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Impact of endosulfan on the profiles of phospholipids at sublethal concentration in the male *Heteropneustes fossilis* (Bloch)

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Abstract: *Male Heteropneustes fossilis were exposed for 30 days at sublethal concentration (0.002 ppm) during different phases of its annual reproductive cycle. Its impact on total phospholipids (TP), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatdylinositol (PI) and phosphatidylethanolamine (PE) were measured in liver, plasma and testes. During preparatory phase, in general, the levels for TP, PC, PS, PI and PE decreased after endosulfan exposure in the above tissues. During prespawning and spawning phases, the phospholipids also showed decreasing trend. The postspawning phase, exhibited decline in hepatic levels of PS and PI only and remained unaffected in the other two tissues. During the resting phase too, the hepatic levels of TP, PS and PI declined and remained unaltered in others. The present results indicate that endosulfan have very selective effects on phospholipids classes during different phases of the annual reproductive cycle interfering with the production of lipid deprived energy i.e. vitellogenin. In general, endosulfan has inhibitory role during reproductive growth affecting phospholipid biosynthesis via hepatic enzyme systems as well as by hormonal imbalance.*

Key words: *Endosulfan, Phospholipids metabolism, Fish, Reproduction.*

Introduction

Aquaculture apart from agriculture is common in India, where fish, the non-target organisms are directly exposed to pesticide endosulfan which is an organochlorine insecticide used for the control of insects and pests. The pesticides affect the survival, growth rate, fecundity and reproductive activity of fish (Hirose, 1975). Toxic substances even in very low concentration which is sublethal have been reported to interfere with basal metabolism and suppressed reproduction (Kondal *et al*., 1989), steroidogenesis (Saxena *et al*., 1986; Wester and Vos, 1994; Singh and Canario, 2004), lipid metabolism (Sangalang *et al*., 1981; Lal *et al*., 1987; Singh and Singh, 1992; Singh and Canario, 2004), degenerative changes in gonadotropin cells and reduction in interstitial cells size (Zutshi, 2005), gonadotropin levels (Van Der Kraak *et al.,* 1992; Singh *et al.,* 1994) act as reproductive biomarkers (Sepulveda *et al*., 2004) and also as endocrine disruptors (Pawlowski *et al*., 2004).

Testicular damage and failure of spermatogenesis (Arora and Kulshrestha, 1984), inhibited male reproductive behavior (Matthiessen and Logan, 1984) and sperm motility (Billard and Cosson, 1992; Njiwa *et al*., 2004) have also been reported in several teleosts after endosulfan exposure. Kime (1998) have reported that long term exposure of pesticide causes extinction of natural fish resources. Earlier reports of Hansen *et al.* (1992) have shown that when [^{32}P] phosphate was added to the water in the holding tank, there was a difference in the incorporation of radioactivity into phosphatidylethanolamine (PE) relative to other phospholipids in gills. Singh and Canario (2004) have reported decreased biosynthesis and release of various phospholipids, sex steroid hormones and variation in gonadotropin levels in *H. fossilis* after γ-HCH exposure during prespawning phase. Lipids are an important source of nutrition in fish providing a significant amount of energy and structural components for reproductive growth (Sargent, 1995). Unless the various classes of phospholipids in liver, plasma and testes are considered simultaneously during annual reproductive cycle, a clear picture of relationship between phospholipids and reproduction can not emerge, because phospholipids undergo rapid breakdown, re-synthesis and inter-conversions with slight change in extero and interoceptic stimuli.

The effects on various classes of phospholipids after endosulfan exposure studies are few during different phases of its annual reproductive cycle. Therefore the present study was performed to assess the effect of endosulfan exposure for various phospholipids on hepatic lipogenesis and their mobilization to testes via plasma during the different phases of its annual reproductive cycle in a tropical teleost, *H. fossilis* of male sex.

Materials and Methods

Experimental fish: The research reported herein was conducted under ethical guidelines for the treatment of animals in behavioral research and teaching (Anonymous, 1998) established for animal usage by Tilak Dhari (PG) College, Jaunpur (UP). Annual reproductive cycle of *H. fossilis*, a seasonal breeder, is divided into five phases: preparatory phase (February-April), prespawning phase (May-June), spawning phase (July-August), postspawning phase (September-October) and resting phase (November-January) by Lamba *et al*. (1983). Male *H. fossils* (weight 65-75g and length 21-22cm) were collected from a local pond (District-Pratapgarh) of the same brood stock during different phases and maintained in a

Table – 1: Gonadosomatic index (GSI) for control and endosulfan exposure during different phases of its annual reproductive cycle in the freshwater catfish, *Heteropneustes fossilis* (Mean ± SEM, n = 5).

