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Effect of Cypermethrin on conjugation of sex steroid hormones during reproductively active and reproductively inactive phases of the annual reproductive cycle in *Heteropneustes fossilis* (Bloch)

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Abstract : *The aim of the present investigation was to assess the impact of cypermethrin exposure for 40 days at sublethal concentration (0.02 ppm) on gonado-somatic index (GSI) and the conjugation of plasma levels of free and conjugate reproductive sex steroids- testosterone (T), 11-ketotestosterone (11-KT), 17-hydroxyprogesterone (17-P) and 11-deoxycortisol (S) during prespawning and post-spawning phases of the annual reproductive cycle in freshwater male catfish, Heteronpneustes fossilis. Results indicate that there is decrease in conjugation (glucuronide and sulfate) after exposure. Histology of testes indicates gross condensation of spermatogenic cells, cytotoxic damage and vacuolization in the tubular epithelium in the testes after exposure. Cypermethrin causes inhibition of free to conjugate form of sex steroids. Obviously such changes and inhibition of free to its glucuronide and sulfate sex steroids causes the disturbances in the equilibrium of sex hormones and affects the pheromonal behavior and reproduction by these conjugates of this species.*

Key Words: Cypermethrin, Histology, Sex Steroids, Conjugation, Catfish, Reproduction.

Introduction

Cypermethrin is a pyrethroid insecticide widely used for pest control programmes in domestic, industrial and agricultural situations because of its low environmental persistence and toxicity. Pyrethroid compounds are considered safe as compared to organochlorine, organophosphate and carbamate compounds owing to its less persistent in nature (Bradbury and Coats, 1989). The wide use of synthetic pyrethroids is increasing worldwide pollution risks. The synthetic pyrethroids are among the most potent and effective insecticides available, account for more than 30% in the world market (Moore and Waring, 2001). We have reported that insecticides decreased the plasma levels of unconjugated (free) sex steroids in fresh water fish (Singh and Singh, 2008 a, b, c; Singh *et al*., 2008) but not for the conjugated (glucuronide and sulfates) sex steroids besides being an important role in pheromonal behavior and spawning (Kime and Singh, 1996; Vizziano *et al*., 2008; Pankhurst, 2008; Rime *et al*., 2010). A Seasonal study of testosterone (T) and 11-ketotestosterone (11-KT) and its glucuronides in male *Salmo trutta* have been described (Kime and Manning, 1982; Barry *et al*., 1990). These hormones are affected by toxicants and affects reproduction as have been reported by several workers (Kime, 1998; Moore and Waring, 1996). Gregry *et al*., (2008) have reviewed the details of effect of endocrine disrupting chemicals on testicular functions. Seasonal reproductive pattern has been found to be changed due to environmental chemicals (Barrett and Munkittrick, 2010; Barrett *et al.,* 2010) and paper pulp effluents on fish health

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(Barrett *et al*., 2010). Recently, Scott *et al*. (2010) have reviewed the role of the maturation-inducing steroid, 17, 20b-dihydroxypregnen-4-en-3-one, in male fishes.

Sundt and Bjorkblom (2011) have reported that the male fish testicular development was altered, showing a rise in amount of spermatogonia and primary spermatocytes and reduction in quantity of mature sperm in produced water discharged from oil industry activities exposed. Recently, it has been reported that pesticide residues of carbaryl, diazinon and methidathion exceeded regularly in apple, strawberry and orange causing a risk for human beings (Gebara *et al*., 2011). Pesticide affects in many ways as endocrine disruption (Han *et al*., 2010; Olujimi *et al*., 2010; Milla *et al*., 2011), growth and reproduction (Hanson *et al*., 2011), biomarkers (Joseph and Raj, 2011), biochemical parameters (Prusty *et al*., 2011).

No information is available about the effect of cypermethrin insecticide on conjugation of sex steroids during reproductive seasons in freshwater Indian food fishes at different sexual maturity besides being the fact that glucuronides and sulfates have very important role in pheromonal behavior and spawning. Above studies are restricted up to carp fish only. Therefore, it prompted us to study the effect of recently introduced pyrethroid insecticides cypermethrin on the conjugated (glucuronides and sulfates) sex steroids of T, 11-KT, 17P and S during reproductively (prespawning) and inactive (post-spawning) phases affecting reproductive physiology in the freshwater male catfish, *Heteropneustes fossilis*.

