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Research article

STUDIES OF CYPERMETHRIN ACTIVITY ON STEROIDOGENESIS IN FEMALE LABEO ROHITA

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ABSTRACT: A growing number of environmental toxicants like synthetic pyrethroids are believed to have deleterious effects on the development of non-target organisms by disrupting hormone sensitive processes. LC_{50} values of different forms of cypermethrin were evaluated by different research groups under laboratory conditions. In this work, the plasma concentrations of Vitellogenin (VTG), 17β-estradiol (E_2), testosterone (T), 17, 20β-dihydroxy-4-pregnen-3-one and Aromatase were evaluated in female Labeo Rohita. *Labeo rohita* were purchased from the local fisheries and fed with fish feed. Six female fish were kept in 500L water tanks and then exposed to 10 and 20 µg/L of Cypermethrin for six days. Vitellogenin, 11-Keto testosterone, Testosterone, 20β-dihydroxy-4-pregnen-3-one and Aromatase were estimated using kits from Biosense laboratories by Enzyme linked immune sorbent assay (ELISA). In female fish, testosterone levels in female have increased with increase in concentration of the toxicant and decrease of 17β-estradiol in exposed fish with increase in cypermethrin concentration. The levels of Vitellogenin and Aromatase have no significant change with increase of Cypermethrin.
Key words: Cypermethrin, Aromatase, Labeo Rohita, Vitellogenin, testosterone

INTRODUCTION

Contamination of the aquatic environment by xenobiotics is presently at the centre of attention in the research community [1]. Hundreds of substances that can adversely affect aquatic organisms can be detected in the aquatic environment. Some chemicals functioning as endocrine disruptors (EDs) are also introduced into the environment that can affect the processes of hormonal regulation in living organisms [2]. Fish are often used as sentinel organisms for ecotoxicological studies because they play number of roles in the trophic web, accumulating toxic substances, responding to low concentration of mutagens [3] and also serve as bio-indicators of environmental pollution playing a significant role in assessing potential risk associated with contamination in aquatic environment since they are directly exposed to chemicals resulting from agricultural production via surface run-off or indirectly through food chain of ecosystem [4]. Sexual reproduction in vertebrates is mediated by steroid hormones and numerous endocrine disrupting chemicals (EDCs) discharged into the environment have been shown to interfere with hormone signaling via several mechanisms [5, 6, 7]. In fish, sex determination and differentiation are particularly susceptible to endocrine disruption and exposures to various synthetic and natural estrogens have been associated with significant effects on reproductive development and function in wild fish populations [8, 9, 10]. Alterations in plasma sex steroid concentrations may have resulted from several different mechanisms of action, including direct effects on steroidogenic enzymes such as aromatase, or indirect modifications associated with altered feedback loops [11, 12]. Sex steroids are transported to sex steroid-binding proteins in plasma resulting in a decreased rate of steroid degradation and regulation of free sex steroids available for receptor binding in different tissues [13, 14].

METHODOLOGY

Procurement and maintenance:

Mature adult female fish, *Labeo rohita* were obtained from the local fisheries department and acclimatized to laboratory conditions for one week. During this time they are fed with commercial fish feed and continuous aeration was provided. All experiments were conducted during the time the fish were reproductively active.

Chemicals

Technical grade cypermethrin, RS-a-cyano-3-phenoxybenzyl, IRS, cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylate was purchased from Sigma-Aldrich (99% pure; St. Louis, Mo, USA).

Experimental Design

The stock solution was prepared in ethanol (99%, Merck). 10mg of cypermethrin was dissolved in 2ml of ethanol and stored at 4^0C. From this dilutions were made and used for daily renewal of the test solutions in the aquaria. Six male fish were kept in 500L water tanks and fed with commercial fish feed and then exposed to 10 and 20 µg/L of Cypermethrin for six days. After the stipulated time blood and gonads were collected for analysis of different hormones and enzymes involved in steroidogenic pathway.

