



Biochemical Modulations in *Duttaphrynus melanostictus* Tadpoles, Following Exposure to Commercial Formulations of Cypermethrin: An Overlooked Impact of Extensive Cypermethrin use

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ABSTRACT

Extensive application of pesticides in agricultural and domestic zones has contributed to environmental contamination globally. With aquatic bodies being ultimate recipients of pesticide residues, the inhabiting fauna are known to be largely affected due to their proximity and inevitable exposure. The present investigation was aimed to examine the effect of sublethal (1.11 µg/L) concentration of cypermethrin on biochemical cluster of tadpoles of *Duttaphrynus melanostictus*. Significant changes were observed in total, soluble and structural protein fractions following cypermethrin exposure to subacute (1, 2, 4 and 6 days) durations in whole animal biochemical composition of *Duttaphrynus melanostictus*. Marked elevation in free amino acid level was observed at all the exposure tenures. Generation of reactive oxygen species with endpoint induction of oxidative stress were evidenced by decrease in activity of catalase, glutathione-S-transferase and increased levels of hydrogen peroxide, reduced glutathione and malodialdehyde levels. The outcome clearly suggests the increased susceptibility of *Duttaphrynus melanostictus* tadpoles to sublethal concentrations of cypermethrin, thus implicating the toxicant to possess detrimental health effects on *Duttaphrynus melanostictus* species. The study may contribute in environmental monitoring and assessment of water bodies with possible cypermethrin contamination.

1. INTRODUCTION

Worldwide decline in amphibian population has become a major issue of global concern [1]. Annual global use of agricultural sprays is estimated to be 11.2 billion kg [2]. With this range of indiscriminate pesticide use, has resulted in inevitable consequence of compromised health status of aquatic fauna with partial contribution to drastic decline in amphibian population as well [3, 4]. According to the first global assessment of the status of amphibian species, more than 40% of the world's 5743 amphibian species have experienced recent declines, a situation far worse than that reported for mammals or birds [5]. With aquatic systems

often being contaminated with pesticides [6], the amphibian susceptibility have been known to reach alarming levels [7, 8]. While pesticides have the potential to affect many non-target aquatic taxa [9], the inexorable conditions of amphibians begs the intervention to counter the excessive pesticide use. The increased permeability of skin and eggs in anurans is known to be one of the crucial cause for facilitating the immediate absorption of wide range of environmental xenobiotics [10, 11]. In addition, amphibians are known to complete a phase of their life cycle in shallow ponds and other freshwater aquatic bodies that may be the recipient of pesticidal residues adjacent to agriculture fields, where the possibilities of pesticidal exposure from agriculture outputs could be certain [12]. Cypermethrin (CY) is a synthetic pyrethroid, and is more suitable for agricultural use because of its improved potency and stability as well as low mammalian toxicity [13]. CY is known to act by disrupting sodium ion channels of the nerve membrane [14] and hence considered a reliable compound for controlling insect pests that could damage crop at large-scale [15].

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The use of CY has been recommended by World Health Organisation (WHO) since, it is found to be very effective against several insect pests of both domestic and agriculture interest [16]. Over the period it has been effectively used in the control of mosquitoes, midgits and several other insect pests. On the contrary, increased use cypermethrin has resulted in ultimate damage to non-targeted aquatic organisms including fishes and amphibians [15]. Hence, it is requisite to investigate the susceptibility of the *Duttaphrynus melanostictus* to commercial formulations of cypermethrin from the ecological point of view and the possible contributions to anuran conservation.

2. MATERIALS AND METHODS

2.1 Collection and maintenance of test species

Amplex pair of *Duttaphrynus melanostictus* was procured during monsoon season and were allowed to breed in well-constructed breeding ponds in the Department of Zoology, Karnatak University, Dharwad, Karnatak, India. Determination of developmental stages of the tadpoles was carried out according to Gosner [17]. Eggs were cultured in 50 litre glass aquaria till embryos reached Gosner stage 20. Tadpoles were transferred to a glass aquaria containing 10 litres of dechlorinated water and physicochemical properties of the water was estimated (Table 1). All experiments were performed at $25 \pm 2^\circ \text{C}$ with a light dark cycle of 14:10 h which was maintained throughout the experimental duration. Tadpoles were fed with commercial food and boiled spinach *ad libitum*. Water in the aquaria was renewed every 24 h and excess food and faeces were removed.

