MATERIALS AND METHODS
Collection of carp and their maintenance

Healthy and active *L. rohita* fingerlings (5 ± 1 g, 7.5 ± 0.25 cm) were procured from the State Fisheries Department, Dharwad, India. Before investigation fish were maintained for 30 days in large cement tanks (22 × 12 × 5 feet). Further, carp were conditioned (acclimatized) to laboratory conditions for 20 d at 22 ± 2 °C in 100 L glass aquaria (120 × 45 × 80 cm) containing dechlorinated tap water of the quality used in the test. Characteristics of the water were determined by the methods mentioned in APHA (2005). Water was renewed every day with 12-12 h dark and light cycle was maintained and fish were fed *(ad libitum)* daily with commercial dry feed pellets (Nova, Aquatic P. Feed) during acclimatization and test periods. But for acute toxicity test, feeding was stopped two days prior exposure to the test medium. During acclimation batches with less than 5% of mortalities were only considered for further experimentations.

Factors influencing pesticide toxicity: The following factors, which are likely to contribute variations in toxicological investigations, were approximately nullified to a satisfactory level.

*Flow of water:* Since, significant difference in the toxicity of pesticides between static and flowing water in fish was reported by Burke and Ferguson, (1969), the experiments of the present investigation were conducted in static renewal media, as suggested by Doudoroff, *et al.*, (1951).
Temperature: It was reported that pesticide toxicity increases with a rise in temperature of the medium (Macek, et. al., 1969). Therefore, throughout the present investigation the temperature of the water was maintained invariably at 29±1°C.

Density of fish: Since, increase in fish density was known to enhance the toxicity of the pesticides (Holden, 1970), a constant ratio of fish bio-mass to water volume was maintained by taking one fish per liter of water.

Water quality: Since it is an established fact that chemistry of water influences the toxicity of chemical (Pickering and Henderson, 1962).

Physico-chemical characteristics of water quality used in the present studies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Datum</th>
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<tbody>
<tr>
<td>Temperature</td>
<td>22 ± 2 °C</td>
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<tr>
<td>pH</td>
<td>7.09 ± 0.10 at 24 °C</td>
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<tr>
<td>Dissolved oxygen</td>
<td>8.90 ± 0.45 mg/L</td>
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<tr>
<td>Carbon dioxide</td>
<td>2.30 ± 0.25 mg/L</td>
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<tr>
<td>Hardness</td>
<td>27.3 ± 3.3 mg as CaCO$_3$/L</td>
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<tr>
<td>Total alkalinity</td>
<td>21.4 ± 5.1 mg as CaCO$_3$/L</td>
</tr>
<tr>
<td>Conductivity</td>
<td>&lt; 10 μS/cm</td>
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<tr>
<td>Specific gravity</td>
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<tr>
<td>Particular matter</td>
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<tr>
<td>Total organic carbon</td>
<td>&lt; 2 mg/L</td>
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<tr>
<td>Unionised ammonia</td>
<td>&lt; 0.87 μg/l</td>
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<tr>
<td>Residual chlorine</td>
<td>&lt; 0.77 μg/l</td>
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<tr>
<td>Total pesticide residues</td>
<td>ND</td>
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Experimental toxicant

Cypermethrin (92.95%) was procured from the Herbana Industries Limited, Borivli (W) Mumbai, India. The stock solution was prepared in acetone, which was found to be nontoxic to fish. Required quantity of cypermethrin was drawn directly from this emulsified concentrate.

Acute toxicity test

The acute toxicity (96 h LC$_{50}$) of cypermethrin for the freshwater fish, *L. rohita* was determined in the laboratory using the semi-static method in OECD (1996). The carp (10 fingerlings in 20 L of test medium in each replicate) were exposed to varying concentrations of cypermethrin with six replicates for each concentration along with the control sets. Concentrations of the test compound used in short term definitive tests were between the highest concentration at which there was 0% mortality (3μg/L) and the lowest concentration at which there was 100% mortality (5.5μg/L). Test medium was renewed for every 24 h with their respective test concentrations of the toxicant without aeration. Exposure concentrations of cypermethrin in the test medium were confirmed by High Performance Liquid Chromatography (HPLC) analysis.

