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ACTIVITY OF CHOLINESTERASE AND ALKALINE PHOSPHATASE IN LIVER, KIDNEY AND BRAIN OF *EUPHLYCTIS CYANOPHLYCTIS* UNDER THE EFFECT OF CHLORPYRIFOS AND DATHRIN

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ABSTRACT

Globally many scientists believe that several common pesticides already exist at levels capable of killing amphibians in the earth. The aquatic biota may be harmed by pesticide-contaminated water. The present study was done to investigate the effects of two pesticide groups organophosphate and pyrethroid, on the activity of cholinesterase (ChE) and alkaline phosphatase (ALP) in the liver, kidney and brain tissue of *Euphlyctis cyanophlyctis*. LD_{50} of each pesticide was determined before the selection of final concentrations of both pesticides. The frogs were treated by two concentrations of chlorpyrifos i.e. 2 and 4%. The effect of these two concentrations on ChE activity in the liver, kidney and brain was estimated. According to results it was decreased upto 30.0 and 45.0% in liver ($F_{2,6}$=116.90, P=0.001), 20.0 and 50.0% ($F_{2,6}$=8.99, P=0.016) in kidney and 33.33 and 55.55 % ($F_{2,6}$=63.96, P=0.001) in the brain, respectively. Under the effect of two concentrations of Dathrin i.e. 0.04 and 0.08% the ChE activity in the liver was decreased upto 15.0 and 45.0% ($F_{2,6}$=14.90, P=0.005), in the kidney it was decreased upto 20.0 and 40.0 % ($F_{2,6}$=7.30, P=0.025), while in the brain the activity decreased upto 22.0 and 44.0 %, respectively ($F_{2,6}$=6.80, P=0.029). The effects of same concentrations of both pesticides were also observed on alkaline phosphatase (ALP) activity in liver, kidney and brain of *E. cyanophlyctis*. In the case of chlorpyrifos it was decreased upto 25.0 and 50.0 % ($F_{2,6}$=7.00, P=0.027), in kidney ALP activity decreased upto 33.33 and 50.0 % ($F_{2,6}$=1.98, P=0.219), while in the brain it decreased upto 22.22 and 44.44 %, respectively ($F_{2,6}$=1.89, P= 0.231). The effect of Dathrin on ALP in liver it was decreased upto 37.50 and 50.0 % ($F_{2,6}$=73.0, P= 0.001), in the kidney the activity was decreased upto 16.66 and 50.0% ($F_{2,6}$=2.02, P=0.214), while in the brain it was decreased upto 44.44 and 55.55%, respectively ($F_{2,6}$=1.98, P=0.219).

Keywords: *Euphlyctis cyanophlyctis,* cholinesterase, alkaline phosphatase, chlorpyrifos, dathrin.

INTRODUCTION

The widespread application of agricultural pesticides has attracted the attention of ecologists to understand the impacts of these chemicals on natural communities (Relyea *et al*., 2005). Amphibians are important to the overall ecosystem balance. The large biomass of amphibians makes them significant prey for other animals (Khan *et al.,* 2007a). Direct contact of sprays of some pesticides can be highly lethal to amphibians (Relyea, 2005). The loss of amphibian populations was first recognized in 1989 as a phenomenon that deserved worldwide attention (Barinaga, 1990; Wake, 1991; Blaustein, 1994; Alford and Richards, 1999). The obvious factor contributing to amphibian population declines are habitat destruction and alteration (Alford and Richards, 1999). A wide array of contaminants may affect amphibian populations which include pesticides, herbicides, fungicides, fertilizers and numerous pollutants (Sparling *et al.,* 2000; Boone and Bridges, 2003). A diversity of pesticides and their residues are present in a wide variety of aquatic habitats (Harris *et al.,* 1998; McConnell *et al.,* 1998; Le Noir *et al.,* 1999; Kolpin *et al.,* 2002). While pesticides have the potential to affect many aquatic species, the impacts on amphibians are of particular concern in the past decade because of the apparent global decline of many species (Blaustein and Wake, 1990; Alford and Richards, 1999; Houlahan *et al.,* 2000; Kiesecker *et al.,* 2001) and the amphibians living in these habitats exhibit physiological signatures of pesticides (i.e. reduced acetylcholine esterase activity; Sparling *et al*., 2001) and declining populations are correlated with greater amounts of upwind agriculture where pesticide use is common (Davidson *et al.,* 2001, 2002).