Table 1: Showing values for quality assessment of water used in the present investigation.

Parameter	Values obtained
Temperature,	$25 \pm 2^\circ \text{C}$
pH,	7.3 ± 0.2
Dissolved oxygen,	$7.7 \pm 0.8 \text{ mg/L}$
Total hardness	$30.4 \pm 3.1 \text{ mg as CaCO}_3/\text{L}$
Salinity,;	Nil
Specific gravity,	1.003
Conductivity	$< 14 \mu\text{S/cm}$
Calcium	$17.31 \pm 0.92 \text{ mg/L}$
Phosphate,	$0.34 \pm 0.004 \mu\text{g/L}$
Magnesium	$0.78 \pm 0.3 \text{ mg/L}$

2.2 Toxicant selected and test solution

Commercial grade cypermethrin 25% E.C was procured from local market Dharwad, Karnataka, India. The stock solution was prepared by dissolving it in double distilled water. The required quantity of cypermethrin was drawn from stock solution for further experimentation. Previously determined value of LC_{50} was considered for present study. This was followed in order to avoid excessive animal kill. $1/3^{\text{rd}}$ ($1.11 \mu\text{g/L}$) of the previously determined 96 h LC_{50} ($3.34 \mu\text{g/L}$) [12] was selected as sublethal concentration for the present investigation. Durations of 1, 2, 3 and 4 days of exposure were carried out under standard semistatic

renewal assay tests. Experiment was carried out in triplicates for each exposure period with (n=10) tadpoles including control set.

2.3 Estimation of soluble, structural and total proteins.

The soluble (SP) structural (STP) and the total proteins (TP) were estimated using the Folin-phenol reagent method as described by Lowry *et al.* [18]. One percentage homogenate (W/V) was prepared in ice cold 0.25M sucrose solution. For soluble and structural proteins 1.0 mL of the homogenate was taken and centrifuged at 3000 rpm for 10 min. The supernatant was separated and to both the supernatant and residue 3mL of 10% trichloroacetic acid (TCA) was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residues were taken for experimentations. For total proteins, 1mL of homogenate was taken; to it 3mL of 10% TCA was added and centrifuged at 3000rpm. Supernatant was discarded and residue was taken for experimentation. All the three residues were dissolved in 5mL of 0.1N sodium hydroxide and to 1mL of each of these solutions, 4mL of reagent - D (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. The samples were allowed to stay for 10 min, at the end of which 0.4mL of folin-phenol reagent (diluted with double distilled water in 1:1 ratio before use) was added. Finally the optical density of the colour developed was measured using spectrophotometer at a wavelength of 600nm. A mixture of 4mL of reagent-D and 0.4mL of folin-phenol reagent was used as blank. Bovine serum albumin was used for the preparation of protein standards. The protein content was expressed as mg/g wet weight.

2.4 Estimation of free amino acids

The free amino acid (FAA) level was estimated using ninhydrin method as described by Moore and Stein [19]. The homogenates (5% w/v) were prepared in 10% TCA and centrifuged at 2000 rpm for 15min. To 0.2 mL supernatant, 2.0mL of ninhydrin reagent was added and the contents were boiled for exactly 5 min. they were cooled under tap water and the volume was made to 10mL with distilled water. The optical density of the colour developed was measured using spectrophotometer at a wavelength of 570nm. A blank using distilled water and amino acid standards were also run similarly. The free amino acid levels were expressed as mg amino acid nitrogen released/g wet weight.

2.5 Estimation of catalase activity (CAT)

Catalase activity was estimated by the method described by Luck [20]. Samples were homogenised in phosphate extraction buffer (50mM, pH: 7.0). The contents were centrifuged and 2.0mL of the supernatant was used along with 1.0mL of phosphate buffer containing with 10mM of hydrogen peroxide as substrate for catalase. The optical density of the sample was measured at 240nm in UV-Visible spectrophotometer (Hitachi, Model No. U-3310), against a blank. The blank consists of 3.0 mL of phosphate buffer solution without hydrogen peroxide. The values were expressed as mM of hydrogen peroxide decomposed/min/mg protein.