Blood collection from the caudal vein/artery

Immediately following anaesthesia, the caudal peduncle is severed transversely, and the blood is removed from the caudal artery/vein with a heparinized microhematocrit capillary tube. Collected blood volumes varied from 4-10µl depending on fish size. An equal volume of Aprotinin buffer (6µg/ml in Phosphate Buffered Saline) was added to the Microcapillary tube, and plasma was separated from the blood by centrifuging at 1500rpm for 5 min. Following that capillary tubes were freeze dried in liquid nitrogen and stored at -80°C until used for analysis of different hormones.

Collection of gonads

Blood collection was followed by removal of gonads by opening the viscera. Dissected ovaries were homogenized and used for aromatase assay as mentioned below.

Vitellogenin assay

The blood plasma Vitellogenin levels in both experimental and control groups were analyzed for individual female fishes. ELISA utilizes specific binding between antibodies and Vitellogenin to quantify VTG in samples from fish. The wells of microplates have been pre-coated with a specific Capture antibody that binds to VTG in standard and sample when added to the wells. A different VTG-specific Detecting antibody is added to create a sandwich of VTG and antibody, which is determined by adding a substrate that gives a coloured product and the colour intensity is directly proportional to the amount of VTG present.

Assay procedure

Dilution of the VTG standard: The content of one vial of VTG standard (vial 1) was dissolved in 1.0 ml cold dilution buffer. Concentration of VTG in this stock solution was calculated based on the VTG amount specified on the vial (µg per vial). Dilution are prepared as mentioned here 1:500 dilution (5µl sample is added to 2495µl cold Dilution buffer), 1:30,000 dilution (10µl of the 1: 500 diluted sample to 590µl cold Dilution buffer), and a 1:1, 800, 000 dilution (10µl of the 1:30 000 diluted sample to 590µl cold Dilution buffer). Dilutions are kept on ice until use.

Incubation with standard and diluted samples: 100µl Dilution buffer was added to each of the two NSB wells and the plates were sealed and incubated at room temperature (20-25 ºC) for 1 hour. Incubation with detecting antibody and Incubation with secondary antibody was carried out at 20-25 ºC for 1 hour.

Testosterone

Sample Extraction Protocol

Ethyl acetate/hexane (50:50) (4x the sample volume) was added to the sample and vortexed for 10 seconds. The layers were allowed to separate. The ethyl acetate/hexane layer (upper layer) was transferred to a clean tube. This step was repeated three times. The ethyl acetate/hexane was evaporated using a vacuum centrifuge. The extract was dissolved in an amount of EIA Buffer equal to the original sample volume. Diluted samples were concentrated by reconstituting the sample and then used for EIA analysis.

Testosterone EIA

Using the ethanol equilibrated pipette tip, 100µl of the Testosterone EIA standard was transferred into a clean test tube and then diluted with 900µl ultra pure water. The concentration of this solution (the bulk standard) will be 1ng/ml. To prepare the standard for use in EIA, 900µl EIA buffer to tube #1 and 500µl EIA Buffer to tubes #2-8 was taken. 100µl of the bulk standard (1ng/ml) was transferred to tube #1 and mixed thoroughly. Then serial dilution of the standard was done by removing 500µl from tube #1 and placing in tube #2. This process was repeated for tubes #2-8. 100 dtn Testosterone AchE Tracer was reconstituted with 6ml EIA Buffer. This reconstituted Testosterone Ache Tracer was stored at 4°C and used within four weeks. 100 dtn Testosterone EIA Antiserum was reconstituted with 6ml EIA Buffer. This reconstituted 11-keto Testosterone EIA Antiserum was stored at 4°C and used within four weeks.

