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Endosulfan induced changes in phospholipids in the freshwater female catfish, *Heteropneustes fossilis* (Bloch)

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Abstract: *The catfish, H. fossilis were exposed to endosulfan for 30 days at sub-lethal concentration (0.002 ppm) during different phases of its annual reproductive cycle. Its impact on total (TP) and different phospholipids- phosphatidylcholine (PC), phosphatidylserine (PS), phosphatdylinositol (PI) and phosphatidylethanolamine (PE) were measured in liver, plasma and ovary. On pesticide exposure, during preparatory phase, the hepatic TP, PC and PE were declined. The plasma levels of TP, PC and PS were declined with the elevation of PE whereas in ovary only PC was lowered after endosulfan exposure. During pre-spawning phase, the hepatic TP, PC and PE declined in liver, plasma and ovary after endosulfan exposure. During spawning phase, only plasma and ovarian phospholipids showed decrease in their levels following endosulfan exposure. In the post-spawning phase, endosulfan elevated the levels of TP, PC and PS in ovary but had no effect on their levels in liver and plasma. During resting phase, the TP, PC and PI were found to be decreasing its levels. Thus it appears that this pesticide interfere with phospholipids metabolism during annual reproductive cycle of this species.*

Key words*: Endosulfan, Insecticide, Phospholipids metabolism, Fish reproduction*
PDF file of full length paper is available with author

Introduction

Paddy-cum fish culture is very common in eastern part of India, where fishes are directly exposed to endosulfan as non target organism. Endosulfan is an organochlorine insecticide used for the control of insect pests. Pesticides affect the survival, growth rate, fecundity and reproductive activity of fish (Hirose, 1975; Park *et al*., 2004; Singh and Singh, 2006). Toxicants even in very low concentration have been reported to interfere with basal metabolism and suppress the reproduction (Kondal *et al*., 1989), steroidogenesis (Wester and Vos, 1994; Singh and Canario, 2004; Sehgal and Goswami, 2005), lipid metabolism (Lal and Singh, 1987; Singh, 1992; Singh and Singh, 1992; Singh and Kime, 1994), gonadotropin levels (Van Der Kraak *et al.,* 1992; Singh *et al.,* 1994) and also act as reproductive biomarkers (Sepulveda *et al*., 2004). The pesticide are reported as endocrine disruptors (Pawlowski *et al*., 2004; Singh and Canario, 2004), contaminant of ground drinking water (Gopal *et al*., 2004; Kannan *et al*., 2005), inducing hyper secretion of gonadotropins causing regression of gonads (Zutshi, 2005), enzyme inhibition (Tilak *et al*., 2005; Agrahari *et al.,* 2006) and reproductive behavioral changes (Prabhakar *et al*., 1988; Prashanth *et al*., 2005). Recently, pesticide residues are reported from milk and butter (Battu *et al*., 2004). Free and conjugated form of sex steroids has also been reported to be affected by pesticide (Kime and Singh, 1996) which play very important role in pheromonal behaviors and spawning during reproductive seasons (Ebrahimi *et al*., 1995; Pavlidis *et al*., 2004).

Kime (1998) has reported that long term exposure of pesticide causes extinction of natural fish resources. Singh and Canario (2004), have recorded decreased biosynthesis and release of various phospholipids in *H. fossilis* after γ-HCH exposure during pre-spawning 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 ovary are considered simultaneously during annual reproductive cycle, a clear picture of relationship between phospholipids and reproduction can not emerge, because phospholipids undergo rapid breakdown, resynthesis and inter-conversions with slight change in regulatory factors. Therefore in the present study, an attempt has been made to explore the effect of endosulfan on the profiles of various phospholipids in relation to reproductive activity of the freshwater female catfish, *H. fossilis* which is abundantly cultured in paddy fields of State of West Bengal, India as well as in the eastern part of Uttar Pradesh.