Mortality was recorded every 24 h and the dead fish were removed when observed, every time noting the number of fish death at each concentration up to 96 h for estimation of acute toxicity (LC$_{50}$). Duncan’s multiple range test (Duncan 1955) was employed for comparing mean mortality values after estimating the residual variance by repeated measures ANOVA (Winner, 1971) for arc sine transformed mortality data (dead
individuals/initial number of individuals). Time of exposure was the repeated measure factor while treatment (concentration and control) was the second factor. The LC$_{50}$ was calculated using probit analysis (Finney, 1953), which has been recommended by OECD guideline as an appropriate statistical method for toxicity data analysis (Lilius, et. al, 1994). After linearization of the concentration response curve by logarithmic transformation of concentrations (log$^2$), 96 h LC$_{50}$ with 95% confidence limits and slope function were calculated to provide a consistent presentation of the toxicity data.

**Experimental design and test concentrations**

**Fixation of lethal and sublethal concentrations:** Taking into consideration of the fact that the effect of a pesticide on fish becomes consistent with 96 hours of exposure for LC$_{50}$ (4.0 µg/l) of cypermethrin was taken as lethal concentration to study the behavioural, physiological and biochemical responses of the fish, *Labeo rohita*. However, knowledge on the concentration of toxicant that kills 50% of the test animals in a fixed period of time could become insufficient to assess various responses of the animal to toxicant. Further, studies on acute toxicity have significant limitations such as the occurrence of adaptation of test animal to the imposed toxicity (Stockner and Anita, 1976). Hence, Perkin, (1979) also viewed the need for sub lethal studies because distinct changes involving a sequence of events in the responses of test animal could occur in sublethal concentration. So, one tenth of the 96 hours LC$_{50}$ (0.40 µg/l) was taken as the sub lethal concentration of cypermethrin for further studies.
Fixation of exposure periods: Since the duration of exposure is having a great influence on the toxicity of a pesticide on an organism. The effects of lethal and sublethal concentration of cypermethrin were studied at different periods of exposure in order to understand the influence of time over toxicity. In the lethal 24, 48, 72, 96 h and in the sub lethal 1, 5, 10, 15 days were chosen to observe the Acute and Subacute effects of cypermethrin on the fish, Labeo rohita.

Experimental design: On determination of 96 h LC$_{50}$ further studies were carried out on the gill, muscle, liver at 24, 48, 72 and 96 h of exposure to lethal and 1, 5, 10 and 15 days of exposure to the sublethal concentration of cypermethrin. Selection of the gill, muscle, liver and testis, ovary (reproductive studies) was to understand the difference in the effects of cypermethrin in different tissues.

Tissues selected

- Biochemical parameters: Gill, Muscle and Liver.
- Histopathology: Liver, Testis and Ovary
- Ultrastructural pathology: Liver and Testis
- Haematology and Hormone assays: Blood

Fishes were exposed to their respective lethal and sublethal concentration of cypermethrin and were maintained in these concentrations up to the stipulated period of exposure. At the end of exposure the fishes were stunned to death and the target organs were dissected out from each animal using sterilized instrument. The organs were weighed accurately on electrical semi-microbalance and transferred into ice-jacketed micro beakers containing fish ringer solution. The fish ringer was prepared as per the
composition given by Ekenberg, (1958). An equilibration time of 15 minutes was allotted to the organs to regain normalcy from a state of shock, if any, due to the handling and dissecting procedures. The experiment was repeated for six times and results were analysed.

**Behavioural studies**

Monitoring of test species is performed at regular time intervals to observe and record the spectrum of behavioral responses at both the control (toxicant free medium) and cypermethrin treated fish as described by Murthy, (1987) adapted (Shivakumar and David 2008).

