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Effect of cypermethrin on estradiol and vitellogenin in the tissues of *Esomus danricus*

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Abstract
Cypermethrin is one of the synthetic pyrethroids which are most widely used for more than three decades as possible alternative to the organophosphate, organochloride and carbamate pesticides. Its toxic effects may include lethal and sublethal concentrations, which may change the growth rate, development, reproduction, histopathology, biochemistry, physiology and behaviour on target organisms and undesirable perturbations in the environment. In these backgrounds of toxicology aspect the present study focussed for the concentration measurement of Estrodiol (17β), Liver and ovarian vitellogenin in the exposed cypermethrin to *Esomus danricus* in 45days experiment in different concentrations. The adverse effects of the Vitellogenin and Estrodiol-17β were decreased when compared to control to less concentrations of cypermethrin exposure to *E. danricus*. The findings were significant as the toxic effect of cypermethrin was found to increase with time of exposure suggesting its deleterious effect on aquatic organisms not only directly but also by bio accumulations.

Keywords: Cypermethrin, toxicity, organochloride, vitellogenin, Estrodiol, *Esomus danricus*

Introduction
A tremendous increase in use of insecticide besides usage of artificial manure's to enhance agricultural production has been witnessed in last three decades. The pesticides and insecticides used for pest control find their way with the runoff water into water bodies and adversely affect non-target organisms in the aquatic ecosystem, especially fishes. The toxicity of various insecticides causes histological, physiological and behavioural changes. Reports pertaining to acute and chronic toxicity of several insecticides to a large number of teleosts are available [1-6]. The study of the effect of sublethal concentration has been forced because of the need to find "Safe" concentration of toxic chemical [2]. Many attempts have been made to study the effect of insecticide on reproductive performance of fishes. Report available describing the effect of insecticide on fish reproduction [7-10].

Pyrethrum is an insecticide was first used at about 1800 in *Transcaucasus* region of Asia. Initially plant sources of pyrethrum were *Chrysanthemum coccineum* and *C. carneum* (Compositae). The pyrethroids are powerful contact insecticides and poor stomach poison in insects. Its common name is (RS)-cyano-3-phenoxybenzyl IRS-cistrans-3-2, 2-dichlorovinyl-2, 2-dimethyl-cyclopropane carboxylate. Cypermethrin is a synthetic pyrethroid it has become one of the most important insecticides in wide-scale use. Since their introduction, synthetic pyrethroid insecticides have generated regulatory concern regarding their toxicity to fish and aquatic invertebrates. The fishes are less sensitive to pyrethroid [11, 12]. Hence, the present study carried out to study the effect of synthetic pyrethroid, cypermethrin on fresh water fish *Esomus danricus*.

Vitellogenin (vtg), a phospholipoglycoprotein, is synthesised by the female liver and incorporated into oocytes by endocytosis after being carried to the ovaries through the blood. Serum vtg levels are directly related to the female reproductive stage. Thus, vtg detection can be used to identify sex of fish, when there is no occurrence of sexual dimorphism, and the measurement of its level in blood gives an index of female sexual maturity. The determination of vtg level was performed by means of indirect measurement, such as the measurement of protein-bound phosphorus (or) calcium binding phospholipid glycoproteins [13]. Vitellogenin (vtg), the precursors of egg yolk protein in oviparous vertebrates, are complex calcium-binding phospholipoglycoprotein in hepatic parenchymal cells under the influence of the female sex steroid, oestrodiol. The production of vtg has proved to be a useful model for the analysis of molecular mechanism of steroid hormone action [14].

So far the level of vitellogenin is studied in the blood serum by injecting the steroid oestrodiol-17β (E2). Only few attempts have been made to study the vitellogenin level in the liver and ovary. Hence the present work conducted to study the vitellogenin level in liver and ovary by an indirect method of finding out protein bound phosphorus in a fish exposed to a synthetic pyrethroid cypermethrin.