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 College, Jaunpur (UP). Annual reproductive cycle of *H. fossilis*, a seasonal breeder, is divided into five phases: preparatory phase (February-April), pre-spawning phase (May-June), spawning phase (July- August), post-spawning phase (September-October), resting phase (November-January). Female *H. fossils* (65-70 g and length 21-22 cm) were collected from a pond of the same brood stock during different phases and maintained in a tank (3500 l) supplied with a constant flow of dechlorinated tap water and enjoyed natural photoperiod and temperature during different phases. They were fed *ad libitum*

Table - 1 : Comparisons of the effect of endosulfan on the concentration of various phospholipids (vehicle control and non-treated / normal control) during pre-spawning phase of the annual reproductive cycle in female catfish, *Heteropneustes fossilis* (Mean ± SE, n = 5)

Phospholipids		Phospholipids concentration: Liver (mg/g tissue)	Plasma (mg/ml)	Ovary (mg/g tissue)
Total Phospholipids (TP)	Vehicle control	4.18 ± 0.07	6.20 ± 0.43	3.20 ± 0.22
	Non-treated control	4.20 ± 0.07 NS	6.46 ± 0.39 NS	3.50±0.23 NS
Phosphatidylcholine (PC)	Vehicle control	1.73 ± 0.04	1.19 ± 0.31	1.29 ± 0.09
	Non-treated control	1.69 ± 0.03 NS	1.03 ±0.22 NS	1.37 ± 0.03 NS
Phosphatidylserine (PS)	Vehicle control	0.27 ± 0.02	0.71 ±0.10	0.15 ± 0.01
	Non-treated control	0.24 ± 0.02 NS	0.79 ± 0.03 NS	0.13 ± 0.01 NS
Phosphatidylinositol (PI)	Vehicle control	0.30 ± 0.01	0.87 ± 0.02	0.22 ± 0.01
	Non-treated control	0.28± 0.01 NS	0.82 ± 0.03 NS	0.21 ± 0.01 NS
Phosphatidylethanol-amine (PE)	Vehicle control	1.03 ± 0.05	1.77 ± 0.20	1.11 ± 0.09
	Non-treated control	1.11 ±0.04 NS	1.87±0.22 NS	1.12 ± 0.07 NS

Vehicle control vs Non- treated control were compared by Student's 't' test, NS = Not significant at the level of 0.05

Table - 2: Gonadosomatic index (GSI) for control and endosulfan exposure during different phases of its annual reproductive cycle in the freshwater catfish, *Heteropneustes fossilis* (Mean ± SE, n = 5)

Reproductive phases	Date of collection of fish	GSI before pesticide exposure	GSI after pesticide exposure	Photoperiod	Temperature (°C)
Preparatory(Feb. - April)	5 March	0.69 ± 0.05	0.58 ± 0.05 NS	11.5L:12.5D	28 ± 2
Prespawning(May - June)	8 May	10.13 ±1.02	3.67 ± 0.06*	13.0L:11.0D	30 ± 2
Spawning(July -Aug.)	10 July	13.68 ±1.41	5.03 ± 0.08*	13.3L:10.7D	31 ± 2
Postspawning(Sept. - Oct.)	8 Sept.	0.91 ± 0.07	0.61 ± 0.06 NS	12.1L:11.9D	26 ± 2
Resting(Nov. - Dec.)	1 Dec.	0.49 ± 0.04	0.41 ± 0.03 NS	10.3L:13.7D	25 ± 2

Level of significance - *p<0.001, NS = Not significant at the level of 0.05

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 performed.

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. 20 cm x 20 cm x 0.20 mm) 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, courtesy Dr R. B. Raizada, ITRC, Lucknow (UP).

Exposure studies: The concentration of endosulfan exposure was the same as tested by Singh and Srivastava (1982) for this species.