Estradiol assay

Extraction protocol

A known amount of each sample was taken into two tubes. First tube was labelled sample 1 and the second sample 2 and spike. Cold spike of Estradiol was added to the sample and spike tubes. Methylene chloride was added and mixed thoroughly with a vortexer. Layers were allowed to separate. Using a Pasteur pipette, transfer 90% of the methylene chloride (lower layer) was separated into a clean test tube. This extraction procedure was repeated two times. The combined methylene chloride extracts were evaporated by heating at 30ºC under a gentle stream of nitrogen. This was dissolved in 0.5ml of EIA Buffer. 50µl of this reconstituted sample was used.

Estradiol EIA

Using the ethanol equilibrated pipette tip, 100µl of the Estradiol EIA standard (item No. 482254) was transferred into a clean test tube and then diluted with 900µl ultra pure water. The concentration of this solution (the bulk standard) will be 40ng/ml. To prepare the standard for use in EIA, 900µl EIA buffer to tube #1 and 750µl EIA Buffer to tubes #2-8 was taken. 100µl of the bulk standard (40ng/ml) was transferred to tube #1 and mixed thoroughly. Then serial dilution of the standard was done by removing 500µl from tube #1 and placing in tube #2. This process was repeated for tubes #2-8. 100 dtn Estradiol Ache Tracer (96- well kit; Item NO. 482250) was reconstituted with 6ml EIA Buffer. 100 dtn Estradiol EIA Antiserum (96-well kit; Item 482252) was reconstituted with 6ml EIA Buffer. This reconstituted Estradiol EIA Antiserum was stored at 4°C and used within four weeks.

17, 20 β-Dihydroxy-4-pregene-3-one (17,20βP):

Preparation of steroid-carboxymethyloxime (CMO)

The preparation of the 3-CMO of 17,20αP was based on the method of Simpson and Wright.

Generation of G4-acetylcholinesterase

Acetylcholinesterase (AChE, 1 mg) in 500 µl 0.1M phosphate buffer pH 7.0 was treated overnight with 25 µl trypsin solution (25µg ml-1 in 0.1 M phosphate buffer, pH 7.0) at room temperature. The solution was dialysed against 100 ml 0.1M borate buffer pH 8.5 using an 80 mm length of dialysis tubing (Sigma D2272) for 24 hours using several changes of borate buffer. The contents of the dialysis tubes were removed and the tubes were washed with clean borate buffer to give a final volume of G4-AChE solution of 1 ml which could be stored at 4°c for at least two weeks. The activity of enzyme was checked with Ellmans reagent as described later.

Preparation of steroid-CMO-AChE conjugate

Four hundred nmol (174 µg) of the steroid-CMO in 38µl of freshly made N-hydroxysuccinimide solution (1 mg.ml-1 in anhydrous dimethylformamide) was treated overnight with 32 µl freshly made N,N'-dicyclohexylcarbodiimide solution (2 mg.ml-1 in anhydrous dimethylformamide) in the dark (Cuisset et al. 1994). It is essential that all traces of moisture are excluded from this step. Thirty microliters of this solution were reacted for 2 hours in the dark with 200 µl of the 1 mg.ml-1 G4-AChE stock and then mixed with 1 ml of steroid assay buffer (0.1M K2HPO4.3H2O, 0.1M g KH2PO4, 0.15M NaCl, 1 mM EDTA, 0.1% bovine serum albumin (BSA), 0.15mM sodium azide, pH 7.4). The product was chromatographed on a 900 x 15 mm column of BioGel A 15-m (BioRad) with elution buffer (0.01M Tris, 0.01M MgCl2, 1M NaCl, 0.15mM NaN3 pH 7.4) at a flow rate of 300 µl.min-1. The eluate was discarded for 5 hours, then eighty 1.5 ml fractions were collected into tubes containing 1 ml steroid assay buffer. Five microliters of every 5th fraction was treated with 200 µl Ellmans reagent (215 mg 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 200 mg acetylthiocholine in 20 ml 1M potassium phosphate buffered saline (PPBS) kept as 400µl frozen stock aliquots for up to six weeks and diluted 1:50 for use and absorption read at 405 nm after 2 hours. In the region of peak activity, 5 µl from all fractions was tested with Ellmans reagent and an activity curve was plotted. Fractions showing >30% of the maximum Ellman activity were combined and stored in stock aliquots at -20°C. The column was flushed overnight with elution buffer, stored at 4°C and flushed again overnight before reuse.

