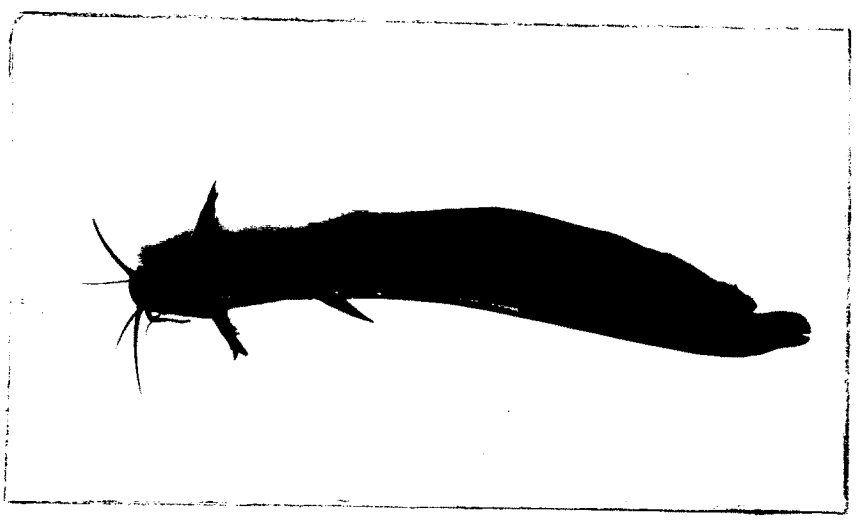
MATERIALS AND METHOD



Heteropneustes fossilis

TEST ANIMAL

Phylum	-	Chordata
Subphylum	-	Vertebrata
Class	-	Pisces
Subclass	-	Teleostomi
Order	-	Siluriformes
Family	-	Heteropneustidae
Genus	-	*Heteropneustes*
Species	-	*fossilis*

The freshwater teleost *Heteropneustes fossilis* locally called "singhi" was used as the test animal. Healthy living specimens of the freshwater teleost *H. fossilis* were collected from the local freshwater ponds or purchased from the fish market at Kesar Bagh, Lucknow. Fish were disinfected with 0.1% potassium permanganate solution and were maintained for three weeks in well-aerated tap water. Prior to experimental they were acclimated to experimental tanks for at least a week. Fish measuring 15 ± 2 cm in length and 30 ± 5 gm in weight were selected. Water samples from the different tanks were routinely analyzed. The water characteristics were temperature 22 ± 4^0C; pH 6.9 ± 2; dissolved oxygen 8.0 ± 1.7; hardness 115 ± 3 ppm (as $CaCO_3$) and alkalinity 35.5 ppm as ($CaCO_3$).

TOXICANT USED

Deltamethrin commonly known as decis is a synthetic pyrethroid used for agriculture, household and forsetry was selected for the present study.

Trade Name	-	Decis
Active ingredient	-	Deltamethrin
Molecular formula	-	$C_{22}H_{19}Br_2NO_3$
Formulation	-	Emulsifiable concentrate 2.8%
Manufacturer	-	Hoechst Schering Agro Evo Limited, GIDC, Ankleshwar-393002, Gujrat.

Chemical formula - (S)-X-cyano-3-phenoxybenzyl (1R, 3R) – 3 -(2 2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate.

TOXICITY TEST

Preliminary toxicity test was conducted under laboratory conditions to determine the LC_{50} values for 96 hr of deltamethrin. Stock solution of the pesticide deltamethrin were prepared in acetone by simple dilution technique. Prior to experimentation, the best fish were examined carefully for pathological symptoms and fish disinfected with 0.1% $KMnO_4$ solution were transferred from the acclimation tank to the experimental containers. From the stock solutions measured quantities of deltamethrin were added separately to each container. Requisite quantity of water was added to each tank for further dilution ensuring the availability of at least one liter of the test solution for each gram biomass of the fish. After 24 hr, dissolved oxygen and pH of the experimental solution were recorded. The fish were considered dead when they did not respond on being prodded with a glass rod and respiration ceased. The mortality data were statistically analyzed by the Trimmed Spearman Karber method (Hamilton *et. al.* 1977). The LC_{50} value for 96 hr was 0.52 mg/L for deltamethrin. In order to observe the chronic effects of the pesticide fish were exposed tc sublethal concentration 0.085 and 0.17 mg/L for 30 days. Water in the tanks was renewed after every 24 hr and fresh solution of the pesticide was added to bring the concentration to the desired level. During the entire tenure of experiments, fish were regularly checked for infection, disease and other unhealthy conditions. At the expiry of each experimental period, control and pesticide exposed fish were processed simultaneously.