Materials and Methods

Experimental fish : The original research reported herein was conducted under ethical guidelines for the treatment of animals in behavioral research and teaching (Animal Behavior, 1998). The catfish *Heteropneustes fossilis* is a seasonal breeder. Its reproductive cycle can be divided into five phases: preparatory (February–April), prespawning (May–June), spawning (July–August), post-spawning (September–October) and resting (November–January) as reported by Lamba *et al*. (1983). The experimental fish, *H. fossilis* (65-70 g and length 21-22 cm) were collected from a pond of the same brood stock during prespawning and post-spawning phase and maintained in cemented tank of size 2 x 1 x 1-m supplied with circulating constant flow of dechlorinated tap water and enjoyed natural photoperiod (prespawning- 13.0L : 11.0D; post-spawning 12.1L : 11.9D) and water temperature (20 ± 2^0C). They were fed *ad libitum* with minced goat liver comprising 20% protein, 5% lipid, 15% carbohydrate the remaining 60% being water, minerals and vitamins etc.

Chemicals : Analytical grade chemicals were obtained from E. Merck, Hi Media (India), Sigma Chemicals Co. (USA) and EIA-KIT of DIA.METRA, Italy (testosterone Lot No. 1233, DKO 002; 17-hydroxyprogesterone- Lot No. 1232, DKO 004; 11-deoxycortisol Lot 1214, DKO 001) and CAYMAN Chemical Company, USA (11-ketotestosterone- Cat. 58275 Lot No. 199913) from local supplier. The technical grade of cypermethrin was a gift from Hindustan Insecticides Ltd. (India) which was used for exposure studies. The weight of technical grade of cypermethrin (94%) was taken accurately on top loading balance Sartorius TE124S (0.01g sensitivity) for exposure studies.

After acclimation, male fish were divided into 2 batches having 5 fish in each glass aquaria having 20 l water during prespawning and post-spawning phases. Total 20 fish were used in this study. The sublethal dose (0.02 ppm) was used for 40 days exposure studies as have been reported for these insecticides in *H. fossilis* (Singh and Singh, 2007). During exposure, fish

were fed on minced goat liver on every 4th day and similar concentration of cypermethrin dose was maintained with freshwater for exposure. After acclimation, fish were bled by caudal puncture and blood was collected to required volume in heparinized (1% heparin sodium salt activity 1,00,000 units 140.3 U/mg) glass tubes and centrifuged at 5,000 rpm for 15 minutes at 4^0C for plasma sex steroid (testosterone, 11-ketotestosterone, 17-hydroxyprogesterone, and 11-deoxycortisol) and their conjugate analysis by enzyme-linked immunosorbent assay (ELISA) reader (Thermo Electron Corporation, Finland fitted with Ascent Software version 2.6, Multiscan EX). After decapitation testes were dissected out, washed in saline (0.6% NaCl) blotted and GSI was calculated (gonad weight x 100/ body weight). Histological sections of testes were also done at 5 ìm thickness to assess the gonadal status.

Extraction of unconjugated and conjugated sex steroid hormones : Extraction of unconjugated and conjugated sex steroid hormones was carried out according to methods described by Singh and Kime (1995) with some modification. Briefly, the 500 µl plasma was extracted twice with 5 ml distilled dichloromethane to give the unconjugated (free) steroid fraction, and the aqueous residue (containing glucuronide and sulfates) was treated with 800 µg â-glucuronidase (Sigma G 0251, from bovine liver, Type B-1, 500 000 units) in 1 ml 0.2M acetate buffer, pH 4.8 for 24 h at 37^0C to hydrolyse glucuronide conjugates. After incubation, steroid moieties of the glucuronides were extracted twice with 5 ml dichloromethane. The aqueous phase was extracted twice with 4 ml of water saturated butan-1-ol, and the extract evaporated. Distilled water (20 µl) was added, votexed, and treated with trifluroacetic acid (TFA-Sigma, T6, 220-0) in ethyl acetate (1/100, v/v, 3 ml) at 45^0 C for 18 h to hydrolyse sulfate conjugates. Distilled water (1 ml) was added to each tube, shaken, and the organic phase containing the steroid moieties of the sulfates pipetted off. The aqueous residue was re-extracted with a further 3 ml ethyl acetate and the extracts combined and evaporated. Loss of hormones in the residual aqueous phase was less than 1.2 %. After extraction, free, glucuronides and sulfate steroids reconstituted in 1 ml phosphate buffer and were assayed for various hormones by ELISA Kit.