Brain plays an important role in the initiation and co-ordination of reproductive functions which shows sexual differentiation in the two sexes [15]. The mature gonads of males and females produce different sex steroids these hormones can pass through blood brain barrier and bind to intracellular receptor in the brain. In addition to steroid hormones binding to intracellular steroid receptor, metabolites of these steroids and steroid hormone production in the brain (neurosteroids) are thought to bind to membrane recognition sites [16, 17]. The gonadotropin appears to act on the brain in what is referred to as short feedback loop, to control the secretion of their respective releasing factor [18]. Estradiol-17β and testosterone receptors are reported in different regions of the brain [19, 20, 21]. The estrogen neurons are not restricted to a hypothetical centre in the hypothalamus (or) the preoptic region but that the presumed hormonally addressed neuron are widespread and occur in different areas of the brain. This may represent an anatomical substrate for the possible multiple effects of the hormone as related to the many facets of reproduction including mating [22]. Estradiol acts directly on the brain to control the secretion of gonadotrophic hormones by the pituitary and to stimulate female sexual behaviour. Hypothalamus is a direct target tissue of estradiol and that the specific trapping mechanism in the target tissue, which retains estradiol in an unconverted form, may serve to initiate a sequence of reaction by which estradiol exerts its feedback control on gonadotropin secretion [23]. The present attempt has been carried out to study the effect of cypermethrin on the levels of Estradiol in brain of *E. danricus*.

Materials and Methods

The fish *Esomus danricus* (Ham.) weighing 1-3g, used for this research work, were collected from Vairamega Thadaagam at Uthiramerur (Kanchipuram) during the month of May 2015. The fishes were acclimatized for two weeks at lab condition before starting the experiment. The male and female fish with a ratio of 1:1 were kept in four glass tanks measuring 2'x1'x1.25'(80lit.), each. During the time of acclimatization and throughout the experiment the fishes were fed with a composition of rice barn, wheat flour, fish meal and groundnut oil cake. All the fish tanks were provided with aerators and the water changed every alternate day.

The synthetic pyrethroid, cypermethrin (commercial grade insecticide-cymbush 10EC (RS) - alpha - cyano - 3 - phenoxylbenzyl (IRS) - cis, trans - 3 - (2-2 -dimethyl - cyclopropane carboxylate) used in this experiment was obtained from local agricultural shop. The composition of the compound: Cypermethrin 10% (w/w), cresloxAE1 5%, cresloxAE2 2.4%, and cresloxAE3 0.61%. The product manufactured by the company Zeneca, Agrochemical Ltd., U K.

Experimental Design

The different concentration of cypermethrin was prepared by diluting the cypermethrin. Various dilutions were prepared to find the safe concentration for the experiment. The commercial product concentration of cypermethrin is 10%, from this 1% of the stock solution of cypermethrin prepared. From 1% of stock solution, different concentrations were prepared to which fishes were exposed. At 5 ppb, 4ppb, and 3ppb concentration fishes showed high mortality at lesser than 24 hours. Further, it was found that the fishes were alive at the concentration of 2ppb for 24 hours. Hence in the present study, three different concentration, 0.02ppb 0.2ppb and 2ppb taken to expose the fish. (i.e., control, Exp.-I 0.02ppb, Exp.-II 0.2ppb and Exp.-III 2ppb). 30 fishes were introduced in each tank with equal number of male and female fish. Cypermethrin was added in the tank at chosen concentration in 40 liters of water. The experiment was conducted for 45 days. After 45 days, the fishes were dissected, and the skin, liver, ovary, testis and brain tissues were removed, weighed and stored in -70 ºC for further analysis.

Vitellogenin Estimation

The vitellogenin production in the liver and ovary were estimated by an indirect method of finding out the protein-bound phosphorus level in the liver and ovary by Ammonium molybdate vanadate method [24].

0.1295gm of analytical grade potassium dihydrogen phosphate (KH_2Po_4) was used as phosphorus standard. The tissue is homogenized using Diacid (Perchloric acid and Nitric acid) with a ratio of 9:4 in a sand bath. Kept in room temperature for 30 minutes till the yellow colour ceases. Precaution was taken as to not to dry the sample. The sample was diluted to the necessary concentration.