Reproductive Phases	Date of collection of fish	GSI before pesticide exposure	GSI after pesticide exposure	Photoperiod	Temperature (^{0}C)
Preparatory (Feb. - April)	March 5th	0.51 ± 0.04	0.50 ± 0.05 NS	11.5L:12.5D	28 ± 2
Prespawning (May - June)	May 8th	9.11 ±1.01	3.14 ± 0.04*	13.0L:11.0D	30 ± 2
Spawning (July -Aug.)	July 10th	12.78 ±1.07	4.33 ± 0.04*	13.3L:10.7D	31 ± 2
Postspawning (Sept. - Oct.)	Sept. 8th	0.71 ± 0.05	0.68 ± 0.06 NS	12.1L:11.9D	26 ± 2
Resting (Nov. – Jan.)	Dec. 1st	0.81 ± 0.03	0.80 ± 0.04 NS	10.3L:13.7D	25 ± 2

GSI before endosulfan exposure vs GSI after endosulfan exposure were compared by Student's 't' test. *p>0.001. NS - Not significant at the level of 0.05.

tank (3500 litre) supplied with a constant flow of de chlorinated tap water with natural photoperiod and temperature during different phases. 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. After 10 days of acclimation, experiments were done.

Chemicals: Analytical grade chemicals were obtained from BDH (India). Solvents were redistilled before use. Thin–layer chromatography (TLC) pre-coated plastic sheets (E. Merck Silica gel G60 F254. 20cm x 20cm x 0.20mm) were obtained from BDH. Phospholipids standards were obtained from Sigma Chemical Co. (UK). Organochlorine insecticide i.e. Endosulfan (=Thiodan-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzadioxathiepin-3-oxide) was obtained, from ITRC, Lucknow (UP).

Exposure studies: For toxicity test endosulfan concentration was used as tested and reported by Singh and Srivastava (1982) for *H. fossilis*.

After acclimation, freshwater male catfish were divided into 2 batches each comprising 6 fish in a plastic aquarium (46 × 25 × 30cm^3) having 20 litre water at room temperature in every phase of the annual reproductive cycle. The endosulfan was dissolved in acetone and diluted with water to the required concentrations. Experimental fish were exposed to endosulfan at the selected sublethal (SL-0.002ppm) concentrations for a month during each phase. Control fish were maintained in plain de-chlorinated tap water containing acetone without the pesticide at the same concentration as the treated groups (20 µl/tank). A separate proper control was also kept without any treatment. During the experiment, fish were fed every 4th day when the aquarium water was changed for freshwater containing the appropriate pesticide concentrations. On 31st day of exposure, fish were bled by caudal incision and blood samples were collected in heparinzed glass culture tubes. Plasma was separated by centrifugation at 5000rpm at 4°C for 15min and stored frozen at –20°C until assayed for various phospholipids. Individual testis weight was taken for calculation of gonadosomatic index (total testis weight × 100 / total body weight) and then kept at -20^{0}C. Livers were individual extirpated, washed in 0.6% saline, blotted and kept frozen at –20°C for further analysis of phospholipids after endosulfan exposure.

Tissue and plasma lipids were extracted by chloroform-ethanol (2:1) following the method of Folch *et al.* (1957). Triplicate samples of each tissue (25mg and 100µl plasma) from a single specimen were taken for analysis. Total phospholipids were separated on TLC using the double solvent system (system I, diethylether : benzene : ethanol : acetic acid, 40 : 50 : 2 : 0.2, and system II, hexane : diethylether, 94 : 6) by Freeman and West (1966) method. R_f values of total phospholipid was 00. Authentic lipids were visualized by exposing the plates to iodine vapor. The total phospholipid was scraped from TLC plate and eluted with chloroform:methanol (2:1) and the solvent evaporated. Samples were re-chromatographed on TLC, and the various phospholipid classes (R_f values for PC, PS, PI and PE were 0.12, 0.20, 0.27 and 0.41 respectively) were separated using methyl acetate: isopropanol: chloroform: methanol: 0.25% aqueous KCl (25 : 25 : 25 : 10 : 9) by volume (Vitiello and Zanetta, 1978). Spots of various phospholipids were made visible by exposing the plates to iodine vapor. Spots of various phospholipids fraction from the samples and the standards, and corresponding areas of the silica gel from the blanks were scraped and transferred to separate test tubes. Quantitative estimation of various lipids was made spectrophotometrically at 375nm by the method of Marzo *et al*. (1971)

Statistical analysis: Data were expressed in mg/g of tissues or mg/ml plasma. Values were expressed as mean ± SEM (n = 5). For statistical analysis of the data, analysis of variance and Student's `t' test at the probability level of 0.05 was employed (Bruning and Kintz, 1977).

