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Differential Induction of Hepatic Cytochrome P450 Isoforms in *Clarias batrachus* Exposed to Ethion and Dicofol

Dawa Bhutia, Joydeb Pal

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ABSTRACT

Pesticides and their byproducts are commonly encountered by aquatic organisms, such as fish. Cytochrome P450 (CYP 450) monooxygenases are widely distributed, multifunctional enzymes essential for the detoxification or activation of pesticides and serve as a biomarker for evaluating the aquatic environment's health. The current study examined the effects of organophosphate (ethion) and organochlorine (dicofol) class of pesticides on CYP 450 enzymes in *Clarias batrachus* after treatment for 5, 10 and 15 days at a 1/3 sub-lethal dosage of the laboratory calculated 96 hour LC50 value. Liver somatic index (LSI), microsomal protein, total CYP 450 content and CYP1A, CYP2B, CYP2E1, CYP3A4 facilitated 7-ethoxyresorufin O-deethylase, N, N- dimethylaniline demethylase, aniline hydroxylase and erythromycin N-demethylase activities in hepatic microsomes were studied. In the pesticide-treated groups, there was a considerable increase in LSI, microsomal protein, and total CYP 450 content. When compared to their respective control groups, the ethion and dicofol-treated groups showed a significant induction ($p<0.05$, $p<0.01$) in CYP1A and CYP3A4 activity. No responses were shown by CYP2E1 activity in either of the treatment groups, but only the ethion-treated group showed a substantial induction in CYP2B activity ($p<0.05$). CYP1A mediated activity was the most pronounced of all the activities.

Keywords Cytochrome P450, Pesticides, Ethion, Dicofol, *Clarias batrachus*, Biotransformation.

INTRODUCTION

Over the past two decades, pesticide use has skyrocketed globally in tandem with shifting farming methods and more intensive agriculture. Pesticides are crucial to maintaining agricultural output because they shield crops from insect infestations and vector-borne diseases (Biswas *et al.* 2019). Pesticide-induced environmental pollution has become a major issue, particularly in aquatic environments.

Dawa Bhutia[1]*, Joydeb Pal[2]

[2]Supervisor

[1]P.G. Department of Zoology, Darjeeling Government College, Darjeeling 734101, West Bengal, India

[2]Ecology and Toxicology Laboratory, Department of Zoology, University of North Bengal, Darjeeling 734013, West Bengal, India

Email: dbhutia16@gmail.com
*Corresponding author

Ethion and Dicofol are organophosphate and organochlorine class pesticides extensively used worldwide to protect agricultural products. Organophosphate and Organochlorine pesticides are neurotoxins used as pest control agents. Contamination of water by pesticides, either directly or indirectly, can lead to fish kills, reduced fish productivity, or elevated concentrations of undesirable chemicals in edible fish tissue, which can affect the health of humans consuming these fish (Naiel *et al*. 2020).

Fish are vulnerable to water contamination from the environment. Therefore, when pollutants like pesticides get into fish organs, they can seriously harm specific physiological and biochemical functions (Okogwu *et al.* 2022). Fish exposed to xenobiotics experience metabolic disruptions as a result of the interactions between these substances and biological systems (Ray and Shaju 2023). Fish can act as bioindicators of environmental pollution and can, therefore, be used for the evaluation of the aquatic system's quality. This is because fish are either directly exposed to these chemicals from agricultural fields through surface runoff of water or indirectly through the food chain of an ecosystem (Okwuosa *et al.* 2019).