Data analysis: Values were expressed as ng/ml plasma (mean ± S.E.M., n = 5). Data were analyzed by Student's t-test and two ways analysis of variance (TW) was employed by Microsoft Excel tool pack data analysis two factor with replication (Bruning and Kintz, 1977).

Results and Discussion

Analysis of variance (TW) revealed that the hormones and phases varied in unconjugated and conjugated sex steroids during prespawning and post-spawning phases. The values have been given in Fig. 1-4.

Effect of cypermethrin exposure on testes: Testes of *H. fossilis* show significant changes when exposed to cypermethrin. Extensive cytotoxic damage, general inflammatory response and other histological abnormalities are quite prominent. Gross condensation of spermatogenic cells, which are evident by clump formation and appearance of inflammatory lesions are also quite prominent. The tubular epithelial vacuolization increased in cypermethrin treated testes of *H. fossilis*. The interstitial cells were found to be degranulated, accompanied by weak chromophobia and vacuolization in the cytoplasm (Fig. 1)

Effect of cypermethrin exposure for 40 days on gonado-somatic index (GSI), testosterone free (TF), testosterone glucuronide (TG) and testosterone sulfate (TS) during prespawning and post-spawning phases in *H. fossilis* : The GSI was decreased during both the phases after

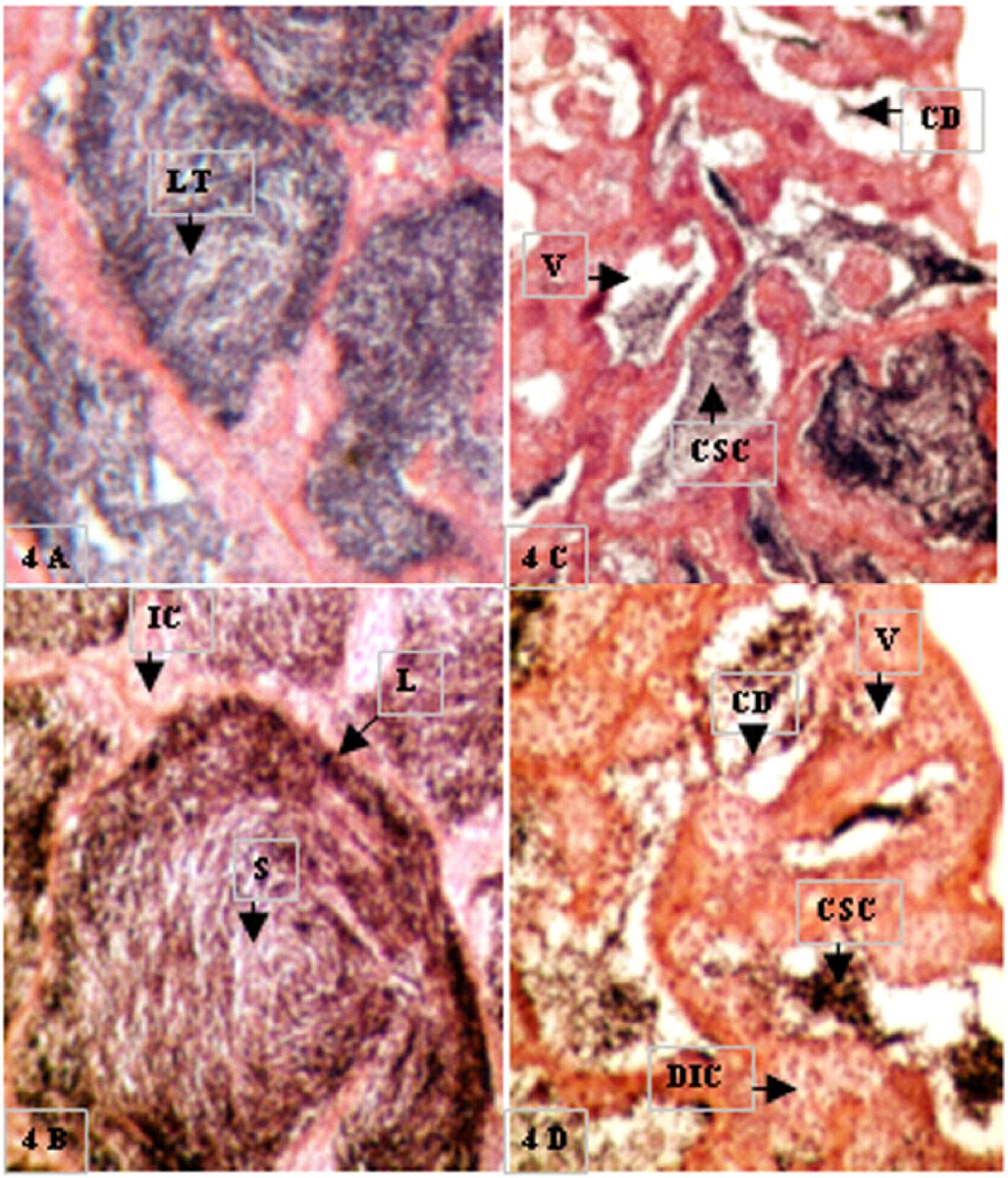

Fig.1. T. S. of testes showing structural differences of control and 40 days exposed with cypermethrin 40 days cypermethrin exposure at sublethal concentration (0.02 ppm) in *H. fossilis*.

A. T. S. of control testes showing lumen of testes (LT) filled with mature sperms during prespermiating stage HE x 40
B. Lobules (L) of testes filled with mature sperms (S) and interstitial cells (IC). HE x 400
C. T. S. testes after 40 days exposure showing cytotoxic damage (CD), condensation of spermatogenic cells (CSC), vacuolization (V) in the tubular epithelium. HE x 40
D. Showing CD, CSC and V and disruption of IC (DIC) in magnification. HE x 400