Standard assay procedure

Serial dilutions of standard steroid (400 to 0.78 pg) in 100 µl assay buffer were made in the first 10 wells of rows A and B of the coated plate. The remaining 2 wells in these rows were reserved for B0 (0 pg) and non-specific bound (NSB). Twenty-five microliters of the extracted steroids from the samples (5 µl incubation media equivalent) were pipetted into the remaining 6 rows in duplicate. Twenty-five microliters of the diluted steroid label was added to all wells, and 25 µl antiserum to all wells except for the NSB at the dilutions determined by the checkerboard titration. All wells were made up to 150 µl in steroid assay buffer; the plates sealed and incubated 2 hours at room temperature in a humid chamber. Plates were then washed three times with wash buffer. 200 µl per well of Ellmans reagent were added, the plates incubated overnight in the dark at room temperature and read at 405 nm. Picograms per well were calculated for the samples from the standard curve using Stingray software (Dazdaq, Ringmer, UK).

Aromatase activity

It was determined by the enzymatic conversion of androstenedione to estradiol, with the release of ^{3}H from the C-1 carbon and subsequent formation of tritiated water (Thompson and Siiteri, 1974). A modification of the assay of Melo *et al.* (1999) was used. Briefly, dissected ovaries from individual fish (10-20 mg wet weight of tissue per fish) were thawed, homogenized in 10 µ1 phosphate buffer (10 mM K_2HPO_4, 100 mM KC1, 1 mM EDTA, 1 mM DTT, pH 7.4) per mg tissue, and certrifuges at 10,000 *g* for 10 min. Forty to 50 µl of supernatant was incubated in phosphate buffer with 4 nM (1,4,6,7,-^{3}H)-androstenedione (Amersham Pharmacia Biochem, Piscataway, NJ; specific activity 100 Ci/mmol) and 1 mM NADPH at 20^{o}C for 3 h. Following the incubation, samples were placed in an ice bath, 150 µl of ethyl ether was added, and samples were held on ice for 10 min. Samples were then held at – 80^{o}C for 10 min to freeze the aqueous fraction.

The ether fraction was discarded, and 300 µl of 5% dextran-coated charcoal (Sigma, St. Louis, MO) slurry was added to each tube and placed on ice for 30 min to remore any remaining steroids. Samples were centrifuged at 1500 *g* at 4°C for 20 min. A 300 µl aliquot of the supernatant was added to 5 ml of scintillation cocktail (Ultima Gold; Packard, Downers Grove, IL) and ^{3}H was determined as dpm, using a Packard 2500-Tr liquid scintillation counter. Data were corrected for background through analysis of samples that had been treated similarly in all respects, except they had been heated for 15 min at 90°C. Protein was determined in 5 µl samples with Bradford reagent (Sigma) and quantified by comparison to a standard curve generated with bovine serum albumin (Sigma).

RESULTS AND DISCUSSION

The plasma vitellogenin in female Labeo Rohita did not shown much change after the 6th day when exposed to 10µg/L and 20µg/L of cypermethrin (Fig. 1 and Table.2).

.Table 1: Levels of Vitellogenin in plasma of Female *Labeo rohita* exposed cypermethrin.