**Assay of respiratory rate**

Respiratory rate (oxygen consumption) of cypermethrin exposed fish was measured besides control by following the method of Welsh and Smith (1953) as described by Shivakumar and David (2008) and the apparatus setup was the same as described by Saroja, (1959). The difference in the oxygen content of the initial and final samples was taken as the amount of oxygen consumed by the fish during the period of experiment. After experiment, the fish were individually weighed and their unit metabolism was calculated and expressed as ml oxygen consumed/g wet weight/h.

**Estimation of Acetylcholine (ACh) content:** The tissue ACh content was estimated by the method of Hestrin as described by Augustinson (1957). After isolating and weighing, they were teased and transferred in tubes already kept in boiling water bath for 10 min to inactivate the enzyme acetylcholinesterase and to release bound ACh. The tubes were cooled and the contents were homogenized in 2.0 ml of distilled water. 2.0 ml of alkaline
hydroxylamine hydrochloride and 1.0 ml of 1:3 diluted hydrochloric acid with water was added. The contents were centrifuged and 1.0 ml of ferric chloride was added to the supernatant. The optical density of the sample was measured at 540 nm in spectrophotometer against a blank. The blank consisted of 2.0 ml of distilled water, 2.0 ml of alkaline hydroxylamine hydrochloride, 1.0 ml of diluted HCL and 1.0 ml of ferric chloride solution. A standard graph was prepared with ACh and the values were expressed as µM of ACh/g wet wt. of tissue.

Estimation of AChE (EC 3.1.1.7) activity: AChE activity was assayed by the method of Ellman, et. al. (1961) by measuring the increase in extinction at 412 nm in a spectrophotometer (Systronics, model no. 169) using acetylthiocholine iodide as substrate. Homogenates (4%) of tissues were prepared in cold 50 mmol Tris-HCl (pH 6.8) extraction buffer using a glass-teflon homogenizer (Remi Motors Ltd., Mumbai, India) and then centrifuged (Remi Motors Ltd., model no. C-24 BL) at 3000 rpm for 15 min. AChE activities were expressed as nmol of acetylthiocholine iodide hydrolyzed/mg protein/min.

Catalase activity (EC 1.11.1.6): Catalase activity was estimated by the method described by Luck (1974). Homogenates (4%) of tissues were prepared in cold phosphate extraction buffer (50 mmol, pH 7) using a glass-teflon homogenizer and then centrifuged at 3000 rpm for 15 min. To the supernatant (1 ml), 10 mmol of hydrogen peroxide in 2 ml of phosphate buffer (50 mmol, pH 7) was added as a substrate for catalase to initiate the incubation. The decrease in absorbance of the sample was measured at 240 nm using UV-visible
spectrophotometer (Hitachi, model no. U-3310). The values were expressed as mmol of hydrogen peroxide decomposed/mg protein/min.

**Hydrogen peroxide levels:*** The tissue hydrogen peroxide level was determined according to the methods of Pick and Keisari (1981) using HRP and phenol red. The values were expressed as nmol of hydrogen peroxide/mg protein.

**Malondialdehyde (MDA):*** Malondialdehyde (MDA) the secondary product of lipid peroxidation was estimated in the tissue homogenates utilizing the colorimetric reaction of thiobarbituric acid (TBA) (Placer, *et al.*, 1966). It gives an index of the extent of progress of lipid peroxidation, since the assay estimates the amount of TBA reactive substance e.g., MDA, it is also known as TBARS (thiobarbituricacid reactive substance) test. Tissue homogenates (16%) were prepared in cold 50 mmol Tris-HCl (pH 6.8) extraction buffer. To 0.8 ml of the homogenate, 2 ml of 15% trichloroacetic acid (TCA) was added and centrifuged at 5000 rpm for 15 min. To the entire supernatant, 0.7 ml of TBA reagent (1%) was added and the test tubes were covered with aluminum foil followed by incubation in a shaking water bath for 60 min at 100 °C. The tubes were then transferred to ice-cold water for 10 min and the absorbencies were read at 532 nm against a reagent blank. The rate of LPO was expressed as nmol TBARS formed/mg of protein using a molar extinction coefficient of $1.56 \times 10^5 \text{mol}^{-1} \text{cm}^{-1}$.