cypermethrin exposure at sublethal concentration as compared to control (Fig. 2). The plasma levels of TF and TG declined during prespawning phase but TS remained below detection limit after cypermethrin exposure when compared with control. The plasma levels of TF declined after cypermethrin exposure but TG remained unaffected. The quantum levels of hormones declined more during prespawning phase as compared to post-spawning phase (Fig. 3).

Effect of cypermethrin exposure for 40 days on 11-ketotestosterone free (11-KT), 11-ketotestosterone glucuronide (11-KTG) and 11-ketotestosterone sulfate (11-KTS) during prespawning and post-spawning phases in *H. fossilis* : After 40 days of exposure by cypermethrin, the plasma levels of 11-KTF and 11-KTG declined as compared to control during prespawning phase. After cypermethrin exposure, 11-KTS remained below detection limit when compared to control during prespawning phase but during post-spawning phase; 11-KTG and 11-KTS remained below detection limit when compared with control. The levels of 11-KTG declined after cypermethrin exposure during post-spawning phase (Fig.4).

Effect of cypermethrin exposure for 40 days on 17-hydroxyprogesterone free (17-P), 17-hydroxyprogesterone glucuronide (17-PG) and 17-hydroxyprogesterone sulfate (17-PS) during prespawning and post-spawning phases in *H. fossilis* : The plasma levels of 17-PF and 17-PG declined after cypermethrin exposure whereas 17-PS remained below detection limit as compared to control during prespawning phase. During post-spawning phase, after cypermethrin exposure GSI and the plasma levels of 17-PF was decreased as compared to control but remained below detection limit for 17-PG and 17-PS (Fig. 5).

Effect of cypermethrin exposure for 40 days on 11-deoxycortisol free (SF), 11-deoxycortisol glucuronide (SG) and 11-deoxycortisol sulfate (SS) during prespawning and post-spawning phases in *H. fossilis* : The plasma levels SF, SG declined as compared to control after cypermethrin exposure during both the phases. The plasma level of SG was below detection limit after cypermethrin exposure during post-spawning phases. The plasma levels of SS remained below detection limit in both the phases (Fig. 6).