Most pesticides are mainly metabolized through phase I biotransformation. The initial enzymatic defence mechanism against xenobiotics is often the phase I detoxification system, mostly made up of the cytochrome P450 (CYP 450) supergene family of the enzyme found in all eukaryotes and certain prokaryotes. Enzymes found mostly in the liver catalyze metabolization reactions, yet there has been evidence of notable activity in other organs as well (Zhao *et al.* 2021). The CYP 450 family of enzymes is primarily responsible for the phase I biotransformation of several xenobiotics in fish. Pesticide metabolism involves a large number of CYP 450s, and the metabolism can lead to activation or detoxification processes. The liver of some freshwater fish has recently been found to contain CYP1A, CYP2B, CYP2E1, CYP2K1 and CYP3A, which are crucial for the detoxification of organophosphate, carbamate, pyrethroid, and organochlorine insecticides (Ferrari *et al.* 2007, Bhutia *et al.* 2015, Bonansea *et al.* 2017, Bhutia and Pal 2023). One significant mechanism contributing to pesticide resistance is CYP 450 mediated pesticide metabolism (Zhang *et al.* 2019). Additional research has also demonstrated that CYP 450 genes have a role in the metabolism of a number of pesticides, including carbamates, pyrethroids, organophosphates, and organochlorine, as well as the bioconversion of chemicals (David *et al.* 2013).

The common pathways of biotransformation of different kinds of pesticides include three CYP 450 mediated reactions: O-dealkylation, hydroxylation, and epoxidation of pesticides (Straus *et al.* 2000, Behrens and Segner 2001). CYP 450 is also continuously used as a biomarker and has been reported to be very informative about an organism's stress response to individual toxicants and mixtures. The oxidative transformation of lipophilic xenobiotics into substances that are more water soluble and hence easily eliminated and detoxified is catalyzed by CYP 450 related enzymes (Melo de Almeida *et al.* 2022).

The metabolism of ethion and dicofol in fish species, as well as any possible impact on phase I enzymes, are, nevertheless, little understood. The present work is focused on studying the impact of ethion and dicofol on hepatic CYP 450 isoforms in *Clarias batrachus*, an economically important fish in India. The study also seeks to ascertain whether CYP 450 is a viable biomarker for exposure to ethion and dicofol.

MATERIALS AND METHODS

Animal treatment and experimental design

Specimens of *Clarias batrachus* were collected from the local fish market. The fish were treated with potassium permanganate solution (0.1%) for 1 min to remove any dermal adherent. The healthy fish (male and female) weighing approximately 35±5 g were transferred to a glass aquarium with 50 L capacity in controlled light (12 hr light/12 hr dark) and aeration conditions and fed regularly. After two weeks of acclimatization, the experiment was started. The experimental procedure was undertaken in compliance with the regulatory rules set by the University of North Bengal Animal Ethics Committee.

Fish were randomly taken in four groups (ten fish in each aquarium), each of control and treated. The experimental fish were treated for 5, 10 and 15 days with a sub-lethal dosage of 16.1 µg/L of ethion and 17.2 µg/L of dicofol (1/3 of LC_{50} value) based on acute toxicity data (not published) acquired from the laboratory trials. The water was renewed every 48 hrs with fresh pesticide for the treated groups and only water for the control group. Homogeneity was maintained in all the groups by providing similar experimental conditions. Liver somatic index (LSI) was calculated as the percentage ratio of liver weight to body weight after livers were removed and weighed at the end of the experiment. The formula LSI = (liver weight/total live weight) × 100 was used to determine the liver somatic index. The liver samples were pooled (ten fish livers apiece) prior to homogenisation since they were too small to be processed separately for enzyme activity.

Microsome isolation

Microsomes were isolated using the procedure described by Chang and Waxman (1998). Livers were perfused with a large volume of ice-cold perfusion buffer (1.15% KCl, 1 mM EDTA, pH7.4) to get rid of unwanted tissues, fat bodies and blood and homogenised in four volumes of homogenisation buffer (1.15% KCl, 1 mM EDTA and 0.05 M Tris, pH 7.4). The homogenate was centrifuged in a cooling centrifuge at 12000 g for 20 min. The supernatant was then centrifuged at 100000 g for 60 min at 4ºC. The resultant pellet was resuspended in two volumes of resuspension buffer containing 0.05 M Tris, 1mM EDTA and 20% Glycerol v/v, pH 7.4 as the hepatic microsomal fraction.

Enzyme assay

Protein in the microsomal fraction was estimated as described by Lowry *et al*. (1951) using bovine serum albumin as standard.