S.No	Sample	Mean ± SD (µg/ml)	DMR-Test
1	Control	220.4 ± 16.9	-
2	10µg/L	201.6 ± 13.8	Not Significant
3	20µg/L	190.8 ± 15.3	Not Significant

Note: Values are mean ± SD (n=6)

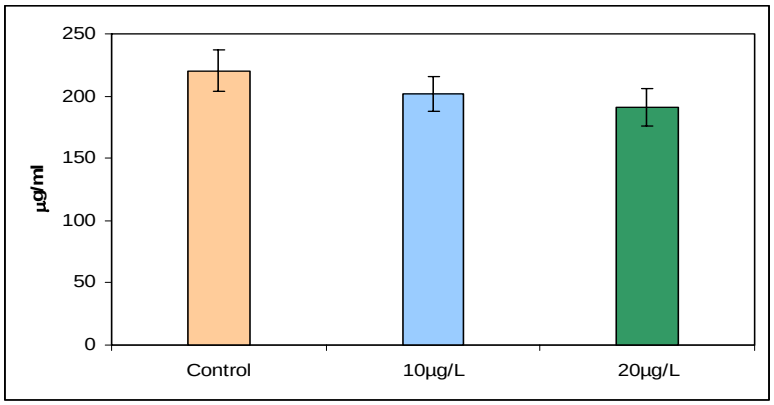

Fig. 1: Levels of Vitellogenin in plasma of Female *Labeo rohita* exposed cypermethrin

In this work, we also examined the effect of two different concentrations i.e. 10µg/L and 20µg/L of cypermethrin on plasma testosterone in female *Labeo rohita* for 6 days. The results presented in Fig. 2 and Table 3 has shown that testosterone level in female has increased with increase in concentration of the toxicant.

Table 2: Levels of Testosterone in plasma of female *Labeo rohita* exposed to cypermethrin.

S. No	Sample	Mean ± SD (ng/ml)	DMR-Test
1	Control	0.4 ± 0.02	-
2	10µg/L	0.8 ± 0.03	Significant over control
3	20µg/L	1.3 ± 0.3	Significant over control and 10µg/L

Note: Values are mean ± SD (n=6)

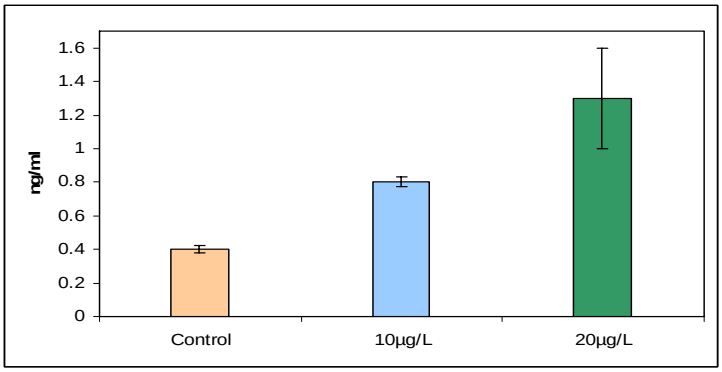

Fig. 2: Levels of Testosterone in plasma of female *Labeo rohita* exposed to cypermethrin

17β-estradiol in female after exposure to two different concentrations of cypermethrin namely 10µg/L and 20µg/L have shown that there was a decrease of 17β-estradiol in exposed fish with increase in cypermethrin concentration.

Table 3: Levels of 17β- Estradiol in plasma of Female *Labeo rohita* exposed to cypermethrin.

S. No	Sample	Mean ± SD (pg/ml)	DMR-Test
1	Control	5923.3 ± 30.1	-
2	10µg/L	4291.6 ± 24.9	Significant over control
3	20µg/L	3666.6 ± 26.2	Significant over control and not Significant over 10µg/L

Note: Values are mean ± SD (n=6)