To the diluted sample 10ml of vanadomolybdate reagent was added and mixed thoroughly to give a yellow colour complex. The absorbance was measured after 30minutes at 420nm. From the standard plot, the relative phosphorus content in the tissue was estimated.

Estradiol-17β Estimation

To study the neurotoxicity of cypermethrin in central nervous system, estradiol-17β was measured by Radio immuno assay (RIA) using the following procedure [21].

Brain of samples of Esomus danricus were taken for steroid extraction, brain samples were homogenized in isotonic saline (0.6%) and then extracted with ethyl acetate, centrifuge at 2800rpm at 10mins. The clear layer of supernatant was collected in fresh tube, again we add ethyl acetate to precipitate for further extraction this same process is triplicates, the clear supernatant was collected then it will evaporated in room temperature further analysis[21]. The standard steroid of estradiol diluted from 0 to 3000 pg in log scale i.e 0, 5, 8, 12, 35, 55, 90, 150, 250, 400, 650, 1100, 1800, and 3000. Duplicates were maintained to get concordant values. RIA tubes set to receive the non-specific binding with β-globulin, specific binding with the specific antisera raised against the estradiol and the different concentrations of cold estradiol, total binding without the cold steroid, and the samples were run together. First the vials received the specific antibodies, after 2 hours of incubation labelled estradiol added to the tubes, overnight incubation at 4 ºC, followed by addition of charcoal, and bound hormone separated by centrifugation. The supernatant of the free labelled hormone measured in β-counter by adding the cocktail. The results obtained from these studies were subjected to statistical analyses of one way (ANOVA).

Results

Phosphorus

The (Table 1) Shows the phosphorus level in liver (Fig.1) and ovary (Fig. 2) shows a dose dependent decrease in treated fishes compared to control fishes (P<0.05). This difference is directly related to vitellogenin accumulation in ovary and liver. It reveals that the cypermethrin affected the vitellogenin production in these organs.

Estradiol

The level of brain estradiol-17β decreased when compared to control fish (Fig. 3). In 0.2ppb group estradiol-17β the level is slightly increased compared to control. In 0.02ppb and 2ppb group estradiol-17β level is decreased. This change might be due to the cypermethrin interference in the brain (P<0.01).

Table 1: Showing the values of the protein - bound phosphorus (Vitellogenin) levels in liver and ovary of *E. danricus*

S. No.	Sample	Phosphorus level in Liver(ng/mg)	Phosphorus level in ovary (ng/mg)
1	Control	0.29	22.4
2	Exp I	0.16	25.9
3	Exp II	0.36	31.1
4	Exp III	0.22	29.5

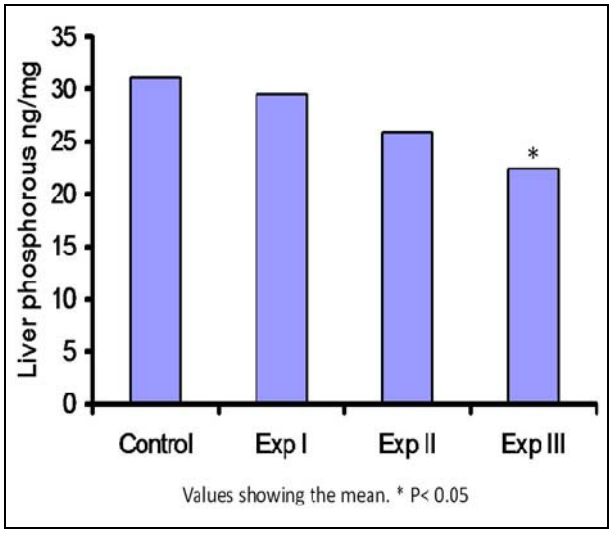

Fig 1: Effect of cypermethrin on protein-bound phosphorous in liver of *E. danricus*