Detection and estimation of CYP 450 was based on the method described by Omura and Sato (1964). The CYP 450 content was determined using the extinction coefficient (ΔE450–490) of 91 mM^{-1} cm^{-1}.

Ethoxyresorufin O-deethylase (EROD) activity in the liver microsome samples was determined spectrophotometrically by the method of Klotz *et al.* (1984). The brown color of resorufin formed at the end of the reaction was measured at 572 nm. The reaction was carried out at 32°C.

CYP2B catalyzed N, N-dimethylaniline demethylase (N, N-DMA) activity was determined by the method of Schenkman *et al.* (1967) with minor modifications. The reaction mixture consisted of N, N-dimethylaniline (100 mM), MgCl2 (150 mM), semicarbazide (100 mM) and microsomes (1.5-2.0 mg). The mixture was pre-incubated for 5 min at 32ºC, and the reaction started by adding NADPH (10 mM) instead of an NADPH generating system. Following aerobic incubation for another 30 min, the reaction was terminated by adding 0.5 mL each of 25% zinc sulfate and saturated barium hydroxide. Formaldehyde formed during the assay was measured by the method of Nash (1953) at 412 nm.

CYP2E1 catalyzed Aniline hydroxylase (AH) activity was determined by measuring the amount of p-aminophenol formed at 630 nm. The method was modified from Schenkman *et al.* (1967) by using aniline (10 mM) as substrate and NADPH (10 mM) instead of an NADPH generating system. In addition, the incubation mixture consisted of 100 mM MgCl2, 120 mM tris (pH 7.4) and 1.5–2.0 mg microsomal protein. After pre-incubation for 5 min at 32ºC, the reaction was initiated by adding NADPH and incubated for 30 min.

CYP3A4 catalyzed Erythromycin demethylase (ERND) activity was determined following the method of Werringloer (1978). The reaction was incubated at 32ºC for 10 min. Formaldehyde formed as the end product was measured by the method of Nash (1953) at 412 nm.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using one-way variance analysis (ANOVA) followed by Dunnett's test. The statistical significance was tested at 1 and 5% levels.

RESULTS

Liver somatic index (LSI), Microsomal protein content, CYP 450 content

Table 1 presents the LSI values, microsomal protein content and CYP 450 content in *C. batrachus* exposed to ethion and dicofol.

All the treated fish groups displayed elevated LSI values after treatment with ethion, however, no significant elevation in LSI values was seen in any treated groups compared to the control. In dicofol treatment, the mean value increased significantly ($p<0.05$, $p<0.01$) in all the treated groups (5, 10 and 15 days), with the highest elevation (54%) seen at 5 days of exposure and then a gradual decrease was to seen at 10 and 15 days of exposure when compared to the control.

All the ethion and dicofol treated fish groups (5, 10 and 15 days) displayed an increase in the microsomal protein content, however, only 15 days treated group showed a significant difference ($p<0.05$) when compared with the control group.

All the ethion treated groups displayed an elevated level of CYP 450 content, but only 10 days (34%) and 15 days treated group (71%) showed a significant difference ($p<0.05$, $p<0.01$) compared to the control group. All the dicofol treated groups displayed a significant difference ($p<0.01$) compared to the control group.

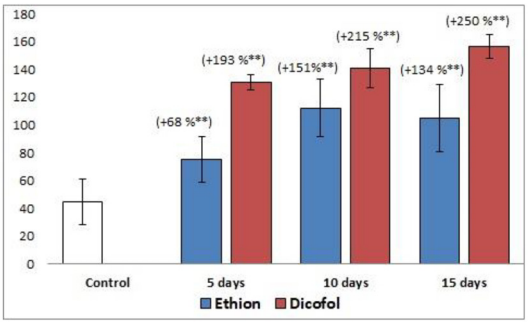

Fig. 1. EROD activity (pmole resorufin formed/mg protein/min) of hepatic microsomes in *C. batrachus* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Values in parentheses represent percent change over control. Significantly different *($p<0.05$), ** ($p<0.01$).