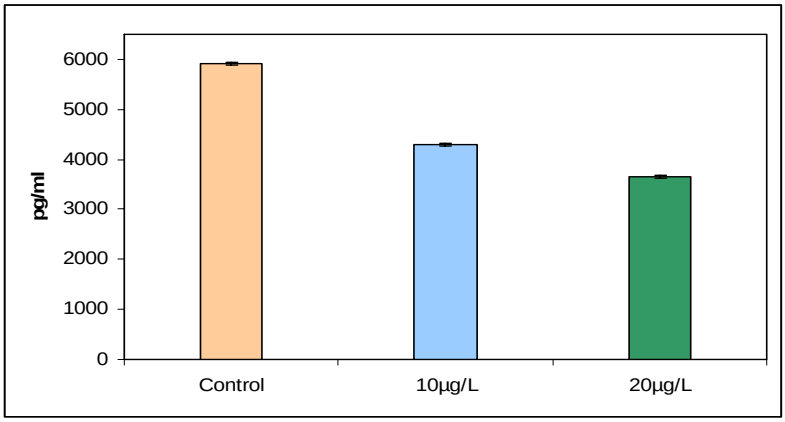

Fig. 3: Levels of 17β-Estradiol in plasma of Female *Labeo rohita* exposed to cypermethrin

17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-diOHprog), the maturation inducing hormone was analysed in female fish to know its level under cypermethrin toxicity. There was a steady decrease in this hormone in fish exposed to 10µg/L and 20µg/L compared to control fish (Fig. 4 and Table 4)

Table 4: Levels of 17,20β Pregnenolone in plasma of Female *Labeo rohita* exposed to cypermethrin.

S. No	Sample	Mean ± SD (ng/ml)	DMR-Test
1	Control	0.8± 0.02	-
2	10µg/L	0.6 ± 0.02	Significant over control
3	20µg/L	0.2 ± 0.02	Significant over control and not Significant over 10µg/L

Note: Values are mean ± SD (n=6)

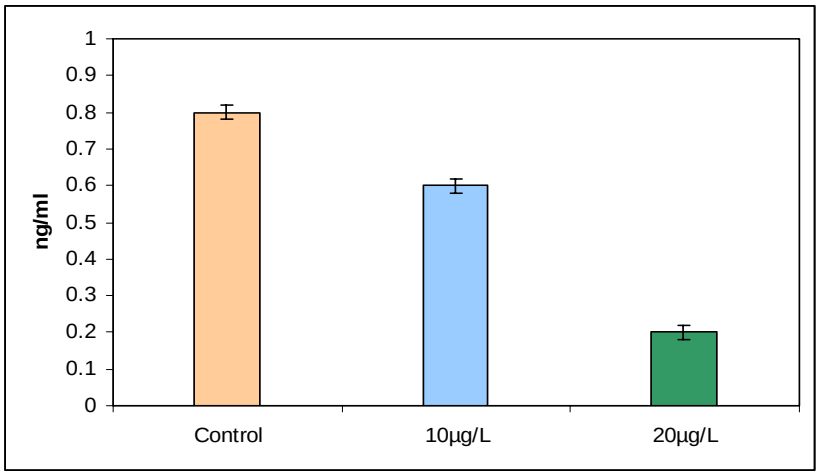

Fig. 4: Levels of 17, 20β Pregnenolone in plasma of Female *Labeo rohita* exposed to cypermethrin

The enzyme aromatase which converts testosterone to 17β-estradiol was also estimated in ovary of female *Labeo rohita* after exposing the fish to 10µg/L and 20µg/L for 6 days. This enzyme has slightly increased in exposed fish compared to control (Fig. 5 and Table 5).

Table 5: Levels of Aromatase assay in ovary of *Labeo rohita* exposed to cypermethrin

S.No	Sample	Mean ± SD (fmol/mg/min)	DMR-Test
1	Control	12.6 ± 1.2	-
2	10µg/L	16.4 ± 1.6	Significant over control
3	20µg/L	19.2 ± 1.8	Significant over control and 10µg/L

Note: Values are mean ± SD (n=6)