Pesticides can severely affect amphibians in a variety of ways, as they destroy the natural biotic balance in agricultural soils and reduce the diversity and abundance of biodiversity with cascading effects at higher trophic levels (Larson *et al.*, 1997). They can kill amphibians directly, affect their behaviour, reduce their growth rates, act as endocrine disrupters or induce immunosuppression (Bishop, 1992; Carey and Bryant, 1995; Alford and

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Richards, 1999). The causes of these declines include human involvement in an effort to increase agricultural products and indiscriminate use of pesticides. The effects of these toxic materials remain to be studied on non-target biodiversity in many regions of the world. (Khan *et al.*, 2002b). The pesticides, organophosphate and carbamate are widely used and have a variety of lethal and sublethal effects on non-target wildlife species (Parsons *et al*., 2000; Khan *et al.*, 2003a). Pyrethroids appear to effect voltage-dependent neuromuscular sodium channels producing tremors, hyperexcitation and convulsions (Van den Bercken, 1977; Vijverberg *et al*., 1982; Ruigt and Van den Bercken, 1986). In Pakistan pesticides have been reported to have reduced enzyme activity of cholinesterase in frog *Rana tigrina* (Khan *et al.*, 2002a,b, Khan *et al.*, 2003a,b) in skittering frog *Rana cyanophlyctis* (Khan *et al.*, 2003b, c,d; Khan and Yasmeen, 2005; Khan *et al.,* 2007a). Khan *et al. (*2007b) determined the induced effect of chlorpyrifos (organophosphate) on skin of *E. cyanophlyctis.* In the present study the effects of Chlorpyrifos (organophosphate) and Dathrin (pyrethroid) were observed on the activity of two enzymes cholinesterase and alkaline phosphatase in the liver, kidney and brain of skittering frog *E. cyanophlyctis.*

MATERIALS AND METHODS

The experimental work was carried out on adults skittering frog *Euphlyctis cyanophlyctis*, collected from selected areas of Province of Sindh. Collected frogs were brought in laboratory and kept in glass aquarium in the Wildlife Lab, Department of Zoology, University of Karachi. Frogs were fed with prawns and insects. Two concentrations of both pesticides were applied, i.e. 2 and 4% of Chlorpyrifos, while 0.04 and 0.08% of Dathrin were injected in the sub-cutaneous abdominal region of frog by using insulin syringe. The effects of pesticide were observed in liver, kidney and brain tissue. The liver, kidney and brain were taken as per Shakoori and Ahmad's (1973) techniques. Organs (liver, kidney and brain) were crushed in 2 ml bidistilled water and homogenized. The homogenates were centrifuged in Labofuge 15000 at 5000 rpm for 20 minutes and placed in cold chamber. Supernatents were taken in separate glass tubes to use in estimation of enzyme activity.

The activity of cholinesterase was estimated by Randox Kit No. CE-190. In colorimetric method (Knedel and Boetteger, 1967) the reagent composition was consisting of Buffer (Phosphate buffer=50 mmol/l having pH 7.7 and DTNB=0.25 mmol/l) and Substrate (Butyrylthiocholine iodide=6 mmol/l). The method based upon the hydrolysis of butyrylthiocholine by the action of enzyme butyryl cholinesterase. The reaction between thiocholine and dithiobis (nitrobenzoate) gave 2-nitro-5-mercaptobenzoate, a yellow compound which was measured at 405 ηm.

