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Effects of chlorpyrifos on ultimobranchial and parathyroid glands of Indian skipper frog, *Euphlyctis cyanophlyctis*

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ABSTRACT: This study investigated effects of chlorpyrifos on ultimobranchial (UBG) and parathyroid glands (PTG) of frog, *Euphlyctis cyanophlyctis*. Frogs were treated with chlorpyrifos for short and long term and sacrificed after 24, 48, 72 or 96 h in short term and after 5.10, 15 and 30 days in long term. Chlorpyrifos exposure provokes decrease in serum calcium levels after 48 h which persists till 96 h. There is slight decrease in the nuclear volume of UBG cells and cytoplasm depict weak staining response after 72 h. After 96 h these changes are more pronounced. PTG of *Euphlyctis cyanophlyctis* exposed to chlorpyrifos exhibit no change till 96 h. Serum calcium decreases on day 10 after chlorpyrifos exposure which continue to fall progressively till 30 days. After 15 days chloryrifos exposure, nuclear volume of UBG exhibit decrease and follicular epithelium displays decrease in height. Follicular epithelium after 30 days chlorpyrifos exposure reduces to the extent that it becomes single layered. Few degenerating cells have been discerned. At this interval nuclear volume of ultimobranchial cells exhibits a further decrease. PTG of chlorpyrifos treated frog depicts increased nuclear volume of PTG at 10 and 15 days. The nuclei of PTG are hyperchromatic and the gland becomes compact at 15 days. After 30 days following chlorpyrifos treatment nuclear volume exhibits further increase. Also degenerating cells make their appearance. Calcium regulating glands UBG and PTG of frogs were adversely affected by exposure to chlorpyrifos which may disturb the physiological functions of the organism.

Keywords: Chlorpyrifos; Ultimobranchial gland; Parathyroid gland; Indian skipper frog; Organophosphate; *Euphlyctis cyanophlyctis*.

1. INTRODUCTION

Organophosphate pesticides are being widely used all over the world with extensive occurring in aquatic ecosystem. Chlorpyrifos ($C_9H_{11}Cl_3NO_3PS$) is a broad spectrum organophosphate used to control various pests of agriculture and in many non-agricultural situations [1]. In some parts of India, Lari et al. [2] reported the chlorpyrifos content in ground water and surface water as 0.21 µg/L and 0.46 µg/L, respectively.

A variety of sub-lethal effects of chlorpyrifos from various non-target organisms have been reported such as histological abnormalities in various organs [3-7], inhibition of acetylcholinesterase activity [8, 9], developmental abnormalities [8, 10] and reactive oxygen species production [9, 11]. Amphibians deserve special attention regarding the effects of pesticides as they breed near agricultural areas where pesticides are extensively used and hence they are exposed to pesticides at all life stages – larvae (in waters) and adults (on land). This study aimed to evaluate the effects of chlorpyrifos on the histological structure of calcium regulating endocrine glands namely ultimobranchial and parathyroid glands of Indian skipper frog *Euphlyctis cyanophlyctis.*

2. MATERIALS AND METHODS

Laboratory bred *Euphlyctis cyanophlyctis* (both sexes, body wt. 14.34±0.45 g) were used in the experiments. Frogs were kept in all glass aquaria (30 L) and acclimatized to the laboratory conditions (under natural photoperiod 11.58-12.38 and temperature 27.2±1.4 ºC) for 15 days. During acclimatization the frogs were fed daily with live insects, 2-3 times per day. Water was renewed daily after cleaning the fecal matter. All care was taken to avoid giving stress to the frogs. Feeding was stopped 24 h before and during the experimental period.

Short-term and long-term experiments have been performed for each toxicant. The handling and care of frogs were approved by Ethical committee of DDU Gorakhpur University, India (F.Sc.2551/Zoology/4-12-06).

2.1. Short-term exposure

In this the frogs (N=24) were subjected to 3.99 mg/L chlorpyrifos i.e. 0.8 of 96 h LC_{50} [12]. 10 frogs were were maintained in 30 L media. A control group of frogs (N=24) was also used. Six frogs were killed on each time intervals from control and experimental groups after 24, 48, 72 and 96 h of exposure period.