FOOD AND FEEDING

The fish were conditioned to feeding on pelleted diet (Prawn powder, fish powder and minced liver in 2:2:1 ratio) at the rate of 3% of

the body weight. Feeding was discontinued during the acute exposure period. For chronic exposure period, the fish were fed twice daily.

HAEMATOLOGICAL STUDY

Blood was drawn from the dorsal aorta into ethylenediamine tetraacetate (EDTA) coated red and white cell diluting pipettes for total erythrocyte, leukocyte and thrombocyte count. The blood was diluted with Toisson's, Turk's and Rees Ecker fluids for erythrocyte, leukocytes and thrombocytes counts respectively. The diluted blood was charged into improved Neubauer Haemocytometer and total number of the cells was calculated (Dacie and Lewis, 1977). The total blood cell counts were also confirmed with the help of Labora Mannhem GmbH laboratory counter (W. Germany). Haematocrit (%) was measured by using 75 X 1.0-1.25 mm capillary tubes and the erythrocytes sedimentation rate (ESR) being measured mm/hr by the Micro-Wintrobe method (Blaxhall and Daisley, 1973) using tubes similar to those used for haematocrit. Clotting time of the blood was determined as described by (Srivastava, 1969) and haemoglobin content (gm %) was determined by Sahli-Hellige method.

Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin content (MCHC) and mean corpuscular volume (MCV) were calculated according to the methods of Dacie and Lewis (1977). The MCH indicates the average weight of haemoglobin in RBC and is obtained from the following formula.

$$\text{MCH (gm \%)} = \frac{\text{Haemoglobin (gm/dL)} \times 10}{\text{No. of RBC/mm}^3}$$

The MCHC is an expression of the average concentration of haemoglobin in RBC. It gives the ratio of weight of haemoglobin to the volume of the RBC. It was calculated as follows:

$$\text{MCHC (\%)} = \frac{\text{Hb (mg/dL)} \times 100}{\text{PCV}}$$

Likewise, MCV exhibits the average volume of the red blood cells and has been calculated by the formula given below:

$$MCV\ (\mu m^3) = \frac{PCV\ X\ 10}{No.\ of\ RBC/mm^3}$$

For differential leukocyte count a drop of fresh blood was placed on a clean dry glass slide and spread to a thin smear of blood. The smear was then dried and fixed by a momentary dip in methanol. The cells were stained with Leishman stain for 2 minutes. The slides were washed, dried and examined under oil immersion lens in a microscope. A total of 100 cells were counted on digital counter and differentiated into neutrophils, small and large lymphocytes, eosinophils, basophils and monocytes.

BIOCHEMICAL STUDY

Blood was drawn from the dorsal aorta in direct penicillin vials containing 0.1 ml of double oxalate mixture for biochemical and ionic parameters. The blood was gently mixed with the oxalate mixture to avoid coagulation.

Blood Glucose Estimation

Blood glucose was determined by the method (Nelson- Somogyi, 1944). To 0.2 ml of blood, 1.9 ml of zinc sulphate and 1.9 ml of barium hydroxide were added. The contents were shaken vigorously and centrifuged for 10 minutes. To 0.5 ml of supernatant, 1.0 ml of alkaline copper was added and the tubes were placed in boiling water bath for 20 minutes. The tubes were then cooled and 1.0 ml of arsenomolybdate colour reagent was added with shaking. The volume of each tube was made up to 10 ml and the optical density was measured at 540 µm with Spectrophotometer. Blank and standard tubes were processed similarly.

Protein Estimation

The quantitative estimation of total protein in tissues and serum of *H. fossilis* was made according to the method of Lowry *et al.* (1951) using

bovine serum albumin (BSA) as standard. Tissue samples were deproteinized with 10 % trichloroacetic acid (TCA). After centrifugation for 10 minutes, the supernatant was discarded and protein precipitate was washed with 5 % trichloroacetic acid (TCA) and centrifuged again for 10 minutes. The precipitates were dissolved in 1 ml of 1.0 N NaOH. Equal volume (0.5 ml) of dissolved precipitates, distilled water and alkaline copper reagents were placed in test tubes. The mixture was kept for 10 minutes for complex formation. Then 0.5 ml of Folin's reagent was added to each tube and left for about 30 minutes at room temperature. Blank and standards were also prepared by the same procedure and the optical density was read at 660 nm with Spectrophotometer.

