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Alterations in the Serum Electrolytes of the Indian Skipper Frog *Euphlyctis cyanophlyctis* caused by an Organophosphate Pesticide: Chlorpyrifos

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Abstract

The aim of the present study is to determine the changes in blood electrolytes (calcium and phosphate) in the Indian skipper frog *Euphlyctis cyanophlyctis* following short-term and long-term treatments with chlorpyrifos. To determine the effects of short-term exposure, the frogs were exposed to 3.99 mg/L of chlorpyrifos (i.e. 0.8 of 96 h LC_{50} value) for ninety-six hours. To investigate the effects of long-term exposure, the frogs were exposed to 0.99 mg/L (0.2 of 96 h LC_{50} value) of chlorpyrifos for 30 days. The frogs were sacrificed after 24, 48, 72 and 96 hours (for short-term experiment) or after 5, 10, 15 and 30 days (long-term experiment). Blood samples were collected and serum calcium and phosphate levels were analyzed. Student's t test was used to determine the statistical significance difference between the experimental group and its specific-time control group. Exposure of the frog *Euphlyctis cyanophlyctis* to chlorpyrifos causes a decrease in the serum calcium levels after 48 hours. This decrease continued up to the end of the experiment (96 hours). The serum inorganic phosphate levels decrease progressively 72 hours onwards following the chlorpyrifos exposure. In the long-term experiment, the first perceivable change has been noticed on day ten in the serum calcium as the levels decreased at this interval. The levels continued to fall progressively till the end of the experiment (thirty days). The serum phosphate levels of the chlorpyrifos-treated *Euphlyctis cyanophlyctis* show a decrease on day ten and fifteen. However, on day thirty, the levels were almost normal. The changes noticed in the blood electrolytes may cause disturbances in the vital physiological functions of the frog, growth and even its ability to survive in nature.

Keywords: Amphibia, chlorpyrifos, organophosphate, serum calcium, serum phosphate, *Euphlyctis cyanophlyctis*

1. Introduction

Organophosphorus pesticides are widely used around the world although they lack target specificity, and have severe effects on aquatic non-target animals (Fulton and Key, 2001; Yan *et al.,* 2008). Chlorpyrifos, a non-systemic organophosphate pesticide, is one of the most widely used insecticides on a variety of crops and in numerous non-agricultural situations (WHO, 2009). Amphibians are sensitive to most pesticides when exposed through direct overspray, pesticide drift, rainfall and run-off into water bodies. Many amphibians breed within or near agricultural areas that are usually exposed to pesticides (Palenske *et al.,* 2010), thus both the larvae and adults are exposed to pesticides at all life stages, either in the waters (larvae) or on land (adults). This can lead to a decline in their global population which is a major concern now-a-days (Sparling, 2003; Relyea, 2005; Hayes *et al.,* 2006; Mccallum, 2007; Todd *et al.,* 2011; Whittaker *et al.*, 2013; Arntzen *et al.,* 2017; Srivastav *et al.,* 2016, 2017).

Chlorpyrifos is highly toxic to amphibians (Davidson *et al.,* 2012). Residues of chlorpyrifos have been found in the Pacific tree frog tadpoles (Datta *et al*., 1998). Jayawardena *et al.* (2011) have noticed profound effects in amphibians after a chronic exposure to chlorpyrifos. Bernabo *et al.* (2011) exposed frog tadpoles to chlorpyrifos and noticed that 20-25 % of the exposed tadpoles became intersex. The exposure to Chlorpyrifos in amphibians resulted in (i) damage to muscles (Colombo *et al*., 2005), (ii) reduced swim speed and activity in tadpoles (Wijesinghe *et al.,* 2011), (iii) reduced body length and mass (Richards and Kendall, 2003), and (iv) increased induction of micronuclei and chromosomal lesions in the erythrocytes (Yin *et al*., 2009).

Agrochemical contaminants, organophosphates and organochlorine pesticides have been reported to cause inhibition of AChE and malformations in frogs (Fort and

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Paul, 2002; Fort *et al*., 2004 a,b; Krishnamurthy and Smith, 2010; Hegde and Krishnamurthy, 2014). Palenske *et al.* (2010) have suggested that physiological studies provide a better understanding regarding the toxic effects of contaminants to aquatic organisms. Although several studies have been performed dealing with the effects of toxicants on amphibians, there exists no information regarding the effects of toxicants on amphibian calcium regulation. Calcium is vital for living organisms, and has been implicated in controlling a wide variety of physiological and biological functions. It seems very difficult to mention a physiological process that does not, in one way or another, depend on calcium. Hence, the present study aim to investigate the effects of chlorpyrifos on blood calcium and phosphate levels of the anuran, Indian skipper frog *Euphlyctis cyanophlyctis.*

2. Materials and Methods

Laboratory reared Indian skipper frogs, *Euphlyctis cyanophlyctis* (both sexes; body wt. 12-17 g) were selected and acclimatized for fifteen days in 30 L all glass aquaria. The frogs were not fed for twenty-four hours before and during the experiment. Short-term and long-term experiments have been performed. This study evaluates the possible effects of chlorpyrifos after acute exposure, i.e. short-term exposure to high doses of chlorpyrifos. The real exposure effects come after a long-term exposure using low-doses of chlorpyrifos which may result in varied effects compared to the acute exposure to high-doses of chlorpyrifos. This could be very useful in understanding the long-term effects of chlorpyrifos with low concentrations and comparing them with the short-term effects of chlorpyrifos at high concentrations.