EROD, N, N-DMA, AH and ERND activity

All the ethion and dicofol treated groups (5, 10 and 15 days) displayed a significant difference in an increasing trend ($p<0.01$) when compared with the control group in EROD activities from liver microsomes (Fig. 1).

The N,N-DMA activity in ethion treatment was significantly ($p<0.05$) induced only in the 5 days treated group compared to that of the control group, while 10 days and 15 days treated groups displayed a negligible response. All the dicofol treated groups displayed a negligible response compared to the

Table 1. Liver somatic index (LSI), microsomal protein content and CYP 450 content of *C. batrachus* exposed to ethion and dicofol (n= 6). Values are the means ± SD. Means were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test. Significantly different * ($p<0.05$), ** ($p<0.01$). Values in parentheses are percent change over control. The sign (+) or (−) denotes percent increase or decrease over control.

	Liver somatic index (LSI)		Microsomal protein content (mg/g liver)		CYP 450 content (nmole/mg protein)	
	Ethion	Dicofol	Ethion	Dicofol	Ethion	Dicofol
Control	0.992±0.237		3.239±0.891		0.316±0.098	
5 days	1.033±0.053 (+4.13)	1.531±0.488** (+54.33)	3.447±0.062 (+6.42)	3.533±1.083 (+9.07)	0.377±0.065 (+19.30)	0.534±0.111** (+68.98)
10 days	1.045±0.024 (+5.34)	1.410±0.284** (+42.13)	3.571±0.111 (+10.25)	3.885±0.380 (+19.94)	0.424±0.133* (+34.17)	0.613±0.105** (+93.98)
15 days	1.068±0.040 (+7.66)	1.263±0.049* (+27.31)	4.110±0.563* (+26.89)	4.380±1.128* (+35.22)	0.542±0.084** (+71.51)	0.705±0.069** (+123.1)
F value	0.672	6.555	2.943	1.746	7.182	29.980
P value	0.580	0.003	0.070	0.203	0.002	0.000

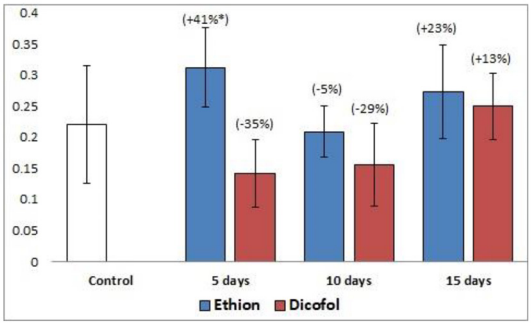

Fig. 2. N, N-DMA activity (nmole formaldehyde formed/mg protein/min) of hepatic microsomes in *C. batrachus* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Values in parentheses represent percent change over control. Significantly different *($p<0.05$), ** ($p<0.01$).

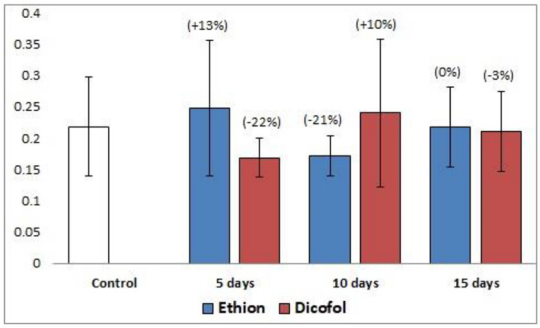

Fig. 3. AH activity (nmole p-aminophenol formed/mg protein/min) of hepatic microsomes in *C. batrachus* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Values in parentheses represent percent change over control. Significantly different *($p<0.05$), ** ($p<0.01$).