**Estimation of protein carbonyl levels:*** Protein carbonyls were derivatised with 2,4-dinitrophenyl hydrazine (DNPH) and measured by spectroscopy.
using the technique of Quinlan, et. al, (1994). Two aliquots (40 µl; 16% homogenate in cold 50 mmol Tris-HCl [pH 6.8] extraction buffer) of each sample were taken and freshly prepared 1 ml of 10 mmol DNPH in 2 mol hydrochloric acid (HCl) was added to the first and 1 ml of 2 mol HCl was added to the second making an individual blank for each sample. These were then incubated with gentle mixing at 37 °C for 90 min. After incubation, 2 ml of 15% TCA was added to each tube, which was then placed on ice for 5 min and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the protein pellet was washed in 5 ml mixture of 50% ethanol and 50% ethyl acetate and centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the washing step was repeated. The tubes were left upside down for 5 min to drain before the protein pellet was dissolved in 1 ml of 6 mol guanidine hydrochloride in 2 M HCl. This was left further in the dark for 60 min to allow colour development and the absorbencies were measured at 360 nm using a UV-visible spectrophotometer. Protein carbonyl concentrations were calculated by subtracting the paired control tubes from the DNPH-derivatized tubes and dividing by an extinction coefficient of 21,000 mol⁻¹ cm⁻¹ for the protein carbonyl-DNPH complex.

**Ionic composition and associated ATPases**

The levels of sodium, potassium and calcium ions and the activities of Na⁺- K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPases were estimated in gill, liver and muscle of fishes.
Estimation of sodium, potassium and calcium ions: The weighed organs were wet ashed in 50:50 (V/V) concentrated perchloric acid and nitric acid (Dall, 1967). After keeping the wet ash solutions for half an hour, until the organs were completely dissolved, they were evaporated at 100°C to 200°C temperature. The residues were dissolved in glass with distilled water and made up to 10 ml. It was filtered through whatman No.1 filter paper. Further, appropriate dilutions were made prior to estimations and the sodium, potassium and calcium ions were estimated with the help of Atomic Absorbance Spectrophotometer (GBC 932). Standard solutions of sodium, potassium and calcium were prepared by using analytical grade chemicals. The values are expressed as µg/g wet wt of the organ.

Na⁺- K⁺, Mg²⁺ and Ca²⁺ ATPase activities (ATPase phosphorylase EC 3.6.1.3.): Na⁺- K⁺, Mg²⁺ and Ca²⁺ ATPase activities were estimated separately in the organs by the method described by Watson and Beamish, (1981) with slight modification. 1% tissue homogenate (W/V) were prepared in ice-cold 0.25 M sucrose solution containing 5 mM EDTA (Prepared in 40 mM tris-HCl buffer at pH 7.5) and 0.01 M imidazole. The homogenates were centrifuged at 2500 rpm for 10 minutes and the supernatants were taken as crude enzyme extract for the assay of the ATPase enzyme activities.

After due standardization of enzyme kinetic parameters, three sets of incubation mixtures were prepared. In a total volume of 2 ml, the first set consisted of 100 mM disodium adenosine triphosphate (Prepared in 20 mM tris-HCl buffer at pH 7.5), 100 mM NaCl, 20 mM KCl, 3 mM MgCl and 0.3 ml
of enzyme extract. The second set consisted of 100 mM disodium ATP (Prepared 2 mM tris-HCl buffer at pH 7.5). 100 mM NaCl, 20 mM KCl, 3 mM MgCl 1 mM ouabain (potent inhibitor of Na⁺-K⁺ ATPase) and 0.3 ml of enzyme extract and the third set consisted of 100 mM disodium ATP (Prepared in 20 mM tris-HCl buffer at pH 7.8), 5 mM CaCl₂ and 0.3 ml of enzyme extract. All the three incubation sets were incubated at 37°C for exactly 15 minutes and then the reaction was arrested by adding 2 ml of cold 10% TCA. The inorganic phosphates liberated were estimated by the method of Fiske and Subba Rao, (1925). The absorbance was measured at 660 nm. Endogenous blanks were prepared to find out the endogenous inorganic phosphates. Another blank was prepared without using the co-factor to deduct the sodium salt stimulated activity as the co-factor used was a disodium salt of ATP.