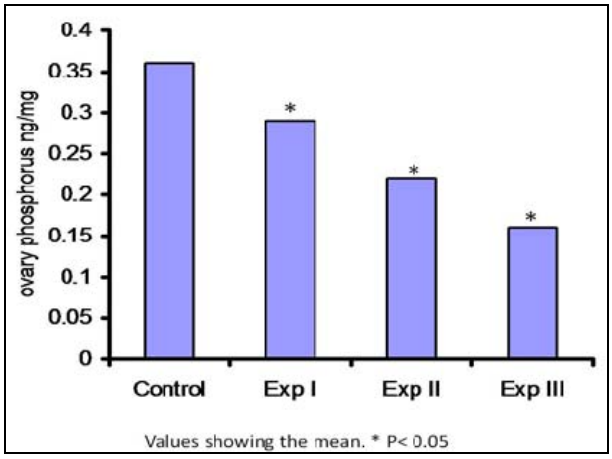

Fig 2: Effect of cypermethrin on protein-bound phosphorous in ovary of *E. danricus*

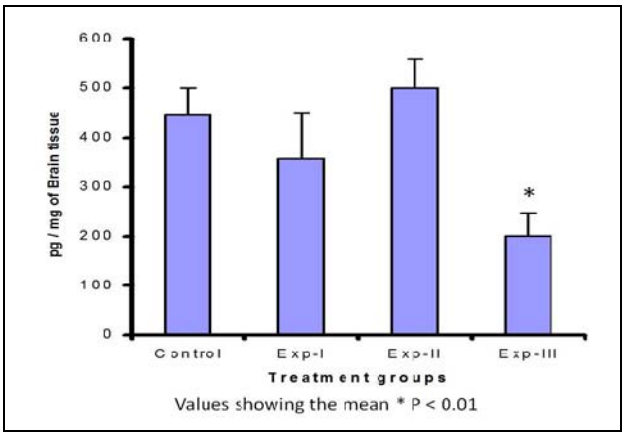

Fig 3: Effect of cypermethrin on the levels of estradiol -17β in the total brain of *E. danricus*

Discussion

Pesticides greatly affect the secondary growth of primary oocyte to the impairment of vitellogenesis. Arrest of follicular development, recrudescence and atresia of follicles were reported in *C. punctatus* exposed to fentrothion and carbonyl [25]. Similar effect was noticed with the treatment of BHC in *C. carpio* [26] and in *S. mossambicus* with Malathion [27] and with methyl parathion [28]. Degenerative changes in ovary were exhibited by stage-I (oogonium) and stage-II (immature/non-vitellogenic) oocyte as marked by perinuclear ooplasmic lysis, clumping and dissolution resulting in disintegration of nuclear material altogether were reported in *C. punctatus* exposed to carbofuran [29].

Partial disruption of the ovarian follicles, vacuolation in the cytoplasm of germinal cells, and secondary oocytes were reported in *H. fossilis* exposed to BHC [30]. Degeneration of follicular walls, connective tissues and vacuolisation in the ooplasm of stage-II and stage-III oocyte was observed in *A. testudineus* treated to carbofuran [31]. This supports the present study that the oocyte were affected with a degeneration of connective tissue, disruption of follicles, vacuolation in the cytoplasm of germinal cells and disintegration of such oocytes were noticed in *E. danricus* exposed to cypermethrin. Pesticide effects on the testis are scarce. In *O. mossambicus* exposed to BHC caused hyperplastic changes in both primary and secondary spermatocytes [32]. Likewise, hyperplasia and vacuolisation in all types of spermatogenic cells have been reported in *S. mossambicus* exposed to diamicron [33].

Clumping of chromatin material and also cytoplasm were quite apparent in primary spermatocytes. Few sperm shows vacuolation in the head region were noticed in *H. fossilis* exposed to endosulfan. Endosulfan also causes testicular degeneration and disappearance of sperm [34]. Testicular deleterious changes included degeneration of spermatogenic element and necrosis of interstitial cells of Leydig were reported in *C. punctatus* exposed to carbofuran [28]. This report supports the present study that the vacuolation, testicular degeneration was observed in *E. danricus* exposed to different concentration of cypermethrin. Liver exhibited varying degrees of histopathological changes including cytoplasmolysis, nuclear pyknosis and necrosis leading to complete exhaustion and disintegration of hepatocytes. In some region of liver, extensive degeneration of proliferated hepatocytes, in close proximity to blood sinuses, looking like darkly stained debris of hepatocytes, necrosis resulting in the complete dissolution of hepatocytes were noticed in *C.*

punctatus exposed to carbofuran [35]. This supports the present study that similar observation has been made in the fish *E. danricus* exposed to different concentration of cypermethrin. This degeneration of hepatocytes could be due to cypermethrin.