(i) *Short-term Exposure***:** In this experiment, the frogs (n =24) were subjected to 0.8 of 96 h LC_{50} (LC_{50} value of chlorpyrifos described earlier by Srivastav *et al*., 2017) value of chlorpyrifos (3.99 mg/L) for ninety-six hours. Simultaneously, a control group (n =24) was also used for comparison. The frogs were kept in groups of ten each in 30 L media. Six frogs of the control and experimental groups were killed on each time intervals after a period of 24, 48, 72 and 96 hours of exposure.

(ii) *Long-term Exposure***:** The frogs (n =24) were exposed to 0.99 mg/L (0.2 of 96 h LC_{50} value) of chlorpyrifos for thirty days. Simultaneously, a control group (n =24) was also used for comparison. Six frogs from the control and experimental groups were sacrificed after 5, 10, 15 and 30 days of the toxicant treatment.

In each experiment, the frogs were slightly anesthetized with ether, and their blood samples were collected by cardiac puncture. The collected blood samples were allowed to clot at room temperature. Sera were separated by centrifugation (at 3000 rpm) and were kept at -20C until analysis for serum electrolytes using commercial diagnostic kits - calcium (calcium kit, Sigma-Aldrich) and inorganic phosphate (Pointe Scientific, USA). All determinations were carried out in duplicates for each sample. Animal handling and sacrifice were carried out in accordance with the guidelines provided by the Ethics Committee of the University (F.Sc.2551/Zoology/4-12-06).

All data were presented as the mean ± S.E. of six specimens, and the Student's t test was used to determine statistical significance. In all studies, the experimental group was compared to its specific-time control group.

3. Results

Short-term exposure of the frog *Euphlyctis cyanophlyctis* to chlorpyrifos results in a decrease in the serum calcium levels after forty-eight hours This decrease continued till the end of the experiment (96 h) (Figure 1). The serum inorganic phosphate levels remain unaffected till forty-eight hours following the chlorpyrifos exposure. The levels decreased progressively seventy-two hours onwards (Figure 2).

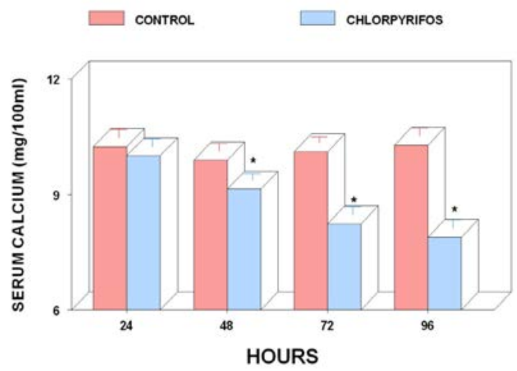

Figure 1. Serum calcium levels of short-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences (*P*< 0.05) from control.

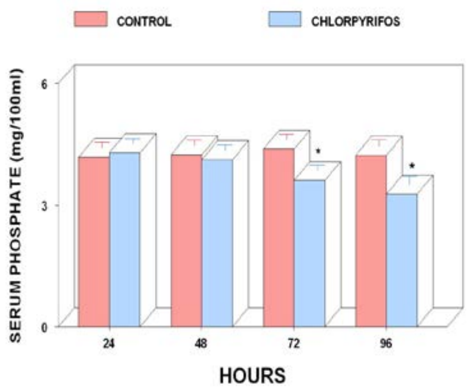

Figure 2. Serum phosphate levels of short-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences (*P*< 0.05) from control.

In the long-term exposure of the *Euphlyctis cyanophlyctis* to chlorpyrifos, the first perceivable change has been noticed in the serum calcium by day ten; the levels decreased at this interval. The levels continued to fall progressively up to the end of the experiment (30 days; Figure 3). The serum phosphate levels of the chlorpyrifos-treated *Euphlyctis cyanophlyctis* showed a decrease on days ten and fifteen. However, on day thirty, the levels were almost normal (Figure 4).

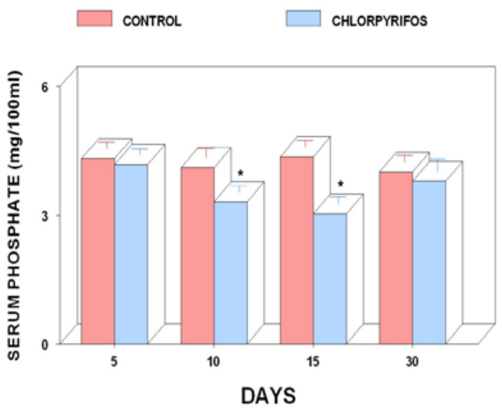

Figure 3. Serum calcium levels of long-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences ($P< 0.05$) from control.