Lactic Acid Estimation

Lactic acid was determined by the method of Barker and Summerson (1963). Blood and tissue homogenates were deproteinized with trichloroacetic acid (TCA) at a 1: 10 dilution. 2 ml of the protein free filtrate, representing 0.2 ml of sample was transferred to a centrifuge tube graduated at 10 ml. In a second similar tube 5 ml of standard lactic acid solution containing 0.01 mg of lactic acid per ml was placed. In a third test tube equal volume of water served as a blank to control the small amount of colour yielded by the reagents alone. To each tube 1 ml of 20 % copper sulphate solution was added and diluted to 10 ml with water. 1 gm calcium hydroxide powder was added to each test tube, stoppered and shake vigorously until the solids were uniformly dispersed. The tubes were allowed to stand for half an hour. 1 ml portion of the supernatant was transferred to thoroughly clean and dried test tubes having an internal diameter of 18 to 23 mm. To each tube, 0.05 ml of 4 % copper sulphate solution was added followed by 8 ml of concentrated sulphuric acid from a burette. The contents of the tube were mixed well during the addition. The tubes were cooled and placed upright in a boiling water bath for 5 minutes, then transferred to cold water at 20 ^{O}C. When the

contents of the tubes were sufficiently cool, 0.1 ml of the parahydroxy diphenyl (p-OH) reagent was added drop by drop to each tube. The reagent precipitates out on entering the concentrated acid. Lateral shaking dispersed it throughout the solution as quickly and uniformly as possible. When the reagent has been added, the tubes were placed in a beaker of water at 30 ^{O}C and allowed to stand for 30 minutes. The precipitate was redispersed in vigorously boiling water for exactly 90 seconds, removed and cooled in cold water to room temperature. The colored solution was transferred to suitable containers and the optical density was determined at 560 nm with spectrophotometer using water as blank.

ENZYME ASSAY

Preparation of Enzyme Extract

For the estimation of the activities of different enzymes, vital tissues were removed immediately from both control and exposed groups of fish. The tissues were separated from the adjoining tissues, and blotted free of blood with filter paper. For the enzyme assay tissues were pooled separately after each exposure period. The tissues were then weighed to the nearest milligram and homogenized in Potter- Elvehjem type homogenizer. During homogenization, the homogeniser tube was packed in ice, so that a temperature near zero was maintained. The homogenates were adjusted to 10% (w/v) strength with 0.25M chilled sucrose solution. The homogenates were centrifuged under refrigeration at 1000xg for 20 minute and the clear supernatant fluids were used as the source of enzyme.

Lactate Dehydrogenase (LDH) Activity

The activity of lactate dehydrogenase was estimated by the method of Srikantan and Krishnamoorthi (1955). 0.5 M solution of sodium lactate was used as the substrate for lactate dehydrogenase. The incubation time was 2 hr at 23 ^{O}C. The reaction was stopped by the addition of 6.0

ml of glacial acetic acid and formazan formed was extracted with xylene at 0 ^{o}C by keeping overnight in a refrigerator. The optical density of the coloured xylene layer was read at 495 nm. The enzyme activity is expressed in ug of formazan formed per mg of enzyme protein per hour.