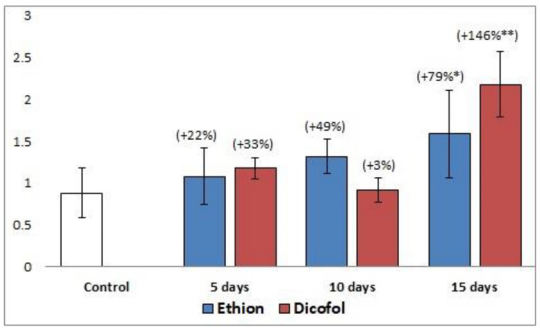

Fig. 4. ERND activity (nmole formaldehyde formed/mg protein/min) of hepatic microsomes in *C. batrachus* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Values in parentheses represent percent change over control. Significantly different *($p<0.05$), ** ($p<0.01$).

control group (Fig. 2).

All the ethion and dicofol treated groups (5, 10 and 15 days) displayed a negligible response in AH activity compared to the control (Fig. 3).

The ERND activity differed significantly ($p<0.05$, $p<0.01$) after 15 days of exposure compared to the control group in both ethion and dicofol treated groups. The 5 days and 10 days treated groups showed an increase in the activity, but was not significant (Fig. 4).

DISCUSSION

During the last few years, several articles on the role of CYP 450s in the metabolism of a wide variety of pesticides have appeared (Jie *et al.* 2011, Bonansea *et al.* 2017, Biswas *et al.* 2019). Many CYP 450s are involved in the metabolism of pesticides and the metabolism may result in activation or detoxification reactions.

A physiological marker that gives details on liver metabolic activity is the LSI. It is especially helpful because generic stressors have a significant impact on them because of a variety of differences, from physico-chemical factors to amounts of exposure to pollutants (Samanta *et al.* 2018). The increase in LSI is commonly seen in fish exposed for longer periods to contaminants in both the laboratory and field (Whatley *et al.* 2010). This increase is not unexpected since the liver is regarded as the main detoxification organ that stores and metabolises toxicants. Fish exposed to environmental toxins can be examined using LSI as a biomarker to determine their physiological status, which can be used to determine their wellbeing and condition (Samanta *et al.* 2018).

The amount of microsomal protein is a critical scaling factor used in toxicology study models to extrapolate *in vitro* metabolism rates to xenobiotic clearance *in vivo*. Given the higher protein content in fish exposed to pesticides, it is likely that any potential protein loss is offset by greater tissue protein synthesis to satisfy the increased need for pesticide detoxification, which calls for increased production of detoxification enzyme proteins (Williams *et al.*

2013). Muley *et al.* (1996) reported increased protein content in the gill, muscles and kidney of *Tilapia mosambica* exposed to endosulfan. Nath *et al.* (2012) also reported an increase in the protein level in the liver of freshwater fish, *Oreochromis niloticus*, after exposure to thiamethoxam. The current study's increased microsomal protein concentration might be the result of examining the impact and attempts to recover from the pesticide stress.

Xenobiotic metabolism in fish occurs mainly in the liver, which displays the highest specific and total enzyme activities of all tissues. Metabolism plays a critical role in determining both the efficacy and the residence time of xenobiotics in the body as well as in modulating the response to toxic chemicals (Li *et al.* 2019). CYP 450 related enzyme assay is currently considered a simple and reproducible tool for assessing the presence of organic compounds in the environment, ranging from severe pollution to low-level contamination.

The CYP 450 catalytic enzyme family is inducible when animals are exposed to toxins. This explains why CYP 450 content was higher in experimental fish exposed to pesticides compared to the control. However, CYP 450 proteins only constitute a limited percentage of total microsomal protein content. Another microsomal enzyme system involved in pesticide metabolism is flavin-containing monooxygenase (FMO) (Hodgson *et al.* 1995). Thus, the difference in microsomal protein content between control and experimental fish may result from the induction of both CYP 450 and FMO systems.