After acclimation, freshwater female catfish were divided into 2 batches, each comprising 6 fish in a plastic aquarium (46 x 25 x 30 cm^3) having 20 l water at 20°C. Both the aquaria were surrounded by a water jacket at 20°C. The endosulfan was dissolved in acetone and diluted with water to the required concentrations. Experimental fish were exposed to endosulfan at the selected sub-lethal (SL: 0.002 ppm) concentration for a month during each phase. The purpose of the present study was not to test the toxicity limits of the endosulfan rather a dose was selected based on previous report at which no mortality is there but fish are in little stress so that the effect of endosulfan could be studied on the lipid metabolism of pesticide exposed fish. Control fish were maintained in plain de-chlorinated tap water containing acetone at the same concentration as for the treated groups (20 μl/tank). A separate control was also kept without any treatment. During the experiment, fish were fed every 4th day when the aquarium water was changed with freshwater containing the appropriate pesticide concentrations. On 31st day of exposure, fish were bled by caudal incision and blood samples were collected in heparinised glass culture tubes. Plasma was separated by centrifugation at 5000 rpm at 4°C for 15 min and stored frozen at -20°C until assayed for various phospholipids. Individual ovarian weight was taken for calculation of gonadosomatic index (total gonad weight x100 /total body weight) and then kept at -20°C. Livers were individually removed, washed in 0.6% saline, blotted and kept frozen at -20°C for further analysis of phospholipids.

Tissue and plasma lipids were extracted chloroform methanol (2:1) following the method of Folch *et al.* (1957). Triplicate samples of each tissue from a single specimen were taken for analysis. Total phospholipids were separated on TLC using the double solvent

Table 3: Effect of endosulfan on the concentration of various phospholipids in liver, plasma and ovary during the different phases of the annual reproductive cycle in freshwater female catfish, *Heteropneustes fossilis* (Bloch). (values are in mg/g tissue or mg/ml plasma, Mean ± SE, n = 5)