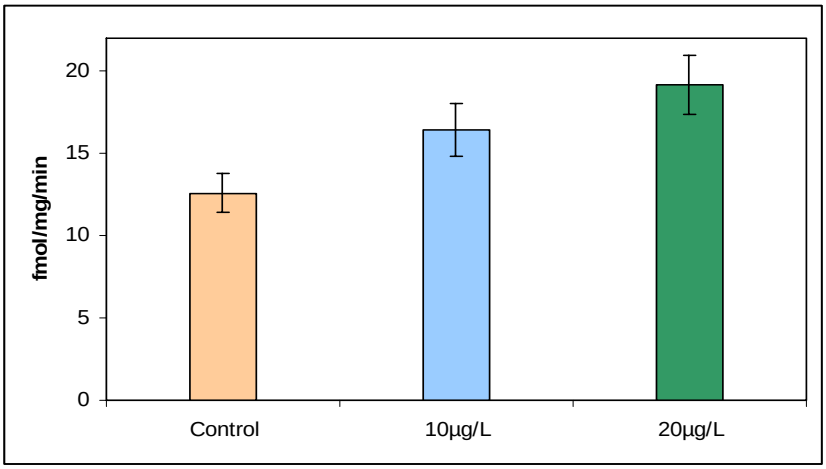

Fig. 5: Levels of Aromatase assay in ovary of *Labeo rohita* exposed to cypermethrin

Summary of the work was given in the Fig 6

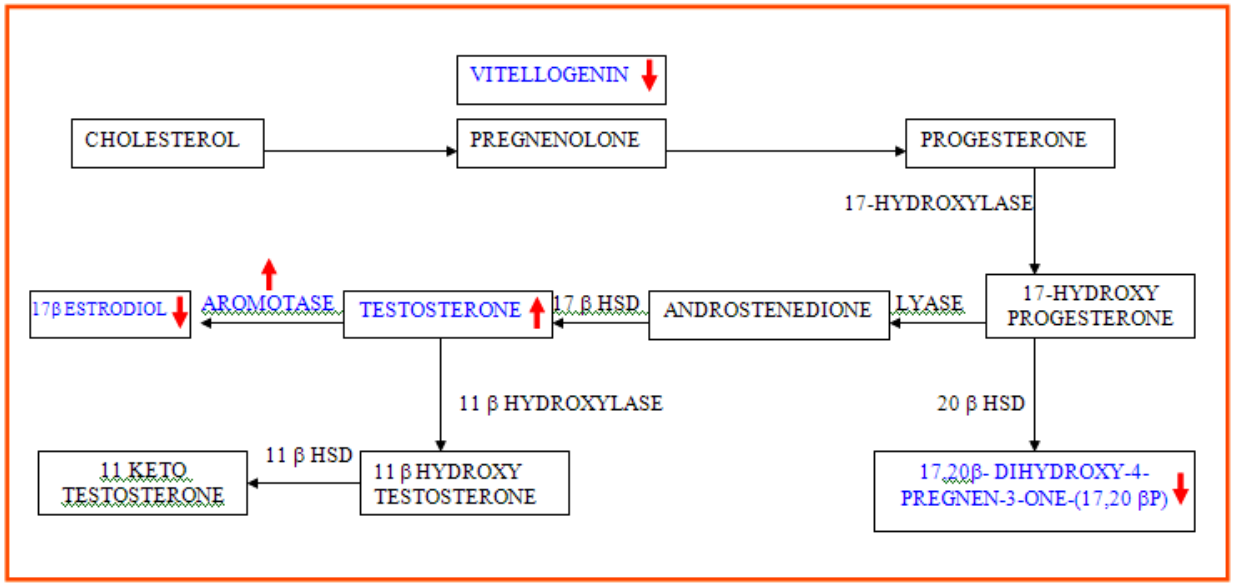

Fig 6: Summary of Findings in Female *Labeo Rohita* Exposed to Cypermethrin

CONCLUSION

Cypermethrin has impact on steroidogenesis in female *Labeo rohita* to the extent studied. Female fish have shown induction of plasma vitellogenin indicating that cypermethrin is estrogenic. Altered levels in testosterone, 17β estrodiol, 11-Keto testosterone could be the possible reason for decreased level of 17, 20β Dihydroxy-4-Pregnen-3-one, which inturn may be impacting fecundity in female fish. Elevation of the enzyme aromotase was not significant and hence its role here cannot be taken into consideration.

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