2.2. Long-term exposure

The frogs (N=24) were subjected to 0.99 mg/L i.e. 0.2 of 96 h LC_{50} value [12] of chlorpyrifos for 30 days. Frogs (N=24) was also used as control group. Six frogs from the control and experimental groups were sacrificed after 5, 10, 15 and 30 days.

Blood from both experiments (short- and long-term) were collected by cardiac puncture under slight ether anesthesia and allowed to clot at room temperature. Sera were separated and analyzed for serum calcium (Sigma-Aldrich). All determinations were carried out in duplicates for each sample.

For ultimobranchial and parathyroid glands, glottis together with a small piece of surrounding tissue were fixed in aqueous Bouin's solution. These fixed tissues were processed through routine histological procedure, embedded in paraffin, sectioned at 6 μm and then stained with hematoxylin and eosin (HE). Photomicrographs were taken with the aid of Olympus CH 20i microscope and Olympus E 420 camera.

2.3. Nuclear volume

Nuclear indices (maximal length and maximal width) of ultimobranchial gland and parathyroidal cells were taken by ocular micrometer and nuclear volume was calculated as – volume = $4/3\ \pi\ ab^2$, where 'a' and 'b' represents major semiaxis and minor semiaxis. Only the indexes of intact nuclei were measured.

Each data represents mean ± S.E. of six specimens and Student's t test was used to determine statistical significance between the experimental group and its specific time control group.

3. RESULTS

3.1. Short-term chlorpyrifos exposure (0.8 of 96 hour LC_{50})

Exposure of the frog *Euphlyctis cyanophlyctis* to chlorpyrifos provokes a decrease in the serum calcium levels after 48 h. This decrease continues till the end of the experiment (96 h) (Fig. 1). The details of ultimobranchial glands (Fig. 2) of control frogs are similar as described earlier by Srivastav et al. [13].

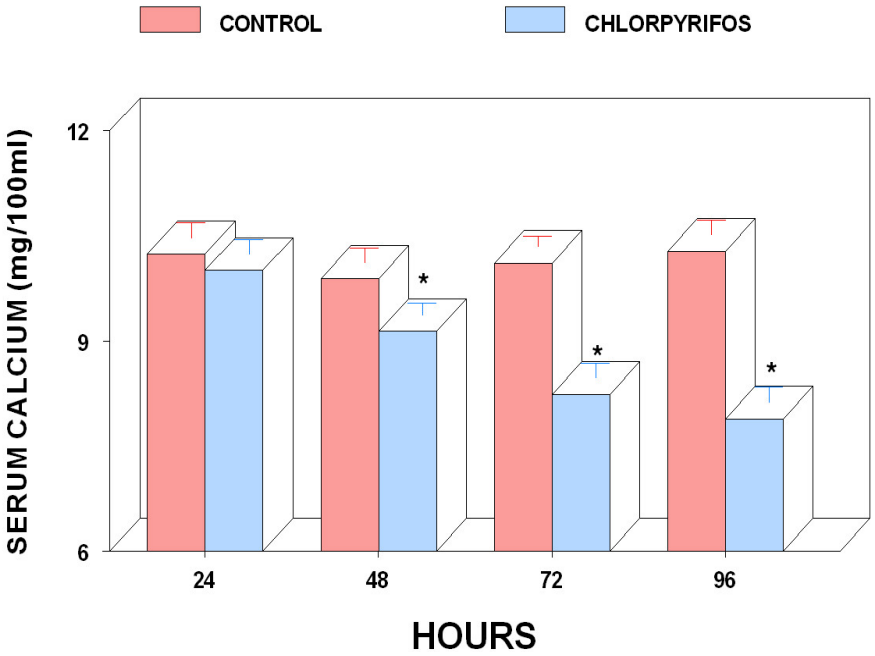

Figure 1. Serum calcium levels of short-term chlorpyrifos treated *Euphlyctis cyanophlyctis*.
Values represent mean ± S.E. of six specimens. * indicates significant differences (P< 0.05) from control.

