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"PESTICIDE-INDUCED ENZYMATIC DISRUPTION IN AMPHIBIANS: A CASE STUDY ON *EUPHLYCTIS CYANOPHLYCTIS* IN SINDH, PAKISTAN"

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Abstract

Amphibians are globally recognized as bioindicators due to their sensitivity to environmental stressors. This study investigates the effects of two commonly used pyrethroid pesticides, β-Cypermethrin and Fenpropathrin, on the enzyme alkaline phosphatase (ALP) in the liver and kidney of *Euphlyctis cyanophlyctis* (skittering frog), collected from the selected areas of Sindh Province, Pakistan. Frogs were exposed to varying concentrations of each pesticide under controlled laboratory conditions, and subsequent ALP activity was measured using spectrophotometry. Results indicated a significant dose-dependent decrease in ALP activity across both organs. The liver showed up to a 50.5% significant inhibition under 0.1% Fenpropathrin exposure, while kidney ALP activity was also inhibited by up to 50%. These findings highlight the susceptibility of amphibians to pyrethroid exposure and the broader ecological risks associated with pesticide overuse. The study underscores the urgent need for sustainable agricultural practices and policy-level intervention to prevent amphibian biodiversity loss in pesticide-intensive regions.

Keywords: Amphibia, pyrethroids, alkaline phosphatase, liver, kidney, *Euphlyctis cyanophlyctis*, Sindh.

Introduction

Amphibians, the ancient vertebrates with a lineage dating back over 300 million years, are currently facing a precipitous decline in populations worldwide. Their permeable skin, unshelled eggs, and dual life stages (aquatic and terrestrial) render them highly susceptible to environmental disturbances. Globally, nearly one-third of all amphibian species are considered threatened, with pollution, habitat destruction, disease, climate change, and pesticide exposure cited as primary causes.

Pakistan, as an agriculturally driven nation, has experienced a significant increase in pesticide use to boost crop yields. While pesticides support agricultural productivity, their environmental toll, particularly on non-target species such as amphibians, remains a critical concern. Among various pesticide groups, pyrethroids like **β**-Cypermethrin and Fenpropathrin are widely used and often labeled "safe," yet studies suggest otherwise.

This research evaluates the enzymatic response specifically the activity of alkaline phosphatase (ALP) in *Euphlyctis cyanophlyctis*, a common amphibian species in Sindh Province, following

controlled exposure to pyrethroids. ALP, a key enzyme in metabolism and membrane transport, serves as an indicator of physiological stress and organ dysfunction in exposed organisms.

Efforts to address the decline of amphibians involve a combination of strategies. Conservationists are working to protect and restore critical habitats, implement strict pollution control measures, and establish captive breeding programs for the most endangered species. Public awareness campaigns and education also play a vital role in rallying support for amphibian conservation.

Amphibians are important to the overall ecosystem balance both, aquatic and terrestrial habitats. The plight of amphibians is a stark reminder of the complex web of interactions that sustain life on Earth. The precipitous decline of these ancient creatures, despite their resilience over millions of years, underscores the urgency of addressing the myriad threats they face. The scientific assessments conducted by various researchers highlight the need for collaborative global efforts to halt and reverse the decline of amphibian species, ensuring their survival for generations to come (Whiles *et al.,* 2006).

The large biomass of these amphibians makes them significant prey for other animals. The global loss of amphibian populations was first recognized in 1989 as a phenomenon that deserved worldwide attention (Barinaga, 1990; Wake, 1991; Blaustein *et al*., 1994; Alford and Richards, 1999). The US National Research Council Workshop in 1990 followed with the first systematic examination of amphibian population declines (Barinaga, 1990; Wake, 1991). By 1993 more than 500 populations of frogs and salamanders on six continents were listed as declining or of conservation concern (Vial and Saylor, 1993; Alford and Richards, 1999). Reports of declining populations persisted at the Third World Congress of Herpetology in 1997, engendering a call for research focused on the question that the threat of amphibians extinction is increasing (Storfer, 2000; Houlahan et al., 2000; Alford *et al*., 2001; Wilson, 2002; Collins *et al.*, 2003). Over the last 5 years, workshops, conferences, symposia and new research findings have greatly improved our capacity to answer this question. They are more threatened and are declining more rapidly than either birds or mammals so there is now a consensus that alarming declines of amphibians have occurred (Corn, 1994; Kuzmin, 1994; Pechmann and Wake, 1997; Waldman and Tocher, 1998).