The first set gave the total ATPase activities of Na⁺- K⁺ and Mg²⁺, whereas the second set gave only the Mg²⁺ ATPase activity as ouabain inhibits Na⁺-K⁺ stimulated ATPase. Hence, the Na⁺-K⁺ activity was derived by subtracting the Mg²⁺ ATPase from total of Na⁺- K⁺ and Mg²⁺, ATPase activities. The third set directly gave the Ca²⁺ ATPase activity. All these three ATPase activities are expressed as µM Pi liberated/mg protein/h.
The levels of soluble, structural and total proteins, free amino acids, ammonia, urea and glutamine were estimated in the gill, muscle, liver of fish under this study.

Estimation of soluble, structural and total proteins: The soluble, structural and the total proteins in the organs were estimated using the folin-phenol reagent method as described by Lowry, et. al, (1951). 1% homogenate (W/V) was prepared in ice-cold 0.25 M sucrose solution. For soluble and structural proteins 1.0 ml of the homogenate was taken and centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and to both the supernatant and residue 3 ml of 10% trichloroacetic acid was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residues were taken for experimentation. For total proteins, 1 ml of homogenate was taken, to it 3 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm. Supernatant was discarded and the residue was taken for experimentation. All the three residues were dissolved in 5 ml of 0.1 N sodium hydroxide and to 1 ml of each of these solutions, 4 ml of reagent -D (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. The samples were allowed to stay for 10 minutes, at the end of which 0.4 ml of folin-phenol reagent (Diluted with distilled water in 1:1 ratio before use) was added. Finally, the optical density of the colour developed was measured using spectrophotometer at a wavelength of 600 nm. A mixture of 4 ml of reagent-D and 0.4 ml of folin-phenol reagent was used as
Bovine albumin was used for the preparation of protein standards. The protein content is expressed as mg/g wet wt of the tissue.

**Estimation of free amino acids:** Free amino acid levels in the tissues were estimated by the ninhydrin method as described by Moore and Stein (1954). Homogenates (4%) were prepared in cold phosphate extraction buffer (50 mmol, pH 7) and 2 ml of 15% TCA was added to 0.2 ml of the homogenate followed by centrifugation at 3000 rpm for 15 min. To the entire supernatant (2.2 ml), 2 ml of ninhydrin reagent was added and the contents were boiled for exactly 5 min. The contents were cooled in ice-cold water and the volume was made up to 10 ml with distilled water and n-propanol in 1:1 ratio. The optical density of the colour developed was measured using spectrophotometer at a wavelength of 570 nm against a reagent blank. The free amino acid levels are expressed as μmol of tyrosine equivalents/g wet wt. of the tissue.

**Estimation of protease activity:** Protease activities in the tissues were estimated by the ninhydrin method as described by Davis and Smith (1955). Homogenate (4%) was prepared in cold phosphate extraction buffer (50 mmol, pH 7) and centrifuged at 3000 rpm for 15 min. To 2 ml of the supernatant, 0.5 ml of 1% casein and 2 ml of 0.1 mol phosphate buffer (pH 5) were added. The contents were mixed well and incubated at 37 °C for 30 min. The reaction was stopped by adding 2 ml of 2% ninhydrin reagent. Again the contents were mixed thoroughly and placed in a boiling water bath for 20 min. The solution was cooled and made to 10 ml with diluents (distilled water
and n-propanol in 1:1 ratio). The optical density of the colour developed was measured using spectrophotometer at 570 nm against a reagent blank. The protease activity is expressed as μmol of tyrosine equivalents/mg protein/h.