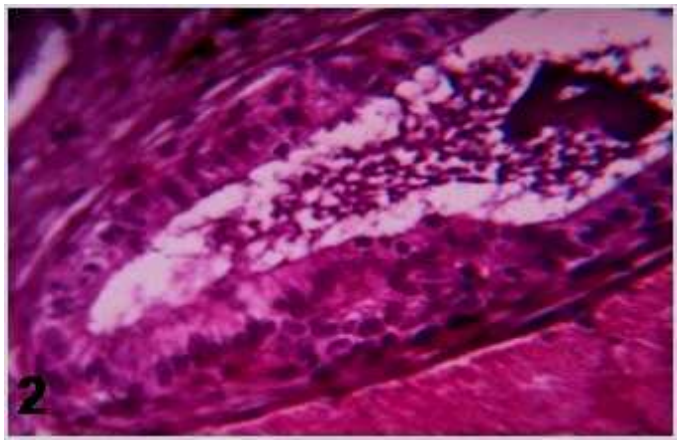

Figure 2. Ultimobranchial gland of control *Euphlyctis cyanophlyctis*. HE x 200.

The ultimobranchial gland of chlorpyrifos treated *Euphlyctis cyanophlyctis* exhibits no histological change up to 48 hours. The cytoplasm of ultimobranchial cells depict a weak staining response after 72 h (Fig. 3). There is a slight decrease in the nuclear volume of these cells (Fig. 4). After 96 hours following the chlorpyrifos exposure, these changes are more pronounced (Fig. 4).

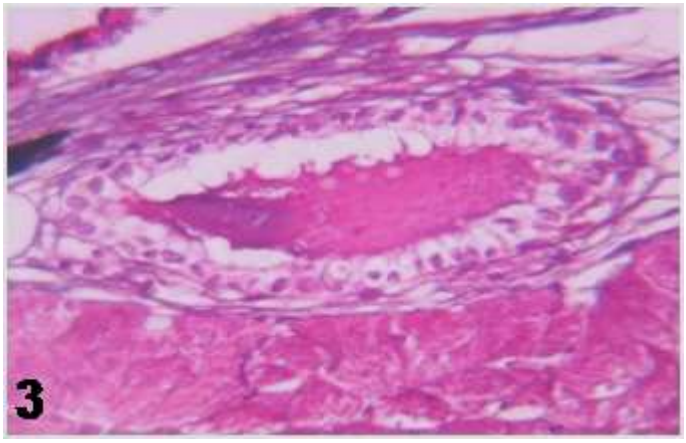

Figure 3. Ultimobranchial gland of 96 h chlorpyrifos treated *Euphlyctis cyanophlyctis* exhibiting weak staining response of the cytoplasm. HE x 200.

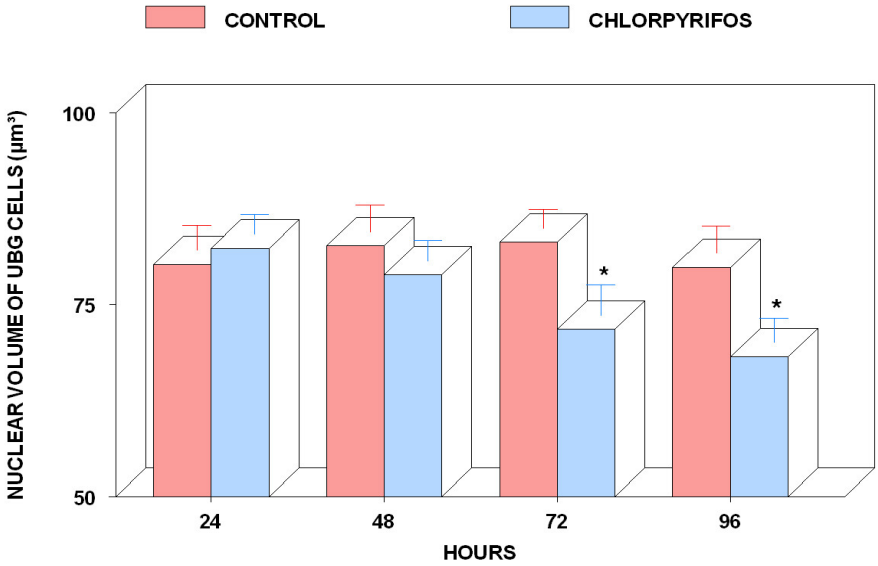

Figure 4. Nuclear volume of ultimobranchial cells of short-term chlorpyrifos treated *Euphlyctis cyanophlyctis.* Values represent mean ± S.E. of six specimens. * indicates significant differences (P< 0.05) from control.