			Reproductive phases				
Tissues	**Phospholipids**	**Group of fish**	**Preparatory**	**Pre-spawning**	**Spawning**	**Post-spawning**	**Resting**
Liver	TP	Control	8.20 ± 0.09	4.18 ± 0.07	3.20 ± 0.05	2.10 ± 0.08	1.02 ± 0.06
		Treated	5.04 ± 0.08^{a}	2.20 ± 0.07^{a}	3.13 ± 0.07^{NS}	2.00 ± 0.04^{NS}	0.80 ± 0.07^{f}
	PC	Control	3.45 ± 0.19	1.73 ± 0.04	1.27 ± 0.10	0.70 ± 0.07	0.31 ± 0.02
		Treated	2.21 ± 0.34^{c}	0.69 ± 0.03^{a}	1.24 ± 0.17^{NS}	0.69 ± 0.05^{NS}	0.25 ± 0.01^{f}
	PS	Control	0.81 ± 0.03	0.27 ± 0.01	0.19 ± 0.02	0.26 ± 0.01	0.11 ± 0.01
		Treated	0.71 ± 0.06^{NS}	0.24 ± 0.01^{NS}	0.17 ± 0.03^{NS}	0.24 ± 0.01^{NS}	0.10 ± 0.01^{NS}
	PI	Control	1.00 ± 0.05	0.30 ± 0.01	0.21 ± 0.03	0.25 ± 0.01	0.12 ± 0.01
		Treated	0.89 ± 0.04^{NS}	0.28 ± 0.01^{NS}	0.19 ± 0.04^{NS}	0.24 ± 0.01^{NS}	0.04 ± 0.01^{a}
	PE	Control	2.01 ± 0.06	1.03 ± 0.05	0.83 ± 0.03	0.49 ± 0.03	0.23 ± 0.03
		Treated	1.00 ± 0.04^{a}	0.53 ± 0.02^{a}	0.80 ± 0.03^{NS}	0.41 ± 0.03^{NS}	0.21 ± 0.03^{NS}
Plasma	TP	Control	3.62 ± 0.31	6.20 ± 0.43	4.20 ± 0.31	1.85 ± 0.31	0.90 ± 0.09
		Treated	1.80 ± 0.22^{b}	3.46 ± 0.39^{b}	2.10 ± 0.31^{b}	1.78 ± 0.30^{NS}	0.80 ± 0.09^{NS}
	PC	Control	1.69 ± 0.05	1.19 ± 0.31	1.63 ± 0.31	0.72 ± 0.10	0.23 ± 0.03
		Treated	0.69 ± 0.03^{a}	1.03 ± 0.22^{NS}	0.71 ± 0.12^{e}	0.62 ± 0.10^{NS}	0.21 ± 0.03^{NS}
	PS	Control	0.26 ± 0.03	0.71 ± 0.10	0.34 ± 0.03	0.25 ± 0.03	0.09 ± 0.01
		Treated	0.10 ± 0.03^{c}	0.19 ± 0.03^{a}	0.21 ± 0.02^{c}	0.23 ± 0.03^{NS}	0.07 ± 0.01^{NS}
	PI	Control	0.32 ± 0.03	0.87 ± 0.02	0.36 ± 0.03	0.27 ± 0.03	0.10 ± 0.01
		Treated	0.30 ± 0.03^{NS}	0.42 ± 0.03^{a}	0.22 ± 0.02^{b}	0.24 ± 0.03^{NS}	0.08 ± 0.01^{NS}
	PE	Control	0.10 ± 0.02	1.77 ± 0.22	1.03 ± 0.12	0.47 ± 0.03	0.16 ± 0.03
		Treated	0.51 ± 0.03^{a}	0.87 ± 0.22^{d}	0.51 ± 0.09^{c}	0.41 ± 0.03^{NS}	0.11 ± 0.03^{NS}
Ovary	TP	Control	2.42 ± 0.22	3.20 ± 0.22	5.18 ± 0.44	1.18 ± 0.03	0.86 ± 0.07
		Treated	2.02 ± 0.22^{NS}	2.50 ± 0.12^{d}	2.80 ± 0.22^{b}	0.91 ± 0.03^{a}	0.80 ± 0.07^{NS}
	PC	Control	0.89 ± 0.03	1.29 ± 0.09	2.09 ± 0.22	0.37 ± 0.01	0.23 ± 0.01
		Treated	0.73 ± 0.03^{c}	0.87 ± 0.03^{b}	1.08 ± 0.03^{b}	0.31 ± 0.01^{b}	0.22 ± 0.01^{NS}
	PS	Control	0.16 ± 0.03	0.15 ± 0.01	0.86 ± 0.03	0.10 ± 0.01	0.13 ± 0.01
		Treated	0.11 ± 0.01^{NS}	0.13 ± 0.01^{NS}	0.22 ± 0.01^{a}	0.04 ± 0.01^{b}	0.08 ± 0.01^{c}
	PI	Control	0.31 ± 0.03	0.22 ± 0.01	0.89 ± 0.03	0.11 ± 0.01	0.10 ± 0.01
		Treated	0.30 ± 0.03^{NS}	0.21 ± 0.01^{NS}	0.24 ± 0.01^{a}	0.10 ± 0.01^{NS}	0.09 ± 0.01^{NS}
	PE	Control	0.59 ± 0.03	1.04 ± 0.03	1.01 ± 0.03	0.22 ± 0.01	0.18 ± 0.01
		Treated	0.56 ± 0.03^{NS}	0.72 ± 0.03^{a}	0.81 ± 0.03^{b}	0.20 ± 0.01^{NS}	0.16 ± 0.01^{NS}

Abbreviations used : TP = Total Phospholipids, PC = Phosphatidylcholine, PS = Phosphatidylserine, PI = Phosphatidylinositol, PE = Phosphatidylethanolamine. Control vs treated were compared by student's 't' test. Level of significance- $^{a}p > 0.001$, $^{b}p > 0.005$, $^{c}p > 0.01$, $^{d}p > 0.02$, $^{e}p > 0.025$, $^{f}p > 0.05$. NS = Not significant at the level of 0.05

system (system I, diethylether : benzene : ethanol : acetic acid, 40 : 50 : 2 : 0.2 and system II, hexane : diethylether, 94 : 6) of Freeman and West (1966). 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 rechromatographed 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 (Vitello 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 375 nm 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 $\pm$ 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 control (Table 1), hence, vehicle treated control values were used for further consideration. Significant difference was noticed in GSI during different reproductive phases as well as following endosulfan treatment (Table 2). Responses of phospholipids to endosulfan exposure varied with tissues and phase of the annual reproductive cycle.

Changes in gonadosomatic index (GSI):

The GSI was significantly decreased during preparatory and prespawning phases in response to endosulfan treatment and the rest of phases did not showed significant differences (Table 2).