Results and Discussion

There was no difference in the values between both the controls i.e. vehicle treated and non-treated proper control, hence, vehicle treated control values were taken into consideration. Toxicity test was confirmed as reported by Singh

Table – 2: Effect of endosulfan on the concentration of various phospholipids in liver, plasma and testes during preparatory phase of the annual reproductive cycle in freshwater male catfish, *Heteropneustes fossilis* (Bloch).(values are in mg/g tissue or mg/ml plasma, Mean ± SEM, n = 5).

Phospholipids concentration				
Phospholipids		**Liver**	**Plasma**	**Testes**
Total Phospholipids (TP)	Control	6.18 ± 0.09	3.00 ± 0.47	2.04 ± 0.22
	Treated	4.22± 0.07*	1.68 ± 0.05****	1.60 ± 0.22NS
Phosphatidylcholine (PC)	Control	2.23 ± 0.32	1.21 ± 0.09	0.71 ± 0.03
	Treated	2.01 ± 0.37NS	0.54 ± 0.03*	0.53 ± 0.03**
Phosphatidylserine (PS)	Control	0.82 ± 0.03	0.15 ±0.03	0.23 ± 0.03
	Treated	0.32 ± 0.02*	0.14 ± 0.03NS	0.20 ± 0.03NS
Phosphatidylinositol (PI)	Control	0.90 ± 0.04	1.18 ± 0.03	0.25 ± 0.03
	Treated	0.51 ± 0.05*	1.16 ± 0.03NS	0.21 ± 0.03NS
Phosphatidylethanol amine (PE)	Control	1.97 ± 0.05	1.00 ± 0.06	0.59 ± 0.03
	Treated	1.01 ± 0.08*	0.45 ± 0.15***	0.41 ± 0.03**

Control vs treated were compared by student's 't' test. *p > 0.001; **p > 0.005; ***p > 0.01 ****p > 0.025. N S - not significant at the level of 0.05.

Table – 3: Effect of endosulfan on the concentration of various phospholipids in liver, plasma and testis during prespawning phase of the annual reproductive cycle in freshwater male catfish *Heteropneustes fossilis* (Bloch).(values are in mg/g tissue or mg/ml plasma, Mean ± SEM, n = 5).

Phospholipids concentration				
Phospholipids		**Liver**	**Plasma**	**Testes**
Total Phospholipids (TP)	Control	3.80 ± 0.08	5.00 ± 0.10	3.00 ± 0.22
	Treated	1.60 ± 0.04*	3.20 ± 0.12*	2.20 ± 0.22***
Phosphatidylcholine (PC)	Control	0.87 ± 0.03	2.09 ± 0.14	1.21 ± 0.09
	Treated	0.51 ± 0.03*	1.29 ± 0.05*	0.71 ± 0.03*
Phosphatidylserine (PS)	Control	0.24 ± 0.01	0.31 ± 0.03	0.11 ± 0.01
	Treated	0.20 ± 0.01**	0.18 ± 0.03**	0.10 ± 0.01NS
Phosphatidylinositol (PI)	Control	0.27 ± 0.02	0.32 ± 0.04	0.16 ± 0.01
	Treated	0.21 ± 0.01***	0.31 ±0.03NS	0.14 ± 0.01NS
Phosphatidylethanol amine (PE)	Control	0.59 ± 0.02	1.01 ± 0.12	1.01 ± 0.03
	Treated	0.43 ± 0.02*	0.95 ±0.12NS	0.61 ± 0.03*

Control vs treated were compared by student's 't' test. *p > 0.001; **p > 0.02; ***p > 0.05. NS - not significant at the level of 0.05.

Table – 4: Effect of endosulfan on the concentration of various phospholipids in liver, plasma and testis during spawning phase of the annual reproductive cycle in freshwater male catfish *Heteropneustes fossilis* (Bloch).(values are in mg/g tissue or mg/ml plasma, Mean ± SEM, n = 5)