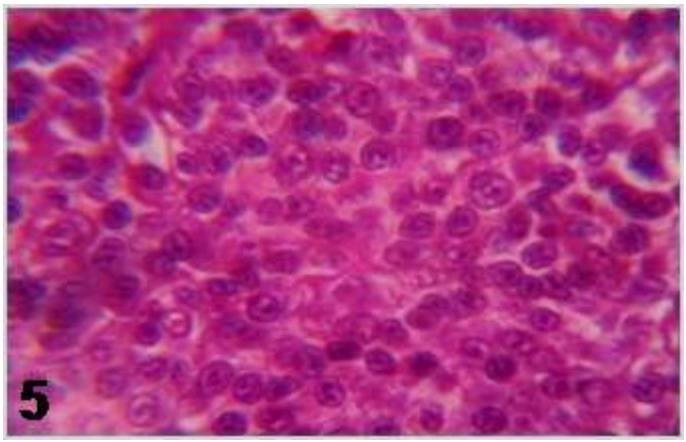

Figure 5. Parathyroid gland of control *Euphlyctis cyanophlyctis*. HE x 500.

The details of paraythyroid glands (Fig. 5) of control frogs are similar as described earlier by Srivastav et al. [13]. The parathyroidal cells of *Euphlyctis cyanophlyctis* exposed to chlorpyrifos exhibit no change (Fig. 6) in the histological structure throughout the experiment.

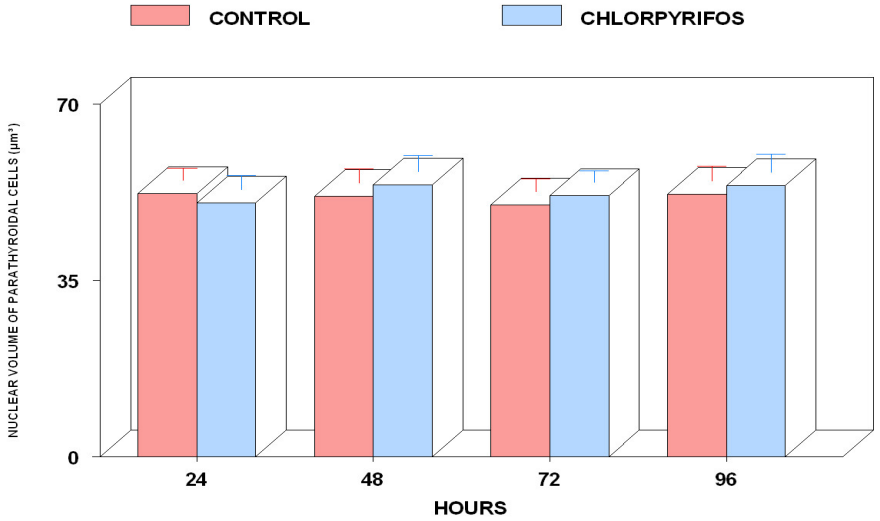

Figure 6. Nuclear volume of parathyroidal cells of short-term chlorpyrifos treated *Euphlyctis cyanophlyctis.* Values are mean ± SE of six specimens.

3.2. Long-term chlorpyrifos exposure (0.2 of 96 hour LC_{50})

After chlorpyrifos exposure to *Euphlyctis cyanophlyctis* the first perceivable change has been noticed on day 10 in the serum calcium as the levels decrease at this interval. The levels continue to fall progressively till the end of the experiment (30 days; Fig. 7).