2.6 Estimation of hydrogen peroxide content

The hydrogen peroxide content was estimated by the method of Luck [20]. Tadpoles were homogenised in phosphate extraction buffer (50mM, pH: 7.0). The contents were centrifuged and 2.0 mL of the supernatant was used along with 1.0mL of phosphate buffer.

The optical density of the sample was measured at 240nm in UV-Visible spectrophotometer (Hitachi, Model No. U-3310), against a blank. The blank consists of 3.0 mL of phosphate buffer solution. The values were expressed as μM of hydrogen peroxide/g wet weight of tissue.

2.7 Lipid peroxidation level (LPO)

Malondialdehyde (MDA), the secondary product of lipid peroxidation (LPO), was estimated by the colorimetric reaction of thiobarbituric acid (TBA) as per the method of Buege & Aust [21]. It gives an index of the extent of progress of lipid peroxidation. Since the assay estimates the amount of TBA reactive substances e.g., MDA, it is also known as TBARS. Trichloroacetic acid (TCA) was added to the homogenate (4%), to precipitate proteins, followed by centrifugation at 3000g for 15min. TBA (1%) was added to the supernatant and kept in a water bath for 60min at 80 °C in test tubes covered with aluminium foil. After incubation the tubes were kept in ice-cold water for 10min. Absorbance of the pink colour thus developed was read against a reagent blank at 532nm.

The concentration of MDA was read from standard calibration curve plotted using 1,1,3,3'-tetra-ethoxy propane. The amount of TBARS was expressed as nmol MDA formed/mg protein.

2.8 Glutathione-S-transferase (GST) activity

Samples were homogenised in ice cold 25 mL sodium phosphate buffer (0.1 mol/L, pH 6.5) containing 20% glycerol, 1.4 mmol/L dithioerythritol and 1mmol/L EDTA. Enzyme extracts were prepared in volume according to Pflugmacher and Steinberg [22] with modifications described by Greulich *et al.*, [23]. Enzyme activity of GST was quantified spectrophotometrically using the model substrate 1-chloro-2,4-dinitrobenzene (Habig *et al.*, [24] and calculated in terms of the protein content of the sample.

2.9 Reduced glutathione (GSH)

The assay for GSH was done according to the method of Ellman [25]. A 10% homogenate (w/v) was prepared with 5% metaphosphoric acid. The homogenate was kept at room temperature for 15 min, centrifuged at 111g for 30 min at room temperature. The supernatant was used for the assay.

The assay mixture contained 0.5mL supernatant, 2.5 mL 0.3mM DTNB. The mixture was incubated for 25min at room temperature and centrifuged for 5min at 4500g. The absorbance was read at 412nm. The level of GSH was calculated from the standard curve and expressed as $\mu\text{g/g}$ tissue.

2.10 Statistical analysis

Data correspond to the average of six replicates. The data obtained were analysed statistically by following Duncan's multiple-range test [26].

2.11 Ethical committee

All procedures implemented in the present study were in accordance to the guidelines of the Institutional Animal Ethics Committee (IAEC). The animals subjected to experimentation were handled as per directions of Committee for the Purpose of Control and Supervision of Experiments for Animals (CPCSEA), New Delhi, India.

3. RESULTS

3.1 Levels of TP, SP and STP

Present study revealed significant decrease ($P \leq 0.05$) in protein fractions (TP, STP and SP) at all periods under sublethal exposure (Figure 1). Maximum decrease was observed on 6th day of exposure. Comparison among different protein fractions revealed SP was affected to a greater extent and TP was affected least (Figure 2).

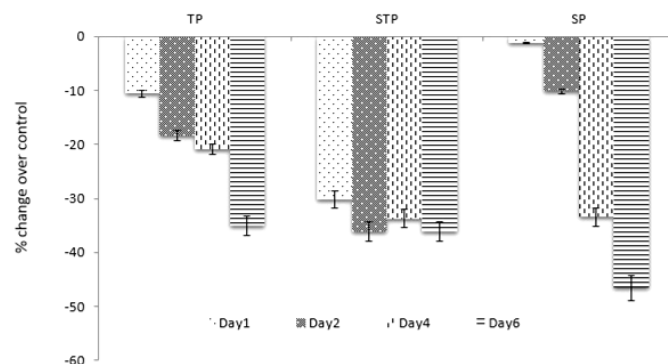


Fig. 1: Percent change in the levels of total protein (TP), structural protein (STP) and soluble protein (SP) fractions of *D. melanostictus* on exposure to sublethal concentration of cypermethrin.