Phospholipids Concentration				
Phospholipids		**Liver**	**Plasma**	**Testes**
Total Phospholipids (TP)	Control	2.90 ± 0.07	3.80 ± 0.31	4.80 ± 0.44
	Treated	2.88 ± 0.07NS	1.87 ± 0.31**	2.00 ± 0.22*
Phosphatidylcholine (PC)	Control	1.21 ± 0.03	1.89 ± 0.11	2.79 ± 0.22
	Treated	1.06 ±0.03***	0.71 ± 0.12*	0.71 ± 0.03*
Phosphatidylserine (PS)	Control	0.19 ± 0.05	0.22 ±0.02	0.36 ± 0.01
	Treated	0.18 ± 0.03NS	0.26 ± 0.03NS	0.24 ± 0.01*
Phosphatidylinositol (PI)	Control	0.21 ± 0.08	0.30 ± 0.04	0.37 ± 0.01
	Treated	0.20 ± 0.06NS	0.27 ± 0.03NS	0.26 ± 0.01*
Phosphatidylethanol amine (PE)	Control	0.81 ± 0.05	1.01 ± 0.12	1.11 ± 0.03
	Treated	0.78 ± 0.04NS	0.45 ±0.09***	0.41 ± 0.01*

Control vs treated were compared by student's 't' test. *p > 0.001; **p > 0.005; ***p > 0.01. NS - not significant at the level of 0.05.

Table – 5: Effect of endosulfan on the concentration of various phospholipids in liver, plasma and testis during postspawning phase of the annual reproductive cycle in freshwater male catfish *Heteropneustes fossilis* (Bloch).(values are in mg/g tissue or mg/ml plasma, Mean ± SEM, n = 5).

Phospholipids concentration				
Phospholipids		**Liver**	**Plasma**	**Testes**
Total Phospholipids (TP)	Control	1.84 ± 0.03	1.50 ± 0.27	0.97 ± 0.03
	Treated	1.80 ±0.03^{NS}	1.42 ±0.26^{NS}	0.89 ±0.03^{NS}
Phosphatidylcholine (PC)	Control	0.63 ± 0.06	0.54 ±0.03	0.30 ± 0.01
	Treated	0.59 ±0.06^{NS}	0.50 ±0.03^{NS}	0.28 ± 0.01^{NS}
Phosphatidylserine (PS)	Control	0.28 ± 0.01	0.13 ±0.03	0.10 ± 0.01
	Treated	0.24 ±0.01**	0.10 ±0.03^{NS}	0.09 ± 0.01^{NS}
Phosphatidylinositol (PI)	Control	0.29 ± 0.01	0.15 ± 0.03	0.12 ± 0.01
	Treated	0.21 ± 0.01*	0.13 ±0.03^{NS}	0.10 ± 0.01^{NS}
Phosphatidylethanol amine (PE)	Control	0.35 ± 0.03	0.32 ± 0.03	0.21 ± 0.01
	Treated	0.33 ±0.03^{NS}	0.31 ±0.03^{NS}	0.20 ± 0.01^{NS}

Control vs treated were compared by student's 't' test. $^{*}p > 0.001$; $^{**}p > 0.02$. NS - not significant at the level of 0.05.

Table – 6: Effect of endosulfan on the concentration of various phospholipids in liver, plasma and testis during resting phase of the annual reproductive cycle in freshwater male catfish *Heteropneustes fossilis* (Bloch).(values are in mg/g tissue or mg/ml plasma, Mean ± SEM, n = 5).

Phospholipids concentration				
Phospholipids		**Liver**	**Plasma**	**Testes**
Total Phospholipids (TP)	Control	0.90 ± 0.08	1.80 ± 0.09	0.70 ± 0.07
	Treated	0.60 ± 0.05**	1.72 ±0.10^{NS}	0.62 ±0.07^{NS}
Phosphatidylcholine (PC)	Control	0.22 ± 0.02	0.20 ± 0.03	0.20 ± 0.01
	Treated	0.19 ±0.01^{NS}	0.19 ±0.03^{NS}	0.18 ± 0.01^{NS}
Phosphatidylserine (PS)	Control	0.12 ± 0.01	0.07 ±0.01	0.05 ± 0.01
	Treated	0.04 ± 0.01*	0.05 ±0.01^{NS}	0.03 ± 0.01^{NS}
Phosphatidylinositol (PI)	Control	0.18 ± 0.02	0.07 ± 0.01	0.06 ± 0.01
	Treated	0.06 ± 0.01*	0.06 ±0.01^{NS}	0.04 ± 0.01^{NS}
Phosphatidylethanol amine (PE)	Control	0.17 ± 0.03	0.15 ± 0.03	0.15 ± 0.01
	Treated	0.14 ±0.03^{NS}	0.14 ±0.03^{NS}	0.13 ± 0.01^{NS}

Control vs treated were compared by student's 't' test. $^{*}p > 0.001$; $^{**}p > 0.02$. NS - not significant at the level of 0.05.

and Srivastava (1982) and it was found that SL concentration was 0.002ppm for *H. fossilis*. Significant decline was noticed for gonadosomatic index (GSI) during prespawning and spawning phases following endosulfan exposure (Table 1). Analysis of data showed that responses of phospholipids to endosulfan exposure varied with tissues and various phases of the annual reproductive cycle.