**Estimation of alanine aminotransferase (EC 2.6.1.1.) activities:** Activities of alanine and aspartate aminotransferase in the organs were estimated using method of Reitman and Frankel (1957). 5% homogenate (W/V) was prepared in 0.25 M ice-cold sucrose solution, centrifuged at 3000 rpm for 10 minutes and the supernatant was used as the source of enzyme. Two sets of incubation mixtures were prepared. The first set (for alanine aminotransferase activity) consisted of 0.5 ml of 0.2 M alanine and 0.5 ml of 0.005 M ketoglutaric acid (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. The second set (for aspartate aminotransferase activity). Consisted of 0.5 ml 0.2 M aspartic acid, 0.5 ml of 0.005 M keto-glutaric acid (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. Mixtures were incubated at 37°C for 30 minutes and then the reaction was stopped by the addition of 1 ml 0.001 M 2, 4-dinitrophenylhydrazine (ketone reagent). Finally, the reaction mixtures were made to 10.0 ml with 0.4 N sodium hydroxide and the optical density of the colour developed was measured using spectrophotometer at a wavelength of 545 nm. A blank taking 0.1 ml of distilled water and controls taking 0.1 ml of boiled enzyme were also run similarly. Pyruvate and oxaloacetate
standards were prepared alongside for comparison. The alanine aminotransferase activity is expressed as μ moles pyruvate formed/mg protein/h and the aspartate aminotransferase activity as μ moles oxaloacetate formed/ mg protein/h.

*Estimation of glutamine dehydrogenase (GDH) (E.C. 1.4.1.) activity:* GDH activity was estimated in the organ using the method of Lee and Lardy, (1965) with slight modification. 5% homogenate (W/V) was prepared in 0.25 M ice-cold sucrose solution and centrifuged at 2500 rpm for 20 minutes at 2°C to remove cell debris. The clear cell-free extract was subjected to dialysis against 0.25 M sucrose at 2°C to 4°C for 24 hours. The incubation mixture in a final volume of 2.0 ml contained, 40 μ moles of sodium glutamate, 100 μ moles of NAD (nicotinamide adenine dinucleotide) and 4.0 μ moles of INT (2-p-Indophenol-3-p-nitrophenyl-5-phenyltetrazolium chloride). The reaction was initiated by the addition of 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes in a thermostatic water bath and then the reaction was stopped by the addition of 5.0 ml of glacial acetic acid. The formozan formed was extracted into 5.0 ml of toluene overnight at 5°C. The optical density of the colour developed was measured using spectrophotometer at a wavelength of 495 nm. A blank by taking 0.5 ml of distilled water and control by taking of 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as μM formozan formed /mg protein/h.
Haematology

The State Fisheries Department, Dharwad, India, provided healthy and active 5 month old *Labeo rohita* for haematology of fish (35.70 ± 5.21 g; 13 ± 2.1 cm). Transportation and acclimation conditions remain same as of fingerlings. But carp (under yearlings) were conditioned in 260 L glass aquaria (160 × 100 × 120 cm). The experimental conditions, test concentrations, experimental periods and replicates remain same as described for fingerlings.

**Sampling of blood:** Fish were euthanized by an overdose of MS-222 and then weighed and measured. Blood was sampled by caudal severance from the disease free test species during early hours of the day and stabilized with 50 IU sodium heparin (anticoagulant)/ml blood.