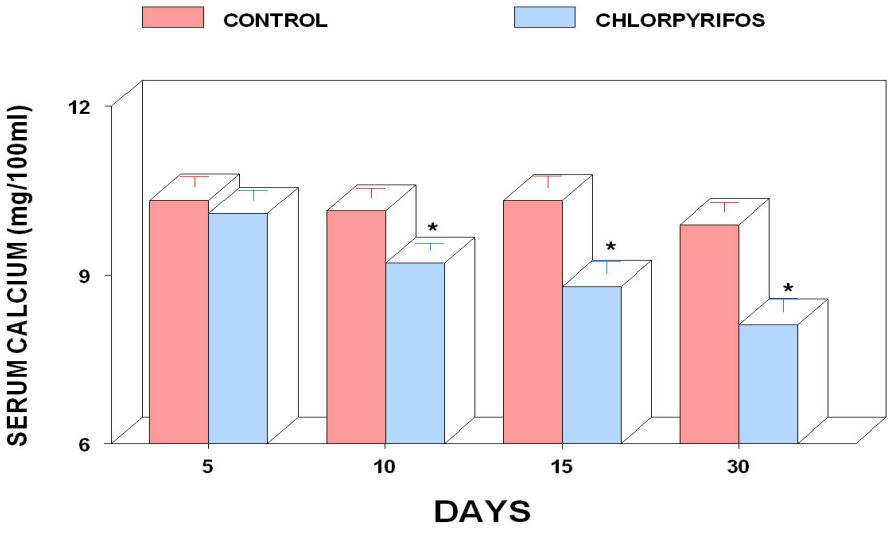

Figure 7. Serum calcium levels of long-term chlorpyrifos treated *Euphlyctis cyanophlyctis*. Values represent mean ± S.E. of six specimens. * indicates significant differences (P< 0.05) from control.

No histological alterations are noticed in the ultimobranchial gland of chlorpyrifos treated *Euphlyctis cyanophlyctis* up to 10 days. After 15 days following exposure to chloryrifos, the nuclear volume of ultimobranchial cells exhibit a decrease (Fig. 8) and the follicular epithelium displays a decrease in height at

certain places. The follicular epithelium after 30 days chlorpyrifos exposure reduces to the extent that it becomes single layered (Fig. 9). Also a few degenerating cells have been discerned (Fig. 9). At this interval the nuclear volume of ultimobranchial cells exhibits a further decrease (Fig. 8).

In the parathyroid glands of chlorpyrifos treated *Euphlyctis cyanophlyctis* no marked changes have been noticed up to 5 days. Thereafter, an increased nuclear volume of parathyroidal cells has been noticed at 10 and 15 days of treatment (Fig. 10). The nuclei of parathyroidal cells are hyperchromatic and the gland becomes compact at 15 days (Fig. 11). After 30 days following chlorpyrifos treatment the gland is more compact. The nuclear volume exhibits a further increase (Fig. 10). Also degenerating cells make their appearance (Fig. 12).

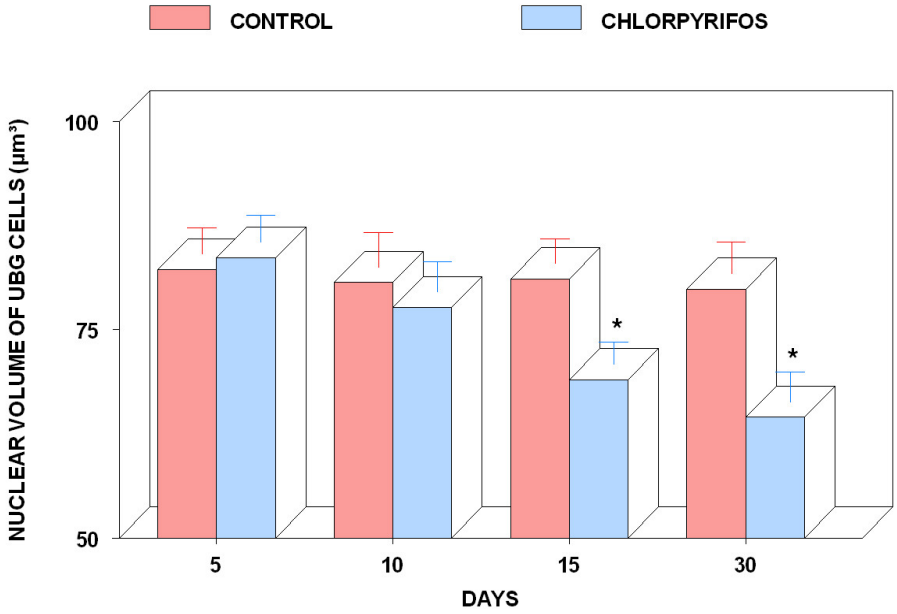

Figure 8. Nuclear volume of ultimobranchial cells of long-term chlorpyrifos treated *Euphlyctis cyanophlyctis.* Values are mean ± SE of six specimens. Asterisk indicates significant differences ($P < 0.05$) from control group.