Na^{+}- K^{+} ATPase Activity

Enzyme activity was determined in reaction mixtures A and B in absence and presence of ouabain (Svobaca and Mossinger, 1981). Reaction mixture A for total ATPase activity contained 0.2 ml of 200 mM KCl, 0.2 ml of 1 M NaCl, 0.1 ml of 100 mM $MgCl_2$, 1 ml of 200 mM tris buffer at pH 7.4, 0.2 ml of distilled water and 0.1 ml of tissue homogenate. The mixture was pre-incubated at room temperature for 5 minutes and then incubated for 15 minutes at ambient temperature after adding 0.2 ml of 25 mM ATP di-sodium salt. For Mg^{2+} ATP activity, reaction mixture B contained 0.1 ml of 100 mM $MgCl_2$, 1 ml of 200 mM tris buffer pH 7.4, 0.1 ml of tissue homogenate, 0.16 ml of water, 0.2 ml of 10 mM ouabain and the reaction mixture was pre-incubated for 5 minutes. The reaction was initiated by adding 0.2 ml of 1 M NaCl and 0.2 ml of 25 mM ATP disodium salt and incubated for 15 minutes at ambient temperature. The reaction in both sets was terminated by adding 1 ml of 10% trichloroacetic acid (TCA) and centrifuged at 3000 RPM for 5 minutes. The supernatant was used for inorganic phosphate estimation (Fiske and Subbarow, 1925). To 0.5 ml of supernatant, 3 ml of distilled water, 0.5 ml of 2.5 % ammonium molybdate in 5N H_2SO_4 and 0.2 ml of 1, 2, 4 aminonaphthol sulphonic acid (ANSA) were added. The mixture was vortexed and optical density was read at 600 nm after 10 minutes. The difference in the inorganic phosphate (Pi) liberated in the two reactions mixtures gave the activity of Na^{+}-K^{+} ATPase. The ATPase activity was expressed as μmole Pi liberated/mg protein/hr.

Ca^{2+} and Mg^{2+} ATPase Activity

Ca^{2+} and Mg^{2+} ATPase activity was assayed by the method of Beutler (1984). To 0.5 ml of tissue (homogenate) in a test tube 0.5 ml of a mixture made up of 100 μl of $MgCl_2$ (0.05 M), 100 μl of $CaCl_2$ (2 mM), 100μl of tris hydrochloric acid buffer (0.5 M, pH 7.4), 200 μl of ATP (15 mM in Tris-HCl 0.5 M, pH 7.4) was added. Incubation took place at ambient temperature in a thermostat and once the reaction had been blocked, the operations described by Beutler (1984) were carried out. The ATPase activity was expressed as μmole Pi liberated/mg protein/hr.

ESTIMATION OF METAL IONS

The levels of sodium, potassium, calcium and magnesium were estimated in plasma and various tissues viz. kidney, gills, liver, brain, muscle and intestine of the catfish.

Plasma

Plasma was deproteinized with perchloric acid and after centrifugation the supernatant was used for the estimation of different metal ions after suitable dilution in 1% HCl on Perkin Elmer Model, 5000, Atomic Absorption spectrophotometer (SP-500).

Tissues

To 1.0 gm of tissue, 5 ml of nitric acid was added and left overnight. The dissolved tissue was then heated at low temperature till evaporation, 2 ml of digestion mixture (nitric acid, sulphuric acid and perchloric acid 6:1:1) was then added and again heated until it became colorless. It was diluted upto desired volume and estimation of different metal ions in different tissues were performed on Perkin - Elmer Model, 5000, Atomic Absorption Spectrophotometer (SP-500).

TRANSMISSION ELECTRON MICROSCOPY

Fixation

Tissues of *H. fossilis* exposed to deltamethrin were fixed in 3 % glutaraldehyde and 2 % paraformaldehyde for 3 hours and washed in 0.1 M sodium cacodylate buffer, three times at 10 minutes intervals each.

PREPARATION OF REAGENTS

Sodium Cacodylate Buffer- Stock Solution (Strength 2 M)

Sodium cocodylate (Na $(CH_3)_2)ASO_4$ $3H_2$) molecular wt.214, dissolved in 500 ml of triple distilled water (TDW) and stored at 4 ^{o}C.

Working Solution- (Strength 0.1 M)

When required, the stock solution was diluted with triple distilled water (TDW) 20 times (0.1 M) or 10 times (0.2 M to be used to prepare osmium tetraoxide solution). The final volumes were made after adjustment of pH to 7.2 ±0.1 using 1 N HCl or I N NaOH.

20 % Paraformaldehyde

20% paraformaldehyde solution was prepared by dissolving 20 gm of paraformaldehyde powder in about 60 ml of distilled water, which was heated upto 60 ^{o}C by constant stirring. To the turbid solution thus obtained a few drops (usually 3-4) of 40 % NaOH (4 gm in 10 ml of distilled water) were added drop by drop with constant stirring to achieve a clear solution and made up to a volume of 100 ml.

Preparation of Fixative

2% paraformaldehyde, 3% glutaraldehyde fixative in 0.1M cacodylate buffer was freshly prepared according to the method of Hoyer and Bucana (1982). Final volume was made upto 33.0 ml with triple distilled water and pH adjusted at 7.2 before making up the final volume.