Concern about amphibians is in large part due to their value as indicators of environmental stress (Blaustein *et al*., 1994; Blaustein and Wake, 1995). They are in close contact with water as larvae and most have some contact with land as adults. Therefore, they experience both aquatic and terrestrial stressors. They have moist, permeable skin and unshelled eggs that are directly exposed to soil, water and sunlight, more sensitive to environmental toxins or to changes in patterns of temperature or rainfall than are other terrestrial vertebrate groups (Blaustein and Wake, 1990; Vitt, *et al.,* 1990). They are important components of several ecosystems where they may comprise the highest fraction of vertebrate biomass through their contribution to trophic dynamics in many communities, a world-wide decline in amphibians could have important impact on other organisms (Blaustein *et al*., 1994). Adult amphibians are important predators as well as prey and larval amphibians may be important herbivores (Blaustein *et al*.,

1994; Gramapurohit *et al.,* 2005). Therefore, loss of amphibians will not only lead to the loss of biodiversity but will also destroy the structure and function of the ecosystem (De Garady and Halbrook, 2006; Kerby *et al*., 2010).

According to IUCN Red List there are 20 countries with the highest number of threatened amphibians species. Significant differences exist among these groups in both species numbers as well as threatened status at the level of taxonomic order and family. They have been completely assessed, having a higher percentage of threatened species. The extinction risk of amphibians may be under estimated as 23% of them are listed as Data Deficient (Baillie *et al.,* 2004). Currently 34 species of amphibians are recorded as having become extinct, 20 of these being endemic to Sri Lanka, most of which has been disappeared over 100 years ago. Nine of the 34 extinctions have taken place since about 1980, plus two others from northern Australia, the Southern Gastric Brooding frog *Reheobatrachus silus* and the southern day frog *Taudactylus diurnus*. Eight of these nine currently extinction were sudden disappearance in suitable habitats, and are probably the effect of fungal disease, chytridiomycosis, probably operating in relation with climatic change (Laurance *et al.,* 1996; Berger *et al.,*1998; Ron *et al.,* 2003). The best-documented declines occurred in Europe and North America, are usually associated with habitat modification (Johnson, 1992; Green, 1997) and are often attributed to interactions among causal factors (Jennings and Hayes, 1994; Kuzmin, 1994; Pechmann and Wake, 1997).

Materials and Methods

Study Area and Species Selection

Adult specimens of *Euphlyctis cyanophlyctis* were collected from wetlands in Thatta (Haleji Lake) and urban water bodies in Karachi. These regions were chosen due to their ecological diversity and proximity to agricultural zones with known pesticide application.

Laboratory Acclimation and Treatment

Frogs were acclimatized in aquaria at the Wildlife Research Lab, Department of Zoology, University of Karachi. Two pyrethroids β-Cypermethrin and Fenpropathrin were prepared in aqueous solutions. Subcutaneous injections (using insulin syringes) were administered in doses of 5% and 10% for β-Cypermethrin, and 0.05% and 0.1% for Fenpropathrin.

Sample Collection and Enzyme Estimation

After 24 hours of the treatment, frogs were sacrificed, and liver and kidney tissues were harvested and homogenized. ALP activity was determined using a standard colorimetric method involving *p*-nitrophenyl phosphate as a substrate, measured at 405 nm in a spectrophotometer.

Estimation of Alkaline Phosphatase activity

The activity of enzyme 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 MgCl2 = 0.5 mmol/l) and Substrate (p-nitrophenylphosphate =10 mmol/l).

The reaction based upon the hydrolysis of p-nitrophenylphosphate by the action of alkaline phosphatase.

Principle:

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

The reagent was prepared to determine the alkaline phosphatase activity. For this purpose 10 ml of the buffer mixed with 10 ml of substrate, then 1.0 ml of this reagent and 0.02 ml of sample were mixed thoroughly, soon after poured into cuvette. The Spectrophotometer UV-160 was used. Temperature of the surrounding and spectrophotometer was maintained at 25 Celsius. The initial absorbance was recorded against air at 405 ηm, simultaneously the timer was started and reading was noted after 30, 60 and 90 seconds. The mean absorbance change per min (ΔA/min) was determined which used in calculation.

Results

Effect of Pyrethroids on Alkaline Phosphatase activity in *Euphlyctis cyanophlyctis*

Significant, dose-dependent reductions in ALP activity were observed in both liver and kidney tissues. The highest inhibition was noted at 0.1% Fenpropathrin exposure.

It was observed that under the effect of both pyrethroids the alkaline phosphatase activities were found to be significantly inhibited in liver and kidney of treated frogs.

Effect of β-Cypermethrin on Alkaline Phosphatase activity in Liver and Kidney

The effect of two concentrations of **β-**Cypermethrin i.e. 5 and 10% on alkaline phosphatase activity in liver of *Euphlyctis cyanophlyctis* was estimated. It was decreased up to 12.5 and 24.0 % respectively (Table1).

