data of O₂ consumption, CO₂ and NH₃ release within a sex (male or females) were compared and analyzed for differences using one way repeated ANOVA according to Arsham (2010) using the web service (StatPages.org) and between sexes (male and female) by two way ANOVA analysis followed by Duncan’s new multiple range test.