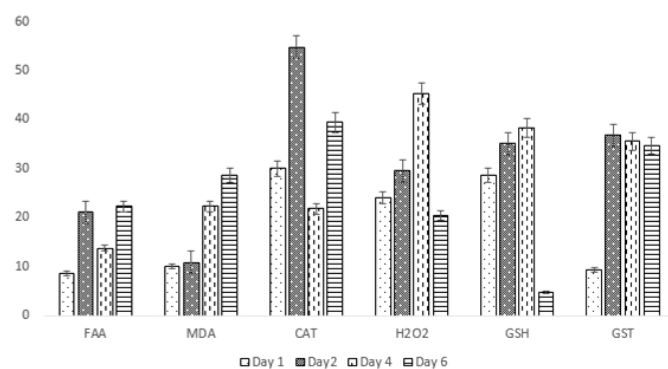


Fig. 2: Responses in the levels of FAA, MDA, CAT, H₂O₂, GSH and GST activities on exposure to sublethal concentration of cypermethrin.

3.2 Levels of free amino acids (FAA)

Elevation in free amino acid levels was observed in all CY exposure periods. Steep increase in the levels of free amino acids was noted (22.291%) at 6th day of exposure period when

compared with control and other exposure periods. Consistent increase in the levels of FAA was significant ($P \leq 0.05$) with increase in exposure periods.

3.3 Effect on the CAT activity

Notable increment was observed in CAT activity among treatment groups. CAT activity was time dependent and tadpoles showed an obvious decline in the activity of CAT at 6th day compared to their respective activities on 2nd and 4th day treatments (Figure 2).

3.4 Effect on hydrogen peroxide

Notable increments were observed in the levels of H₂O₂ among cypermethrin treated tadpole groups. The results were significant when compared to their respective controls at 1, 2, 4th and 6th day. Maximum increment (45.31%) was observed at 4th day exposure period over control ($P \leq 0.05$). Whereas, this increment was almost suddenly vanished to preceding levels at 6th day of exposure (Figure 2).

3.5 MDA level

The levels of MDA were contrastingly intensified in treated groups in comparison to control at all exposure periods and were time dependent. Increments in the levels of MDA were observed on day 4 and day 6 with approximately 2.2 to 2.9 fold increase.

3.6 Effect on GST and GSH level

Time dependent enzymatic changes in response to CY toxicity to *D. melanostictus* revealed that levels of GST increase considerably (up to 36.81% on day 2) at all time periods as compared to controls. The GST activity was decreased from day 2 through day 6. However the activity differed significantly compared to controls at all period of time.

4. DISCUSSION

Present study was undertaken to address the impact of sublethal concentration on oxidative enzymes as a biomarker for CY toxicity in tadpoles of *Duttaphrynus melanostictus*. According to metabolic cost hypothesis, major energy reserves in the organism are affected by exposure to toxic substance [27]. Proteins are the major energy reserves in aquatic organisms and the possibility of toxicants to affect protein reserve as primary target is wide [28]. Alteration in protein content could also indicate a physiological adaptability to overcome toxic stress [29]. Present investigation revealed significant decrease in all protein fractions and this could only mean catabolism of proteins for the total energy production by successive protein oxidation during the course of experimental time period [30]. Alterations in protein fractions might even suggest false protein synthetic mechanism. The decreased protein content might also be attributed to the impairment in protein synthetic machinery [31]. Progressive increase in FAA was observed with decrease in protein fractions at

all-time intervals during the study (Figure 2). This considerable increment in FAA levels may also be affirmative to the fact that it's active involvement in the maintenance of osmotic acid base balance. Further, high level of FAA can also be considered for decreased utilisation of these in anabolic processes. It has also been observed that, progressive decline in all protein fraction was to compensate for the problems of osmoregularity occurring due to loss of ions and ATPase during pyrethroid stress [32]. Further it has also been observed that lipoprotein formation was increased with decrease in protein fractions for the greater good of damaged cells and cellular organelles [33]. Possibly, this helps the animal to adapt to the imposed toxic stress. Thus, the introduction of toxicant either damages proteins directly, and/or upregulates the production of specific proteins.