Changes in phospholipids (PL) :

Preparatory phase: The hepatic TP, PC, and PE were decreased in response to endosulfan exposure while PS and PI were unaffected. The plasma levels of TP, PC, PS and PE were also declined whereas in ovary only PC was lowered (Table 3).

Pre-spawning phase: The hepatic levels of TP, PC and PE were suppressed significantly after endosulfan treatment during this phase, whereas in the plasma all the studied PL were reduced. The ovarian TP, PC and PE decreased significantly.

Spawning phase: During spawning phase, TP, PC, PS and PI and PE levels were unaffected in liver in response to endosulfan treatments whereas their plasma and ovarian levels showed remarkable decline.

Post-spawning: The hepatic and plasma levels of TP, PC, PS and PI and PE were unaffected after endosulfan exposure. The ovarian TP, PC and PS decreased significantly.

Resting phase: During this phase, endosulfan lowered the levels of hepatic TP, PC and PI significantly but had no effect on their plasma levels. The ovarian PS decreased significantly and other remained unaffected after endosulfan treatment.

In the present study, endosulfan significantly decreased total phospholipids in liver, plasma and ovary during preparatory, pre-spawning and resting phases suggesting that endosulfan exposure affects the hepatic enzymes due to which synthesis of phospholipids were affected. Lal and Singh (1987), have reported decreased levels of total PL in ovariectomized catfish, *Clarias batrachus* using microsurgical technique during pre-spawning phase. They have suggested that estradiol-17β (E_2) and testosterone (T) are amongst the factors which influence the lipid metabolism. Thus it appears that sex hormones may be responsible for phospholipids decline during reproductive growth following endosulfan treatment. The reports of Singh and Singh (1992), who have reported decline in the levels of E_2 from preparatory to pre-spawning phases in this species after γ-HCH exposure, and recently, Singh and Canario (2004), who have also demonstrated decline in E_2 as well as PC, PS, PI and PE in the catfish following γ-HCH treatment during prespawning phase (vitellogenic), support our above contention.

Lipids are an important source of nutrition in fish providing a significant amount of energy and structural components for reproductive growth (Sargent, 1995). Some studies have corroborated the elevation in hepatic PL to the production of vitellogenin (Schwalme and Mac Kay, 1991; Schwalme *et al*., 1993). Recently, it has been reported that vitellogenin exists as charge isomers (Sehgal and Goswami, 2005) and are stimulated *in vitro* by estradiol-17β isolated hepatocytes of catfish, *Clarias gariepinus* (Rajendra Phartyal *et al*., 2005). Elevated PL and vitellogenin synthesis are related since PL is the predominant lipid in vitellogenin (Ng and Idler, 1983; Tocher *et al*., 1985) and would be requiring for the proliferation of hepatic rough endoplasmic reticulum for the synthesis of female-specific lipoprotein (Henderson *et al*., 1984). Fremont and Riazi (1988) have indicated that fish vitellogenin comprises 18% total lipid, of which approximately two-third is phospholipid and the remaining fraction is largely triacylglyceride, sterol and sterol esters. Hence on the basis of these reports and present results, it can be suggested that the secretion of E_2 by the ovary (under maturational GtH control) which stimulates the liver for the secretion of phospholipid components or vitellogenin, ultimately to be deposited in growing oocytes (under the influence of vitellogenic GtH) during reproductively active prespawning phase, is disrupted by the endosulfan.

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. Further studies on the individual enzyme involved in the synthesis of different PL and E_2 synthesis during reproductive growth simultaneously are necessary to determine which may be affected by endosulfan exposure in this species.

There are insufficient reports for various classes of phospholipids available in the catfish due to which interpretation for decrease or increase in PI and PE is not possible. The magnitude of effects may be due types and grades of pesticide concentration during different phases in different species.

The present finding clearly demonstrated that endosulfan significantly altered the phospholipid metabolism and have very selective and specific effect on different phospholipids in *H. fossilis* during different phases of its annual reproductive cycle. The mobilization of various hepatic phospholipid to the ovary was also restricted by endosulfan. Thus it appears that this pesticide interfere with the production of lipid deprived, vitellogenin, presently judged by PL, which ultimately results in affecting the reproductive physiology of this species.

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