**Haematological examination:** The haematological variables analyzed were red blood cells count (RBCC), haemoglobin (Hb), white blood cells count (WBCC), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

**Determination of red blood corpuscles (RBC) count:** RBC count was determined with an improved Neubauer crystalline counting chamber as described by (Shaperclaus, 1979). The blood was sucked up to 0.5 mark on the RBC pipette and immediately, Hayems solution as a diluent stain was drawn up to 101 mark and the pipette was rotated between the thumb and the forefinger to facilitate adequate mixing of the solution (dilution: 1:200).
The counting chamber and the cover glass were cleaned thoroughly and the cover glass was placed in position over the ruled area. The fluid from the stem of the pipette was expelled as it contains only the diluting fluid. The pipette was then held at an angle of 45° with the tip of the pipette at the junction of the edge of cover glass and the counting chamber. A drop of blood was placed from the tip of the pipette on the central platform near the edge of the cover slip, so that the drop was sucked up between the central platform and the cover slip by the capillary force. The cells were allowed to settle for 2 to 3 min. The ruled area of the counting chamber was focused under the microscope and the numbers of RBC were counted in 80 small squares (4 squares of 16 at the four corners and one of 16 at the center). The cells touching the upper and left hand lines were counted. The cells touching the lower and the right hand lines were omitted.

The numbers of RBC per sq mm were calculated as follows:

The area of a small square : 1/400 sq mm
The depth of the counting chamber : 1/10 mm
Therefore the volume of a small square is : \( \frac{1}{400} \times \frac{1}{10} = \frac{1}{4000} \) cu mm

The dilution of the blood is 1:200

Total no. of RBC = \( \frac{n \times 4000 \times 200}{80} \)

\( n = \) No. of cells counted in 80 small squares
**Determination of white blood corpuscles (WBC) count:** WBC count was determined by following the method described by Donald and Bonford (1963). The blood was drawn up to 0.5 mark of WBC pipette and immediately the diluting fluid was drawn up to the 101 mark above the bulb (the dilution fluid consists of 1.5 ml of glacial acetic acid and 1 ml of aqueous gentian violet solution and made up to 100 ml with distilled water). The solution was mixed thoroughly by shaking gently and allowed to stand for 3 min. Cleaned Neubauer counting chamber and cover glass were placed over the ruled area. Excess solution was expelled and a drop of fluid was allowed to flow under the cover slip by holding the pipette at an angle of 40° and allowed to stand for 2 to 3 min. The WBC was counted in the four corner square millimeters and the number of WBC per cubic millimeter was calculated.

**Estimation of haemoglobin (Hb):** Hb concentration in the blood was estimated by cyanmethaemoglobin method as described by Blaxhall and Daisley (1973). Hb is converted into cyanmethaemoglobin by the addition of potassium ferricyanide (KCN) and the colour was read in a spectrophotometer at 540 nm against a reagent blank.

**Determination of packed cell volume (PCV) or haematocrit value:** Packed cell volume was determined by micro haematocrit method of Schalm, *et al.*, (1975). The heparinised blood was filled up to the mark 100 of the haematocrit tube with the help of Pasteur pipette and centrifuged at 3000 rpm for 30 min. The relative volume of the height of the RBC's packed at the bottom of the haematocrit tube was recorded as packed cell volume in terms of percentage of total blood column taken in the haematocrit tube.
Determination of mean corpuscular volume (MCV): MCV indicates the average size of the red blood cell in a given sample of blood. MCV was calculated by the following formula and expressed as femtoliter (fL).

\[
MCV = \frac{\text{Haematocrit} \times 10}{\text{RBC count}}
\]

Determination of mean corpuscular haemoglobin (MCH): MCH represents the average content of the Hb in each red blood cell. MCH is influenced by the Hb concentration and the number of RBCC. MCH was calculated by the following formula and expressed in picogram (pg).

\[
MCH = \frac{\text{Haemoglobin} \times 10}{\text{RBC count}}
\]

Mean corpuscular haemoglobin concentration (MCHC): MCHC reflects the average concentration of the haemoglobin in the red blood cells in a given volume of the blood. MCHC was obtained by the following formula and expressed in terms of gram percent (g%).

\[
MCHC = \frac{\text{Haemoglobin} \times 100}{\text{Haematocrit} \times 100}
\]