Table 1. ALP Activity in Liver of *E. cyanophlyctis* (U/L)

Treatment	Mean ± S.E.	% Inhibition
Control	7.5±1.84	00
5% ***β***-Cypermethrin	6.45±2.43	12.5
10% ***β***-Cypermethrin	5.75±1.59	24.0

The activity of alkaline Phosphatase in kidney of *E. cyanophlyctis* treated with 5 and 10% concentration of β-Cypermethrin was estimated. It was decreased up to 16.5 and 33.5 % respectively (Table 2).

Table 2. ALP Activity in Kidney of *E. cyanophlyctis* (U/L)

Treatment	Mean ± S.E.	% Inhibition
Control	5.5±1.59	00
5% ***β***-Cypermethrin	4.6±0.92	16.5
10% ***β***-Cypermethrin	3.6±0.92	33.5

Effect of Fenpropathrin on Alkaline Phosphatase activity in Liver and Kidney

The effect of two concentrations of Fenpropathrin i.e. 0.05% and 0.1% on alkaline phosphatase activity in liver of *Euphlyctis cyanophlyctis* was estimated. It was decreased up to 38.5 and 50.5 % respectively (Table 3).

Table 3. ALP Activity in Liver of *E. cyanophlyctis* (U/L)

Treatment	Mean ± S.E.	% Inhibition
Control	7.4± 1.84	00
0.05% **Fenpropathrin**	4.5±1.84	38.5
0.1% **Fenpropathrin**	3.5±0.92	50.5

The activity of alkaline phosphatase in kidney of *E. cyanophlyctis* treated with Fenpropathrin was decreased up to 16.5 and 50.0 % respectively. (Table 4)

Table 4. ALP Activity in Kidney of *E. cyanophlyctis* (U/L)

Treatment	Mean ± S.E.	% Inhibition
Control	5.5± 1.6	00
0.05% **Fenpropathrin**	4.6±0.9	16.5
0.1% **Fenpropathrin**	2.8±0.0	50.0

Similar inhibitory trends were observed with **β**-Cypermethrin, although Fenpropathrin proved more potent in suppressing enzymatic activity.

Discussion

The alkaline phosphatases are a small family of isozymes expressed in diverse species, including bacteria, insects, nematodes, amphibians, reptiles and mammals. The alkaline phosphatases have been postulated to be involved in a range of other processes, including cell adhesion and cell signaling (Manara *et al*., 2000).

The observed decline in ALP activity suggests cellular stress and potential organ dysfunction in exposed amphibians. As ALP is critical for metabolic regulation and membrane transport, its inhibition implies compromised physiological integrity.

These findings align with previous studies highlighting pesticide-induced enzymatic disruptions in amphibians. The results also corroborate earlier conclusions that *E. cyanophlyctis* is a sensitive bioindicator of environmental toxicity. Notably, the liver exhibited a higher reduction in ALP activity than the kidney, possibly due to its primary role in detoxification.

Given the ecological importance of amphibians in pest regulation, nutrient cycling, and food webs, their decline could trigger cascading effects across ecosystems. Moreover, the widespread application of pyrethroids in agricultural zones poses a persistent threat to amphibian health and biodiversity.

Pritchard and Ruzicka, (1950) observed the thermo-labile nature of alkaline phosphatase enzyme in frog. It was found that alkaline phosphatase was destroyed during incubation at 60 degrees Celsius in the process.

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 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 acids.

Latker *et al.,* (1987) investigated the effect of Levamisole (an ALP inhibitor) and localization of alkaline phosphatase (ALP) in the peripheral and central nervous systems of the frog *Rana pipiens* and rat. The biochemical analysis indicated a sevenfold higher ALP activity in the frog perineurium over the endoneurium, whereas in the rat, threefold more activity was measured in the endoneurium over the perineurium. Levamisole, decreased the enzyme activity by 95% in rat tissues, and by 70% in frog tissues and in plasma from both animals. Similar decrements were observed cytochemically. This study suggested that the distribution of ALP varied between species, but that it was always present in at least one component of the blood-brain and blood-nerve barriers, because barrier tissues of the nervous system had enzymatic activity, they might biochemically modifiy the adjacent environment. The vesicular profiles and caveolae in the blood vessels and perineurium might functioned as microenvironments for enzymatic activity. In rat and frog, different isozymes of AP might be present. In present study, it was also observed that alkaline phosphatase activity was inhibited under the effect of selected pesticides Cypermethrin and Permethrin.