Principle:

$$\text{Butyrylthiocholine} + H_2O \xrightarrow{\text{Cholinesterase}} \text{thiocholine} + \text{butyrate}$$

$$\text{Thiocholine} + \text{DTNB} \rightarrow \text{2-nitro-5-mercaptobenzoate}$$

DTNB = Dithiobis (nitrobenzoate)

The activity of alkaline phosphatase was estimated by Randox Kit No.AP-307. In colorimetric method (Rec.GSCC, 1972) the reagent composition was Buffer (Diethanolamine buffer=1 mol/l, pH=9.8 and $MgCl_2$= 0.5 mmol/l) and Substrate (p-nitrophenylphosphate=10 mmol/l). The reaction is based upon the hydrolysis of p-nitrophenylphosphate by the action of alkaline phosphatase.

Principle:

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{phosphate} + \text{p-nitrophenol}$$

All statistical analyses were conducted by Micro Soft Excel and Minitab (Minitab Inc,1996). Data presented as percentages were arcsine-square-root transformed before analyses. One way analysis of variance (ANOVA) was used to compare the effect of two concentrations of each pesticide on activity of enzymes cholinesterase and alkaline phosphatase.

RESULTS

In this study, effect of two concentrations of chlorpyrifos on cholinesterase in the liver, kidney and brain was determinate. The enzyme level decreased up to 30.0 and 45.0% in liver ($F_{2,6}$=116.90, P=0.001) (Table 1), 20.0 and 50.0% ($F_{2,6}$=8.99, P=0.016) (Table 2) in kidney and 33.33 and 55.55 %, respectively ($F_{2,6}$=63.96, P=0.001) (Table 3) in the brain.

Under the effect of two concentrations of Dathrin the cholinesterase activity in the liver was decreased up to 15.0 and 45.0% respectively ($F_{2,6}$=14.90, P=0.005) (Table 4), in the kidney it decreased up to 20.0 and 40.0 % ($F_{2,6}$=7.30, P=0.025) (Table 5), while in the brain the activity decreased up to 22.0 and 44.0 %, respectively ($F_{2,6}$=6.80, P=0.029) (Table 6).

The effects of same concentrations of both pesticides were also observed on alkaline phosphatase in liver, kidney and brain. In the case of chlorpyrifos it was decreased up to 25.0 and 50.0 % ($F_{2,6}$=7.00, P=0.027) (Table 7), in kidney ALP activity decreased up to 33.33 and 50.0 % ($F_{2,6}$=1.98, P=0.219) (Table 8) and in the brain it decreased up to 22.22 and 44.44 % respectively ($F_{2,6}$=1.89, P= 0.231) (Table 9).

Table 1. Activity of cholinesterase in liver of *E. cyanophlyctis* treated with Chlorpyrifos.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	18.40	10.449	6.039	6.561 - 30.238	00
2%	12.88	5.745	3.321	6.370 - 19.389	30.0
4%	10.12	5.745	3.321	3.610 - 16.629	45.0

$F_{2,6} = 116.90$, P = 0.001

Table 2. Activity of cholinesterase in kidney of *E. cyanophlyctis* treated with Chlorpyrifos.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	9.20	6.373	3.684	1.978 - 16.421	00
2%	7.36	5.745	3.321	0.850 - 13.869	20.0
4%	4.60	3.186	1.842	0.989 - 8.210	50.0

$F_{2,6} = 8.99$, P = 0.016

Table 3. Activity of cholinesterase in brain of *E. cyanophlyctis* treated with Chlorpyrifos.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	8.28	2.76	1.595	5.153 -11.406	00
2%	5.52	2.76	1.595	2.393 - 8.646	33.33
4%	3.68	1.593	0.921	1.874 - 5.485	55.55

$F_{2,6} = 63.96$, P = 0.001

Table 4. Activity of cholinesterase in liver of *E. cyanophlyctis* treated with Dathrin.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	18.4	10.449	6.039	6.561 - 30.238	00
0.04%	15.64	10.449	6.039	3.801 - 27.478	15.0
0.08%	10.12	4.215	2.436	5.343 - 14.896	45.0

$F_{2,6} = 14.90$, P = 0.005

The effect of Dathrin on alkaline phosphatase in the liver was evident by a decrease upto 37.50 and 50.0 % ($F_{2,6}$=73.0, P= 0.001) (Table 10), in kidney the activity decreased upto 16.66 and 50.0% ($F_{2,6}$=2.02, P=0.214) (Table 11), while in the brain it was decreased up to 44.44 and 55.55% respectively ($F_{2,6}$=1.98, P=0.219) (Table 12).