Though EROD activity was significantly induced in all the treated groups (5, 10 and 15 days), the induction level was of lesser magnitude with ethion than dicofol exposure, probably due to EROD's higher detection sensitivity. CYP1A was classically recognised as being induced explicitly by polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs), it is now known that it is not particularly responsive to one unique class of pollutants and can also be induced by pesticide exposure. Many studies have reported an induction of EROD activity in liver microsomes in fishes exposed to chlorpyrifos and parathion (Straus *et al.* 2000), methoxychlor (Stuchal *et al.* 2006), chlorpyrifos (Rai *et al.* 2010) and DDT (Lemaire *et al.* 2010). Somnuek *et al.* (2012) reported that chlorpyrifos and carbaryl at high concentrations significantly elevated CYP1A gene expression. CYP1A subfamily is the most studied CYP 450 isoform and is responsible for a wide range of xenobiotic biotransformation whose catalytic activity is expressed as the activity of EROD. It can be used as a biomarker to monitor the ecological risk of various environmental pollutants (Whatley *et al.* 2010). EROD activity is best viewed as an indicator of contaminant exposure rather than of effect, and this biomarker may also serve as a predictive tool for contaminant risk assessment (Kartal and Bildik 2022).

As CYP2E1 is involved in the metabolism of several low molecular-weight xenobiotic substances, it did not receive attention as a major biotransformation enzyme as other CYP 450 isoforms. However, investigations have shown that exposure to the pesticides cypermethrin (Bhutia *et al.* 2013), atrazine (Lang *et al.* 1997), carbaryl (Tang *et al.* 2002) and parathion (Mutch and Williams 2006) stimulates CYP2E1 activity. On the other hand, dicofol has been reported not to affect CYP2E1 activity in *Channa punctatus*, while the activity was significantly induced in *Heteropneustes fossilis* (Bhutia and Pal 2023).

Organophosphate pesticides are activated in vertebrate liver by CYP 450 mediated oxidative desulfuration to form toxic oxygen analogues (oxons) and also nontoxic dearylation reaction that degrades the parent compound (Banni *et al.* 2011). CYP2B was previously regarded as a minor hepatic CYP 450 enzyme in fish, as the induction of CYP2B isoforms through interaction with the constitutive androstane receptor has not been well established, but this view has changed over the last few years. It has been shown that CYP2B mediates the oxidative metabolism of many phosphorothioate organophosphate pesticides, producing the highest desulfuration activity, while at the same time, CYP3A4 has high activity for both dearylation and desulfuration (Sams *et al.* 2000, Tang *et al.* 2001).

CYP3A4 isoform is highly active and is involved in the metabolism of many pesticides. The fact that CYP3A4 is the most abundant CYP isoform in the liver suggests that this isoform plays a significant *in vivo* role in both desulfuration and dearylation (Tang *et al.* 2001). Contrary to organophosphate pesticides, organochlorine pesticide is seen to be highly metabolised by CYP1A and CYP3A4 than by CYP2B isoform. Channel catfish (*Ictalurus punctatus*) exposed to methoxychlor (Stuchal *et al.* 2006), and *Channa punctatus* and *Heteropneustes fossilis* exposed to dicofol (Bhutia and Pal 2023) reported an induced level of CYP3A4 activity in liver microsomes.

Fish exposed to ethion and dicofol showed a differential response pattern in biotransformation biomarkers. Whenever CYP 450 enzymes are used as biomarkers in monitoring programs, one should be cautious with metabolic differences, even among fish species. Though specific CYP 450s are necessary to metabolise xenobiotics, pesticides can simultaneously induce one or more forms of CYP 450. It is reported that fish respond to exposure to pollutants by altering or adapting their metabolic functions (Melo de Almeida *et al.* 2022). Alterations found in the activity of antioxidant enzymes upon pesticide exposure suggest that the changes observed could be adaptive.

CONCLUSION

In the present investigation, it was evident that in *Clarias batrachus*, CYP 450 isoforms were more responsive towards dicofol than ethion, and CYP1A was the most sensitive isoform. It was also apparent that fish have the capacity to increase their detoxifying power when exposed to pesticides. CYP 450 plays a vital role in protecting tissue from oxidative stress. The increase in this enzyme activity in the liver indicates the development of a defensive mechanism to counter the effect of pesticides and may reflect the organism's ability to provide more efficient protection against pesticide toxicity. Our studies suggest that pesticide exposed fish may improve their detoxification capacity by enhancing the amount of CYP 450 enzyme system.

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