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. These results suggested that a single phenotype of ALP exist in all tissues of bullfrog in contrast to two or three isoenzymes in mammals.

Sawarkar and Navagiri, (1993) studied alkaline phosphatase activity in various representative classes of vertebrates. It was found that the enzyme activity in mucosa was feeble in frog as the muscle coat also lacks the enzyme activity.

Fenoglio *et al.,* (1996) observed the mesonephros of *Rana esculenta* during activity and the hibernation period and reported that the activity of some membrane-transport enzymes (5' nucleotidase and K+-p-nitrophenyl phosphatase) and of energetic metabolism (succinic dehydrogenase) was reduced. However, the alkaline phosphatase activity was not changed significantly, and this suggests that some metabolic activities were preserved in the hibernating samples. These results indicated morpho-functional adaptations of the kidney cells that preserved their role in osmoregulation and some metabolic processes, even during unfavourable seasons.

Josephjohn *et al.,* (2004) conducted a biochemical study on effect of thiodan on *Euphlyctis hexadactylus*. The frogs were exposed to sub-lethal concentration of 0.1 ppm and 0.2 ppm of thiodan for 5, 10, 15 and 20 days respectively and the fluctuation of total proteins, glycogen in

liver, muscles, brain and kidney were estimated. Along with this, the variations in acid and alkaline phosphatases levels in blood serum and RBC and WBC counts were also recorded. The observations showed progressive decrease of total proteins and glycogen as well as in RBC and WBC count. The alkaline phosphatase activity increased with increasing dose and duration of thiodan treatment. But, acid phosphatase decreased initially but later on showed increasing trend. It was concluded that the chronic stress by sub-lethal doses of thiodan become more and more severe with increasing dose and duration of exposure. While in the present finding it was observed that alkaline phosphatase activity was found to be decreased under the effect of pyrethroid pesticides. The main difference is the dose and duration of the treatment also the group of pesticide as thiodan belongs to organochlorine group.

Fenoglio *et al.,* (2005) examined the liver of *Rana esculenta* adult frogs collected at two sample rice fields, one was heavily polluted and the other was relatively unpolluted. Water pollution was determined by chemical analysis on tadpoles. The specific activities of some enzymes (glucose-6-phosphate dehydrogenase (G6PDH), acid and alkaline phosphatases (AcPase and AlkPase), succinic dehydrogenase (SDH), and catalase were studied in the liver of adult frogs to identify the possible changes induced by contamination in the metabolic processes which depend on the function of the liver. The production of reactive oxygen species (ROS) were also evaluated through histochemical techniques. In the polluted samples, hepatocytes showed variations in the activity of G6PDH, AlkPase, and SDH and a moderate to intense ROS expression. Prominent changes were observed in Kupffer cells (KCs) and melanomacrophages, both showed intense reactivity for AcPase, catalase and variations in melanin content and distribution. Results thus indicated a general adaptive response of liver parenchyma to environmental pollution.

Fenoglio *et al.,* (2006) also observed the activities of some enzymes of adult frog *Rana esculenta*, collected at two different rice fields, relatively unpolluted and heavily polluted. The acid and alkaline phosphatase, nitric oxide synthase-related nicotinamide adenine dinucleotide phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, catalase, nonspecific esterases, and succinic dehydrogenase that involved mainly in membrane transport, xenobiotics, and oxidative metabolism. Results suggested the changes in most enzyme activities in keratinocytes and mitochondria-rich cells particularly glucose-6-phosphate dehydrogenase and esterases, both important to counteract oxidative and toxic stress.

In general the present results are in agreement with earlier reports. On the basis of present findings it is concluded that selected pesticides β-Cypermethrin and Fenpropathrin (pyrethroids) decreased the alkaline phosphatase (ALP) activity in the liver and kidney of *E. cyanophlyctis.*

Conclusion

This study demonstrates that pyrethroid pesticides significantly inhibit alkaline phosphatase activity in *Euphlyctis cyanophlyctis*, suggesting toxicological stress and potential organ impairment. These findings reinforce concerns about the ecological safety of "low-risk"

pesticides and emphasize the need for stringent regulations on pesticide application, particularly in amphibian-rich habitats. Conclusively, the present study contributes to the body of scientific knowledge by reaffirming the detrimental effects of β-Cypermethrin and Fenpropathrin, two pyrethroid pesticides, on the ALP activity in the liver and kidney of *Euphlyctis cyanophlyctis*. This consistency with prior research underscores the significance of these findings and emphasizes the importance of evaluating the ecological impact of pesticide usage on amphibian populations. Such insights are critical for fostering sustainable practices that safeguard amphibian biodiversity and the overall health of ecosystems.

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