DISCUSSION

On the basis of lab experiments, amphibians are known to be vulnerable to pesticides and they are cholinesterase inhibitors (Wang and Murphy, 1982). There is some indication, that field application of these chemicals may be deleterious to amphibians (Jolly *et al*., 1978; Thybaud, 1990; Berril *et al*., 1993; Materna *et al*., 1995). A number of non-target species can be affected when pesticides are used because of their affect on cholinesterase activity. The enzyme inhibition occurs in a number of species and reduction can result in sub-lethal toxicity and death (Cooper, 1991). Anticholinesterase pesticides function by binding with this enzyme in animals and disrupting nervous system activity, usually causing death by respiratory failure. Decreased cholinesterase activity can indicate exposure to some commonly used pesticides and can be harmful to wild animals (Catherine and Gloria, 2000).

In the present study, the estimation of two enzymes i.e. cholinesterase and alkaline phosphatase were carried out in the liver, kidney and brain of *E. cyanophlyctis* under

Table 5. Activity of Cholinesterase in kidney of *E. cyanophlyctis* treated with Dathrin.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	9.2	6.373	3.684	1.978 - 16.421	00
0.04%	5.745	3.321	6.509	0.850 - 13.869	20.0
0.08%	5.52	4.780	2.763	0.103 - 10.936	40.0

$F_{2,6} = 7.30$, $P = 0.025$

Table 6. Activity of cholinesterase in brain of *E. cyanophlyctis* treated with Dathrin.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	8.28	2.76	1.595	5.153 - 11.406	00
0.04%	6.44	4.215	2.436	1.663 - 11.216	22.00
0.08%	4.60	1.593	0.921	2.794 - 6.405	44.00

$F_{2,6} = 6.80$, $P = 0.029$

Table 7. Activity of alkaline phosphatase in liver of *E. cyanophlyctis* treated with Chlorpyrifos.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	7.36	3.186	1.842	3.749 - 10.970	00
2%	5.52	4.780	2.763	0.103 - 10.936	25.00
4%	3.68	1.593	0.921	1.874 - 5.485	50.00

$F_{2,6} = 7.00$, $P = 0.027$

Table 8. Activity of alkaline phosphatase in kidney of *E. cyanophlyctis* treated with Chlorpyrifos.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	5.52	2.76	1.595	2.393 - 8.646	00
2%	3.68	1.593	0.921	1.874 - 5.485	33.33
4%	2.76	00	00	-	50.00

$F_{2,6} = 1.98$, $P = 0.219$

the effects of Chlorpyrifos and Dathrin as compared with lab standard (control).

The organophosphate can affect cholinesterase activity in both red blood cells and in blood plasma, and can act directly, or in combination with other enzymes, on cholinesterase in the body. The first notable studies to examine the effects of organophosphates on amphibians were reported in the early sixtees (Edery and Schatzberg-Porath, 1960; Mulla, 1962; Mulla *et al.,* 1963). More recently several studies indicated that standard field application rates of organophosphates insecticides may have a deleterious effect on amphibian population (Anguiano *et al*., 1994; Berril *et al.,* 1993 and1994; Schuytema *et al.*, 1995; Sparling *et al.,* 1997). While pyrethroid have gained a reputation as "safe insecticides" and are widely used in agricultural, aquatic and house hold products (Elliot *et al*., 1978; Smith and Stratton 1986). During this study, the effect of 2 and 4% concentration of chlorpyrifos (organophosphate) on cholinesterase activity in the liver, kidney and brain showed significantly decrease i.e. upto 30.0 and 45.0% ($P<0.001$) in liver, 20.0 and 50.0% ($P<0.016$) in kidney and 33.33 and 55.55 % ($P<0.001$) in the brain, respectively while in the case of Dathrin the effect of 0.04 and 0.08% concentrations on cholinesterase activity in the liver, kidney and brain were found significantly decreased upto 15.0 and 45.0% ($P<0.005$) in the liver, 20.0 and