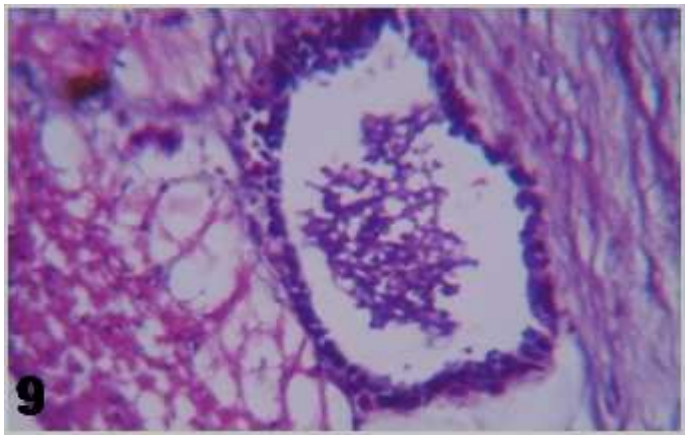

Figure 9. Ultimobranchial gland of 30 day chlorpyrifos treated *Euphlyctis cyanophlyctis* exhibiting single layered follicular epithelium and degeneration. HE x 200.

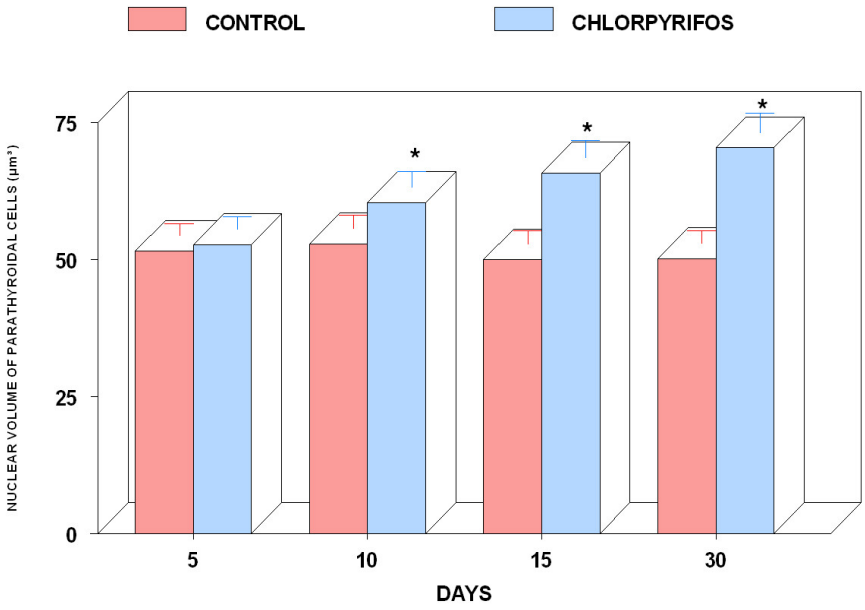

Figure 10. Nuclear volume of parathyroidal cells of long-term chlorpyrifos treated *Euphlyctis cyanophlyctis.* Values are mean ± SE of six specimens. Asterisk indicates significant differences (P < 0.05) from control group.

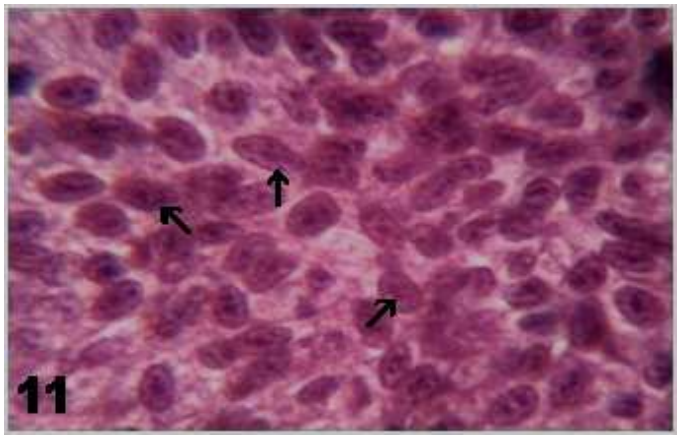

Figure 11. Parathyroid gland of 15 day chlorpyrifos treated *Euphlyctis cyanophlyctis* exhibiting elongated (arrows) and hyperchromatic nuclei. HE x 500.