Reduction in protein level in liver and ovary were observed in *E. danricus* exposed to different concentration of cypermethrin. Similar reduction has been reported in the protein content of the liver tissue of the fish *C. carpio* treatment with cypermethrin [36], and in *A. testudineus* exposed to β-BCH (lindane). The reduction was very high in the higher concentration of lindane. Cypermethrin could have affected the protein synthesis that resulted in the lower production of protein in the liver and ovary. The Ascorbic acid plays an important role in steroid hormone synthesised and utilisation in various action of the body of the fish. If the ascorbic acid production is high in the serum is been sensed in the hypothalamo-hypophyseal region, immediately it reduces the production of steroid hormone which inturn affect the reproduction of the fish. This ascorbic acid level also affects the production and accumulation of vitellogenin in the liver and ovary. If the ascorbic acid level is high or present in the serum of the fish which has a direct influence in the E_2 and vitellogenin level. The large yolky oocyte of fish is the product of the regulatory activities of the ovarian follicle wall, which produce hormone controlling vitellogenesis and through vitellogenin molecules must pass before sequestered in the oocyte. The yolk protein precursor is a phospholipoglycoprotein, which exhibit in the form of dimer of two dissimilar polypeptides.

Each vitellogenin polypeptide consists of phosphate-rich and a lipid-rich portion, and once taken up by the oocyte these two portions are cleaved enzymatically to lipid-rich (lipovitellin) and phosphate-rich (phosvitin) protein. Reduction in vitellogenin (Protein-bound phosphorus) level was noticed in liver and ovary of the fish *E. danricus* exposed to different concentration of cypermethrin. Similar effect was noticed in the hepatocytes of *C. carpio* exposed to Chloro-s-triazine, and O, P-DDT on *Paralichthys dentatus* shows induction of vitellogenesis and in 17β-estradiol treatment high level of vitellogenin is noticed. Injection of estradiol-17β increases the plasma vitellogenin level in male and female chub (*Leuciscus cephalus*) and also showed an effect on the organisation of testis. There is little report indicating that the role of ascorbic acid in production of vitellogenin and estradiol-17β in the serum of the fish *Salmo gairdneri*. Adult rainbow trout which are fed on a diet devoid of ascorbic acid shows a reduction in serum vitellogenin and estradoil-17β [37]. The reduction in the vitellogenin level could be due the following reasons, reduction in ascorbic acid, decrease level of estradiol and the toxic effect of cypermethrin. All this plays role in the reduction of vitellogenin level in the liver and ovary.

Brain plays an important role in the initiation and co-ordination of reproductive function. The appearance of the estradiol-17β in the brain may be accounted for two ways. First, the mature gonads of male and female produce different sex steroids and these steroids can pass through blood brain barrier and bind to intracellular receptor in the brain. Hypothalamus is the direct target tissue for estradiol-17β (E_2). Second, the estradiol synthesis has been noted in brain tissues along with other steroids. Due to the synthesis of the steroids occur in brain; these steroids are called as "neurosteroids". Reduction in the estradiol-17β level in the brain of *E. danricus* exposed to different concentration of cypermethrin was noticed. The cypermethrin might have directly act through neural pathway to decrease the neurosteroids or it could act through reproductive interaction. Earlier studies in our lab reported the reduction in the epidermal skin pigment, lipid patterns in the skin, HSI, GSI, protein profile, ascorbic acid were found less in the higher concentration of cypermethrin exposed to *E. danricus* [38]. Similar reductions were noticed in the, vitellogenin (Protein-bound phosphorus) level in the liver and ovary. The brain estradiol-17β also shows a difference in the higher concentration of cypermethrin exposure to the fish. The cypermethrin act as contact toxic compound as well as the neurotoxic compound to reduce the reproduction in the freshwater fish *E. danricus*.

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