We for the first time reported the effect of pyrethroid insecticide cypermethrin on conjugation of reproductive sex steroids in male catfish, *H. fossilis* and found that after exposure conjugates have been decreased during prespawning phase at or of stage of steroidogenesis. The conjugates (glucuronides and sulfates) play very important role in pheromonal behavior and spawning in fish. Singh *et al*., (1994) have reported that after γ-HCH exposure in the carp, *Carassius auratus* there is decrease in production of 17,20βP but elevation of 11-deoxycortisol obtained *in vitro*. These authors have demonstrated that T and TG and 11-KT were all significantly suppressed while production of SS increased. Our results indicate that when catfish, *H. fossilis* were exposed with cypermethrin there was decrease in Ss production. The reason is not clear why S increase in vitro in the carp *C. auratus* by γ-HCH and decreases *in vivo* exposure by cypermethrin in catfish, *H. fossilis*. Our results suggest an inhibition of androgen synthesis, possibly due to inhibited side chain cleavage (17, 20-lyase) activity at the testicular level. These results also suggest that the pesticide act predominantly on the early stage of steroidogenesis. The above finding is further supported by the report of Singh and Kime (1995) in *Rutilus rutilus* when testicular fragments were treated with γ-HCH. These authors have demonstrated *in vitro* that the major metabolites of 17-hydroxyprogesterone (17-P) in the testis of *Rutilus rutilus*, 17,20âP, showed a significant increase in yield in response to γ-hexachlorocyclohexane (γ-HCH) together with a decrease in production of its glucuronide suggesting that pesticide affects predominantly the stages of steroidogenesis. The reports (Singh and Singh, 2008 a, b, c) have indicated that fish living in polluted water have low levels of T, 11-KT and E2 when compared to the results with fish inhabiting in clean water. This indicates that a pollutant causes decrease in production of conjugates ultimately affecting the reproductive physiology. The decrease synthesis of 11-KT after cypermethrin exposure may also be due to the

preferential conversion of its precursor to the TG. The effect of cypermethrin have been reported to decline the plasma levels of free estradiol-17β and 11-KT production with the decrease in gonadotrophic cells and condensation of sperms in *H. fossilis* after cypermethrin exposure.

Decrease in unconjugated (free) and conjugated (glucuronides and sulfates) sex steroids after 40 days exposure with cypermethrin indicate that the equilibrium of steroids is perturbed by inhibiting synthesis and release by the testes. The decrease in 11-KT and T indicates that the synthesis of T is decreased and its conversion to 11-KT is also inhibited which may be due to loss of aromatase activity. The conversion of androstenedione to T and 11-KT has been reported earlier (Condeca and Canario, 1996). In the present findings, the decrease in 11-KT and vacuolization in testes and condensation of sperms after exposure indicate that cypermethrin inhibits the androgen biosynthesis. The productions of 11-KT and 17,20βP *in vitro* by testicular fragments and isolated sperms have been reported in *Salmo gairdeneri* (Udea *et al*., 1983; 1984). Biosynthesis of steroid glucuronides from testes of zebra fish playing very important role in pheromonal behavior and spawning have also been reported (Van Den Hurk *et al*., 1987; Waring *et al*., 1996).

The decrease in 17-P and its glucuronide after cypermethrin exposure for 40 days indicates that the conversion of 17-P to 17,20βP is reduced. The 17,20βP which play important role in pheromonal and sexual behavior in carp have also been reported (Sorensen, 1992; Berselius *et al*., 1995; Pinnilos *et al*., 2002; Stacey *et al*., 2003). The results show that the balance of steroid production by testes of *H. fossilis* during prespawning phase is significantly perturbed by cypermethrin and suggest that its effect is predominantly at the stages of steroidogenesis. The results show that cypermethrin directly perturbs gonadal receptor sites involved in steroidogenesis. The perturbation in production of T, 11-KT and Ss suggest that cypermethrin may have a profound effect on reproductive activity. Although Singh *et al*. (1994) have demonstrated that long term exposure of goldfish to γ-HCH affects both gonadotropin secretion and steroidogenesis. The present study shows that even 40 days exposure may cause serious perturbations, and this may be of particular importance at crucial phase of the reproductive cycle. The 11-KT and its conjugate have also been shown by several workers to be potent pheromones in some species, so cypermethrin may also affect the behavioral patterns which are essential for successful spawning (Kime and Manning, 1982; Van Den Hurk *et al*., 1987; Singh *et al*., 1994; Waring and Moore, 1997; Vizziano *et al*., 2008).

The present study demonstrates for the first time in male catfish, *H. fossilis*, the production of S steroid in large amounts; however until further information on its biological activity becomes available, it is difficult to interpret the effect of cypermethrin-induced changes of it synthesis on reproductive activity. However, Colombo *et al*. (1973), who obtained 11-deoxycorticosteroneand 11-deoxycortisol from ovarian incubations of three other teleost, have proposed these steroids may be involved in oocytes maturation and ovulation. The decrease in free SS and low production of SS in male catfish in response to cypermethrin may, in fact, result from the less conversion of the steroid in its conjugate. Decrease of hormones or its conjugate may be due to disturbances in aromatase activity as demonstrated by Higley *et al*. (2010). The result suggests that cypermethrin causes inhibition of free to conjugate form of sex steroids, which causes the perturbances in the equilibrium/ balance of sex steroid hormones affecting the reproductive physiology.

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