POST FIXATION TREATMENT

Specimens collected were then post-fixed in 1 % osmium tetraoxide in 0.1 M cacodylate buffer at 4 ^{O}C for 1-2 hr.

Preparation of Osmium Tetraoxide Solution Stock Solution- (Strength 4%)

As described by Polade (1952) a stock solution of 4 % osmiumtetra oxide was prepared by dissolving the contents of 1 g vial (Sigma, USA) in triple distilled water in a thoroughly cleaned stoppered bottle and making up the volume to 25 ml. The solution is usually kept overnight for complete dissolution and stored in dark place covered with black paper.

Working Solution

The required amount of 1 % working solution was prepared with 1 ml triple distilled water and 2 ml of 0.2 M sodium cacodycate buffer.

Preparation of Suspended Materials Polylysine Schedule

Samples of suspended material like blood or any other particulate materials are prepared by Poly-L-Lysine schedule. Poly-L-Lysine, molecular wt. 70,000 to 1,50,000 (LKB) was used for this purpose. Small pieces of cover glasses were taken and one drop of 0.1% Poly-L Lysine is kept on that for 10-15 minutes at 4 ^{O}C. After that the treated surface of the cover glass was washed thoroughly in running tap water. Then the specimen is kept on the cover glass for 5 min. to allow the substance to adhere perfectly on it and after 5 minutes fixative was poured on its surface and processed further for transmission electron microscopy.

HISTOPATHOLOGY BY LIGHT MICROSCOPY

Two fish from each experimental and control group were sacrificed after 30 days.

Autopsy Procedure

A long intudinal incision was made in the abdominal wall so as to expose liver, gills, kidney, and intestine. These tissues were removed and thoroughly examined microscopically for size, shape, colour, texture and other pathological alterations. The liver, gill, kidney, and intestine were cut into small pieces and fixed in 10 % formaldehyde for 24 hr. After washing, the tissues were dehydrated in ascending grade of alcohol, cleared in xylene and embedded in paraffin wax (melting point 60-62 ^{o}C). Sections of 5 μm thickness were cut on a rotary microtome and stained with haematoxylin and eosin for routine histopathological study (M_C Manus and Lowry, 1965).

Endocrine Glands

The area adjoining the heart along with the oesophagus for ultimobranchial gland, corpuscles of Stannius along with the adjoining portion of kidney and the pituitary gland along with the brain was fixed in aqueous Bouin's fluid. Tissues were routinely processed in graded series of alcohol, cleared in xylene and embedded in paraffin wax. Serial sections were cut at 5 μm. The pituitaries were stained with Herlant tetrachrome and Heidenhain's azan techniques. Ultimobranchial gland was stained with hematoxyline-eosin (HE). For corpuscle of Stannius aldehyde fuchsin (AF) and HE were used.

Nuclear Volume

Nuclear volume of ultimobranchial gland (UBG), corpuscles of Stannius (CS) and prolactin cell indexes (maximal length and maximal width) were recorded with the help of ocular micrometer and stage micrometer, and the nuclear volume was calculated.

Volume = $4/3\ \Pi\ ab^3$

Where 'a' is the major semiaxis and 'b' is the minor semiaxis.

STATISTICAL CALCULATION

Standard Deviation

$$S = \sqrt{\frac{\Sigma Y^2 - (\Sigma Y^2)^2 / N}{(N-1)}}$$

Standard Error

$$SEM = \frac{S}{\sqrt{N}}$$

The standard error of the mean is a measure of the reliability of the mean calculated from a set of observations. `S' is the standard deviation, N is the number of observations.

Students `t' test given by Wardlaw (1985) was employed to calculate the significance of the difference between control and experimental means and `P' values of 0.05 or less were considered significant.

$$t = \frac{(\overline{Y}_2 - \overline{Y}_1)\sqrt{N}}{\sqrt{(S_1)^2 + (S_2)^2}}$$

In cases where the number of observations in two groups were different, the formula followed was:

$$t = \frac{(Y_2 - Y_1)}{S\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

Newman-Keuls multiple range test (Zar, 1974) was applied to calculate the significance of difference between groups in an exposure

period and between exposure periods within a group. The test considers the null hypothesis Ho:μB = UA, versus HA:μA, where the subscripts denote any possible pair of groups. For K groups, K(k-1)/1 different pairwise comparisons can be made.