Histopathology

The histopathology studies were carried out in principal metabolic organ Liver of fish, *L. rohita*, exposed to lethal (24, 48, 72 and 96h) and sublethal (1, 5, 10 and 15 days) concentrations of cypermethrin. To study the histopathology of tissue, the method described by Humason (1972) was followed. Treated and control organs of fish were isolated and fixed in Bouin's fluid for 24 hours at room temperature. Tissues were repeatedly
washed with 70% alcohol till all the traces of Bouin’s fluid were removed. Dehydration process was carried out by washing the tissue with alcohol (90% and 100%), alcohol-benzene in different ratios (3:1, 1:1 and 1:3) followed by pure benzene and benzene-paraffin wax (1:1). After this process, the organs were embedded in paraffin (58-60°C). Sections were taken (5-6 micron thickness), stained with Mayer’s haematoxylin (Mayers and Hendricks, 1985) and counter stained with eosin. All sections were mounted with DPX and histopathological changes were observed under light microscope.

Ultrastructure of liver

Treated and control liver of fish were isolated by perfusion and fixed in buffered glutaraldehyde (2.5%; pH 7.2). The liver was stored in the sodium cacodylate buffer at 4 °C (pH 7.4, 0.1M), washed in buffer and post fixed in the 1% osmium tetraoxide for one and half to two hours. Then again washed with buffer, dehydrated in alcoholic series for 1 h, stained enblock in 2% uranyl acetate, in 90% methanol for 1 h and cleared in propylene oxide for 10-15 min and infiltered with araldite : propylene (1:1) mixture for over night. Then infiltered again with fresh araldite (3 changes with a gap of 3-4 h) and embedded in the same media in a beam capsule. The blocks were cut in Leica LKB Broma ultramicrotome. Ultrathin sections were cut at 100-300 Å, mounted on copper grids and stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963). The stained sections were scanned in Jeol – TEM 100 C X II electron microscope for ultrastructural observations.
Reproductive studies

Collection of test animals and maintenance: Adult male and female (1600-1800 g; 40-50cm) fish, *L. rohita*, collected at the local ponds in and around Dharwad, India, were used in this study. All procedures implemented in this study on fish were according to the guidelines of the Institutional Animal Ethics Committee (IAEC) for the Purpose of Control and Supervision of Experiments for Animals (CPCSEA), New Delhi, India. The fish were collected using cast nets three months prior to experimentation and were acclimatized to laboratory condition for 15 d at 24°C. They were held in dechlorinated tap water in large cement tanks previously washed with potassium permanganate to free the walls from any microbial growth. Fish were fed regularly with specially prepared fish food with 12-16 h of photoperiod daily during acclimation and test periods. Water was changed weekly, whose physico-chemical characteristics were analyzed following the methods mentioned in APHA (20005).

Collection tissues: Treated and control organs of fish were autopsied, testis and ovary isolated and fixed in Bouin’s fluid for 24 hours at room temperature. Tissues were processed for histopathology by method described by Humason (1972). For ultrastructural studies tissues were fixed in buffered glutaraldehyde (2.5%; pH 7.2) and processed for electron microscopy.

Collection blood samples: Fish were euthanized by an overdose of MS-222 and then weighed and measured. Blood was sampled by caudal severance.
from the disease free test species during early hours of the day and stabilized with 50 IU sodium heparin (anticoagulant)/ml blood. The blood was drawn from the caudal veins. Immediately after collection, the blood samples were kept under 4°C. Collected blood samples were centrifuged and the serum was decanted and frozen.

**Hormonal assays:** Centrifugally separated plasma was subjected to hormonal (Testosterone, 11 Keto testosterone, Vittellogenin, Estradiol) assays using solid phase enzyme linked immunosorbant assay (ELISA) according to the methods described in the ELISA kits by the suppliers. For testosterone kit was obtained from united biotech Inc Canada (UBI), Magiwel™. Vittellogenin, Estradiol and 11 Keto testosterone kits were supplied by Cayman™ chemicals company Inc, UK.

**Statistical analysis of data:** Data of oxygen consumption, biochemical studies and haematology, correspond to the average of six replicates except hormonal assays (average of three). The data obtained were analyzed statistically by following Duncan’s multiple range test (Duncan, 1955).