Pyrethroids are lipophilic in nature, hence alter phospholipid orientation and fluidity of the cell membrane by penetrating into the lipid bilayer of the tissues [34]. It has also been reported that one of the cascade of events in toxicity is due to alteration in antioxidant activities of cells exposed to cypermethrin. Cyanides and aldehydes are the major biproducts of cyanohydrins in physiological solution. Cyanohydrins in turn produced by cleavage of cypermethrin and other pyrethroids. Former produces free radicals leading to oxidative stress and other harmful conditions [35].

The elevation in CAT activity is due to oxidative stress caused by cypermethrin exposure. This elevation is to provide protection from CY induced free radical stress because it is obvious that during the process of dismutation of superoxide anion radicals, antioxidants get generated and in turn these reduce peroxidation [36]. However, it is also been observed that CAT activity may get inhibited by superoxide radicals and this leads to increased H₂O₂ levels causing inhibition of SOD activity [37]. Significant reduction in the CAT activity may be indicative of utilization of enzyme in neutralizing the free radicals generated by CY. Initial increase in the CAT activity is the efforts of the endogenous antioxidant enzymes to remove the continuously generated free radicals due to an induction but later enzyme depletion resulting in oxidative cell damage [38].

The production of ROS such as H₂O₂ and superoxide radical ion is evident in causing oxidative stress and subsequent cell death [39]. During present study, initial increase (24%) in H₂O₂ content and further increase (29.52%) at 1st and 2nd day of exposure clearly suggest CY to be responsible for condition of oxidative stress. Further, steep increment (45.31%) in H₂O₂ was followed at 4th day which is well above the 2nd day of exposure. In this regard, many studies have reported the excessive production in ROS can render a cell in serious risk of loss of ions, membrane peroxidation and also DNA aberrations [40, 41]. Subsequent decrease in H₂O₂ levels at 6th day well below 1st day of treatment is evident that there is an induction of CAT and antioxidant defence mechanism can still eliminate the generation of H₂O₂ as a response to CY stress. Our data suggests that induction of reactive oxygen species could be associated with the metabolism of CY causing peroxidation of lipid membranes leading to cell apoptosis. Studies

have shown pesticides like endosulphan and cypermethrin could induce lipid peroxidation in fishes [42, 43].

GST is considered to be xenobiotics scavenging enzyme which acts by reducing glutathione through catalysing the conjugation of variety of electrophiles [44]. Regular increments in the levels of GST and GSH were observed initially and later with extended time periods the values reduced drastically. This indicates the initial resistance towards pesticide stress and protection against free radicals of CY toxicity. Reports shows GST and GSH induction in metabolic detoxification of butachlor in *Rhamdia quelen*. These results suggest that protective and scavenging mechanisms are indeed necessary in warding off of pesticide induced reactive oxygen species. However, depletion in GST and GSH (6th day) activity refers to excessive production of reactive oxygen species.

The overall study suggests the devastating tendency of cypermethrin against *D.melanostictus* tadpoles by altering its biochemical make up that consisted of antioxidant enzyme status along with protein and free amino acid levels. Even though pesticides and similar toxic chemicals have been reported for causing adverse effects on fishes [45, 46, 47, 48, 49], their role in declining trend of amphibian population is of highest concern [50], the need for stricter laws in controlling indiscriminate use of highly toxic pesticides still awaits its initiation.

5. CONCLUSION

Our study finds CY to be highly toxic to tadpoles of *D.melanostictus* at environmentally relevant concentration. The effect was evident to be both time and dose dependent and induces oxidative damage of biomolecules in presence of reactive oxygen species through membrane peroxidation and even alters protein metabolism in tadpoles. However, the role of antioxidant enzymes as a defence mechanism against ROS induced oxidative stress has also been demonstrated in the present study.

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