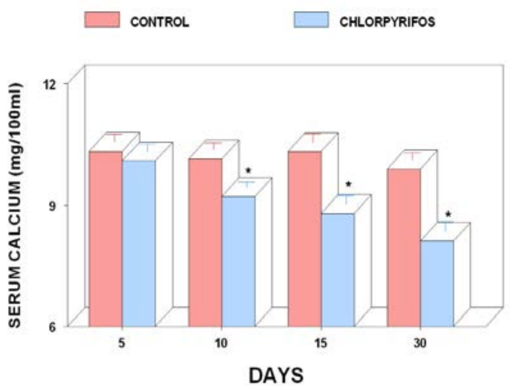

Figure 4. Serum phosphate levels of long-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences ($P< 0.05$) from control.

4. Discussion

Chlorpyrifos exposure provoked hypocalcemia and hypophosphatemia in *Euphlyctis cyanophlyctis.* This study presents the first report regarding the effects of toxicants on the blood electrolytes of amphibians; No report preceding this study which tackles this issue has been found. The present study derives support from the reports of earlier workers who have also noticed hypocalcemia in other vertebrates after the exposure to chlorpyrifos (fish – Srivastav *et al.,* 1997 a; rats – Tripathi *et al.,* 2013), deltamethrin (fish – Srivastav *et al.,* 1997 b, 2010), cypermethrin (fish – Mishra *et al.,* 2011), lead (fish – Rai *et al.,* 2010, 2013), botanical pesticides (fish – Kumar *et al.,* 2011 a, b; Prasad *et al.,* 2011,2013) and cadmium (fish – Larsson *et al.,* 1981; Pratap *et al.,* 1989; Rai and Srivastav, 2003; Rai *et al.*, 2009; rabbits – Kenny, 1966; rats – Tripathi and Srivastav, 2011). Contrary to these reports, few studies have noticed either no effect (Oner *et al.,* 2008; Velisek *et al.,* 2009) or hypercalcemia (Sharma *et al*., 1982; Suzuki *et al*., 2006) after the exposure of fish to toxicants.

In the present study, hypophosphatemia has been noticed in the chlorpyrifos- treated *Euphlyctis cyanophlyctis.* This is in conformity with the reports of other investigators who have also noticed similar effects after the exposure of various fish and other species to toxicants (chlorpyrifos – Srivastav *et al.*, 1997 a; cadmium – Rai and Srivastav, 2003; deltamethrin – Srivastav *et al*., 1997 b; azadirachtin – Kumar *et al*., 2011 a; *Euphorbia tirucalli* – Kumar *et al.*, 2011 b; *Nerium indicum* – Prasad *et al.*, 2013; *Euphorbia royleana* – Prasad *et al.*, 2011); chicken (gammabenzene hexachloride and quinolphos –Agarwal *et al.*, 2009) and rats (cadmium – Tripathi and Srivastav, 2011; chlorpyrifos – Tripathi *et al*., 2013). In the present study, the serum phosphate levels in the frogs after a thirty-day chlorpyrifos-exposure increased approaching the control values. This could be explained as a redistribution of phosphate between the extracellular fluid and intracellular fluid.

Few researchers have noticed degeneration in kidney tubules after the treatment of amphibians with the toxicant (Hanafy and Soltan, 2007), fish (Srivastava *et al*., 1990; Akram *et al*., 1999) and mammals (Chmielnicka *et al.*, 1989; Prozialeck *et al*., 2009; Tripathi and Srivastav, 2010). Mahmood *et al.* (2016) have reported increased metal concentrations in *Euphlyctis cyanophlyctis*, and also noticed degeneration in the kidney cells. The observed hypocalcemia and hypophosphatemia in the chlorpyrifos-treated *Euphlyctis cyanophlyctis* could be attributed to the kidney damage. It has been suggested that toxicant-induced renal lesions may cause hyperfiltration in the kidneys thus causing increased efflux of the electrolytes (Chmielnicka *et al.*, 1989; Prozialeck *et al.*, 2009). Schutte *et al. (*2008) have noticed increased calciuria in cadmium-exposed women. In the past, it has been suggested that renal tubule damage might be one of the main reasons for provoking hypocalcemia/hypophosphatemia in toxicant-exposed animals (Koyama and Itazawa, 1977; Roch and Maly, 1979; Larsson *et al*., 1981; Haux and Larsson, 1984; Rai and Srivastav, 2003; Srivastav *et al.*, 1997 a, b; Kumar *et al*., 2011 a, b; Prasad *et al*., 2011, 2013). Moreover, Patel *et al.* (2006) have also suggested that lead-induced ionoregulatory toxicity in rainbow trout is not exclusively a branchial phenomenon, but is in part a result of disturbances in the ionoregulatory mechanism of the kidneys.

In conclusion, the present study has revealed the consequences of the exposure to chlorpyrifos on alterations in the vital electrolytes of the frog *Euphlyctis cyanophlyctis.* The physiological capabilities of chlorpyrifos raise severe concerns regarding its danger to aquatic organisms. Further studies are needed to explore the biological consequences after the exposure of frogs to chlorpyrifos, and to formulate future strategies for encountering the amphibian population decline.

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