Table 9. Activity of alkaline phosphatase in brain of *E. cyanophlyctis* treated with Chlorpyrifos.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	8.28	7.302	4.220	0.006 - 16.553	00
2%	6.44	4.215	2.436	1.663 - 11.216	22.22
4%	4.6	3.186	1.842	0.989 - 8.210	44.44

$F_{2, 6} = 1.89$, P = 0.231

Table 10. Activity of alkaline phosphatase in liver of *E. cyanophlyctis* treated with Dathrin.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	7.36	3.186	1.842	3.749 - 10.970	00
0.04%	4.6	3.186	1.842	0.989 - 8.210	37.50
0.08%	3.68	1.593	0.921	1.874 - 5.485	50.00

$F_{2, 6} = 73.0$, P = 0.001

Table 11. Activity of alkaline phosphatase in kidney of *E. cyanophlyctis* treated with Dathrin.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	5.52	2.76	1.595	2.393 - 8.646	00
0.04%	4.6	1.593	0.921	2.794 - 6.405	16.66
0.08%	2.76	00	00	-	50.00

$F_{2, 6} = 2.02$, P = 0.214

Table 12. Activity of alkaline phosphatase in brain of *E. cyanophlyctis* treated with Dathrin.

Treatment	Mean (U/l)	S.D. ±	S.E. ±	Range at 95% Confidence limit	Inhibition %
Control	8.28	7.302	4.220	0.006 - 16.553	00
0.04%	4.6	3.186	1.842	0.989 - 8.210	44.44
0.08%	3.68	1.593	0.921	1.874 - 5.485	55.55

$F_{2, 6} = 1.98$, P = 0.219

40.0% (P<0.025) in kidney, 22.0 and 44.0% (P<0.029) in the brain, respectively.

Tilak *et al.* (2003) investigated the effect of fenvalerate in Indian bullfrog to sublethal dose (1/3 of LC_{50} I.E. 1.166 mg/kg) and the effect was studied on the specific activity of acetyl-cholinesterase in the different tissues of frog liver, kidney, brain, muscle, and testis at different time periods viz., 3,6, 12, 24, 48 and 72 hours. It was found that the inhibition in activity of acetyl-cholinesterase was in the order of kidney > brain > muscle > liver > testis. A significant inhibition was noticed in kidney at 12 hours (-64.33%) and no effect was noticed at 3 hours in testis (+0.67%). It was suggested that the AChE activity was inhibited in first three hours of administration of fenvalerate in all the tissues tested. The inhibition continued upto 6 hours or 2 hours in different tissues but the recovery started by 24 hours and almost completed by 72 hours.

Another study, Khan *et al*. (2003a) investigated that the effect of cypermethrin and permethrin on cholinesterase in *Rana tigrina* and reported that the ChE activity decreased in the treated frogs. The present findings are generally in accordance with the previous reports. Khan *et al*. (2003b) compared the effect of two pyrethroids lambda cyhalothrin with permethrin on cholinesterase in *R. cyanophlyctis* and *R. tigrina* and reported that the amphibian in general are sensitive and *R. cyanophlyctis* is more sensitive to *R. tigrina,* and lambda cyhalothrin is