Changes in phospholipids (PL): Changes during different phases are.

Preparatory phase: The hepatic TP, PS, PI and PE recorded a decrease in there level in response to endosulfan exposure during preparatory phase and remained unaffected for PC. The levels for plasma TP, PC and PE recorded decline in its level whereas testes indicated reduction in PC and PE only (Table 2).

Prespawning phase: The hepatic levels of TP, PC, PS and PE decreased significantly after endosulfan exposure during this phase whereas plasma indicated its reduced levels for TP, PC and PS only. The testicular TP, PC and PE decreased significantly and remained unaffected for PS and PI (Table 3).

Spawning phase: During spawning phase, PC was noticed to be decreased whereas TP, PS, PI and PE remained unaffected in liver. The plasma and testes showed decline levels of TP, PC, PS, PI and PE (Table 4).

Postspawning: The hepatic levels of PS and PI decreased and remained unaffected for TP, PC and PE, whereas plasma and testicular levels for all studied phospholipids remained unaffected (Table 5).

Resting phase: During this phase, hepatic TP, PS and PI decreased significantly and rest of phospholipids remained unaffected for plasma and testes after endosulfan exposure (Table 6).

The present experiments which were done during different reproductive phases in male sex of *H. fossilis* indicated that various phospholipids in general, decreased after

endosulfan exposure. This result is in agreement to the other reports for *Carassius auratus* (Singh and Kime, 1994) and *H. fossilis* during prespawning phase (Singh and Canario, 2004). Pesticide induced decrease in gonadal growth as judged by GSI, which has been reported in a number of teleosts (Saxena and Mani, 1987). High levels of various PL (especially PC) in liver, plasma and testes during the reproductively active phases suggest the physiological role of PL in reproduction in the present species probably by building materials to membrane system of proliferating, developing male germ cells as well as maturation of sperms.

In the present study a decrease in total phospholipids in liver, plasma and gonads in response to endosulfan exposure during all the phases revealed that this pesticide arrested the phospholipid synthesis in the liver and translocation of hepatic lipid to testes subsequently. In other reports such as that of Saxena *et al.* (1986) suggested an increase in the total phospholipids level in both ovaries and testis after 30 days exposure whereas carbofuran treated fish did not show increase in significant levels. In our experiments, in response to endosulfan exposure, significant decrease in total phospholipids in liver plasma and gonads during spawning phase was observed while other phases remain unaffected. Here it is concluded that endosulfan exposure affects the hepatic enzymes during anabolic pathway due to which synthesis of phospholipids is affected. The magnitude of effects may be due to types and grades of pesticide concentration in different species.

Mercure *et al.* (2001) have reported that estradiol-17β (E_2) injected *Oncorhynchus mykiss* elevated polar and neutral lipids. Fremont and Riazi (1988) have indicated that the fish vitellogenin comprises 18% of total lipid, of which approximately 2/3 is phospholipids and the remaining fraction is largely triacylglycerides, sterols and sterol esters. In our results, above lipids decreased significantly in response to endosulfan exposure, suggested that endosulfan also affects the synthesis and release of estrogen. Decrease in E_2 and 11-ketotestosterone in response to γ-hexachlorocyclohexane exposure has also been reported in *H. fossilis* by Singh and Canario (2004) during prespawning phase. Recent report of Zutshi (2005) has demonstrated degenerative change in pituitary gonadotropin cells and reduction in interstitial cells size of testis during breeding phase of *Glossogobius giuris* in response to fenthion exposure supporting above contention.

Leslie and Buckley (1976) have reported that in the goldfish liver, PC was the major component of total PL, the rest being PE, PI and PS in decreasing order. They have also shown that liver microsomal choline phosphotransferase enzyme activity decreased as the temperature increased from 10°C to 30°C. Further studies on the individual enzyme involved in the synthesis of different PL are necessary to determine which enzymes are affected by endosulfan exposure in this species. In both the depressed PC (being major component of PL) and PE and elevated activity of PS and PI in *H. fossilis* indicates that the synthesis of PC and PE were reduced in response to endosulfan during these phases. Similar observation has also been recorded in *C. auratus* (Singh and Kime, 1994). There are insufficient reports for various classes of phospholipids available in the catfish due to which interpretation of results i.e. decrease or increase in PI and PE is not possible. In conclusion it is affirmed that endosulfan has inhibitory role for PL biosynthesis during reproductive growth affecting via enzyme systems as well as by hormonal imbalance.

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