more toxic among the pesticides tested. In the present finding also, it was observed that Dathrin inhibited the ChE activity in the liver, kidney and brain. Khan *et al.*, 2003c determined the effect of two pesticides lambda cyhalothrin and monocrotophos on ChE in the liver, kidney and brain of *R. cyanophlyctis*, two different concentrations were used and ChE activity was observed. It was decreased up to 34.6 and 46.3 % in the liver, 25.08 and 57.1% in the kidney and 31.64 and 50.7% in the brain under the effect of lambda cyhalothrin. In the case of monocrotophos treatment, cholinesterase decreased up to 37.7 and 57.7% in liver, 57.5 and 67.5% in kidney and 47.6 and 65.9% in brain, respectively. The brain cholinesterase activity of *R. cyanophlyctis* was decreased up to 4.10 and 13.84 % under the effect of sandaphos and 5.16 and 23.28% under the effect of β-cypermethrin, respectively (Khan and Yasmeen, 2005). Khan *et al*. (2006) reported that the effects of sandaphos and β-cypermethrin on ChE activity in liver and kidney of *E. cyanophlyctis*, under the effect of sandaphos, ChE activity decreased upto 41.28 and 51.46% in the liver and 4.43 and 22.85% in the kidney, respectively. Under the effect of β-cypermethrin, ChE activity decreased upto 24.46 and 26.34 in the liver and 21.46 and 26.63% in the kidney, respectively.

The alkaline phosphatase is a unique enzyme that is usually present in the tissues involved in transport function and regeneration (McGomb *et al*., 1979). This enzyme has been extensively studied in liver, bone, placenta, intestine and serum. The localization of this enzyme in plasma membrane strongly suggests its involvement in membrane functions (Fishman, 1974).

Yora and Sakagishi (1986) studied the activity of alkaline phosphatase isozymes in fish, amphibians, reptiles, birds and mammals. The alkaline phosphatases from the liver, kidney and intestine in various vertebrates were strongly inhibited by beryllium, 2-mercaptoethanol, potassium cyanide and EDTA. The enzymes showed various sensitivities to the inhibition by zinc and to heat denaturation at 56 degrees Celsius for 5 min at pH 7.0. The liver and kidney enzymes showed higher sensitivity to the inhibition by L - homoarginine than by L - phenylalanine. The intestinal enzymes in higher vertebrates were more sensitive to the inhibition by L - phenylalanine than by L - homoarginine, whereas the intestinal ones in lower vertebrates showed quite similar sensitivities to both amino acid. Goseki *et al.* (1990) examined the enzymatic and immunological properties of alkaline phosphatase (ALP) in several tissues of bullfrog *Rana catesbeiana.* The inhibition and thermal inactivation studies showed that bullfrog ALP in kidney, liver and intestine had similar enzymatic properties. In addition, mouse antiserum against bullfrog liver ALP cross-reacted with kidney and intestine enzymes as well as with liver enzyme.

The present study with reference to Pakistan is the first attempt to determine the effects of pesticides on ALP activity in *E. cyanophlyctis* and findings have showed the effect of 2 and 4% concentration of chlorpyrifos on alkaline phosphatase in the liver, kidney and brain decreased upto 25.0 and 50.0% ($P<0.027$) in the liver, 33.33 and 50.0% ($P<0.219$) in kidney, 22.22 and 44.44% ($P<0.231$) in the brain, respectively. Under the effect of Dathrin the effect of lower 0.04 and 0.08% concentrations on alkaline phosphatase in the liver, kidney and brain was found significantly decreased upto 37.50 and 50.0% ($P<0.001$) in the liver, 16.66 and 50.0% ($P<0.214$) in kidney, 44.44 and 55.55% ($P<0.219$) in the brain, respectively. In general the present results are in agreement with earlier reports.

CONCLUSION

On the basis of present findings it is concluded that the selected pesticides Chlorpyrifos (organophosphate) and Dathrin (pyrethroid) decreased the cholinesterase and alkaline phosphatase activity in the liver, brain and kidney of *E. cyanophlyctis.* The organophosphate group is more harmful to *E. cyanophlyctis* as compared to pyrethroid group. It is therefore, suggested that pyrethroid group could be a better pesticide if used at lower doses in agricultural fields and other places.

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