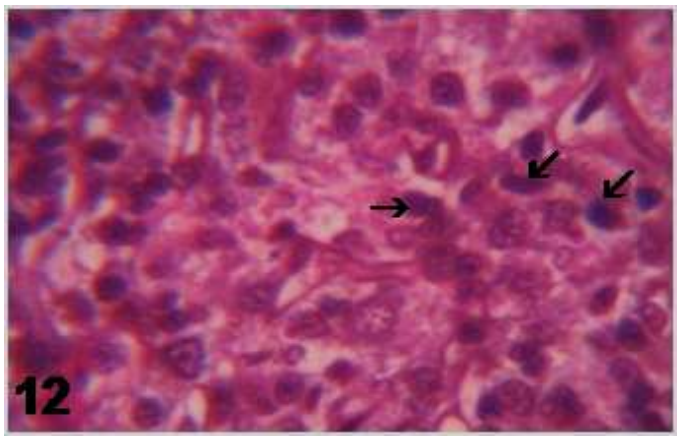

Figure 12. Parathyroid gland of 30 day chlorpyrifos treated *Euphlyctis cyanophlyctis* exhibiting degeneration (arrows). HE x 500.

4. DISCUSSION

In *Euphlyctis cyanophlyctis* chlorpyrifos exposure caused inactivity of ultimobranchial gland. There has been noticed weak staining response, decreased nuclear volume and reduced height of follicular epithelium showing degeneration and vacuolization. This is first report regarding the effects of organophosphate on calcium regulating endocrine glands of amphibians as there exists no study regarding this aspect. The observed inactivity in ultimobranchial gland of chlorpyrifos treated frogs is in agreement with the reports of other workers who have noticed inactivity of ultimobranchial gland in toxicant exposed amphibian [13] and fish [14-18]. The inactivity of the gland noticed in chlorpyrifos exposed *Euphlyctis cyanophlyctis* also derives support from the studies of earlier investigators who have also recorded inactivity of ultimobranchial gland after provoking hypocalcemia by calcitonin treatment to the fish (*Anguilla anguilla* [19]; *Gasterosteus aculeatus* [20]; *Clarias batrachus* [21]; *Heteropneustes fossilis* [22]); amphibian (*Bufo viridis* [23]; *Rana tigrina* [24]) and reptiles (*Natrix piscator* [25]; *Calotes versicolor* [26]). The observation of Anderson and Capen [27]) strengthens the present study as in *Iguana iguana* fed on low calcium diet hypocalcemia was noticed which caused less activity of ultimobranchial gland.

Chlorpyrifos [28] and other toxicants [14, 16-18] caused inactivity of ultimobranchial gland in lower vertebrates whereas hyperactivity of calcitonin cells (which secrete a hypocalcemic hormone calcitonin in mammals) has been noticed in mammals after treatment with chlorpyrifos and other toxicants [29-31]. Increased circulating calcitonin levels has been reported from cadmium exposed rats [32]. It is of interest that chlorpyrifos provoked opposite effects (inactivity or hyperactivity) on the hypocalcemic hormone producing glands (ultimobranchial gland in non-mammals; calcitonin cells in mammals). In non-mammals during embryonic development ultimobranchial cells remain separate as a discrete organ (ultimobranchial gland) whereas in mammals these cells fuse with thyroid gland and remain there as diffused calcitonin cells [33]. Thus, more investigations are required to understand the mechanisms of action of chlorpyrifos in non-mammals and mammals regarding the release of hypocalcemic hormone calcitonin.

Reduced height of follicular epithelium, degeneration and vacuolization of ultimobranchial gland has been noticed in the chlorpyrifos treated *Euphlyctis cyanophlyctis*. Prolonged hypocalcemia noticed in the chlorpyrifos exposed *Euphlyctis cyanophlyctis* might be the possible reason as it rendered continuous disuse of the ultimobranchial gland thus causing its degeneration.

Increased nuclear volume and elongated hyperchromatic nuclei has been discerned in the parathyroid gland of chlorpyrifos treated *Euphlyctis cyanophlyctis.* In the literature there is no report regarding the effects of organophosphate on parathyroid gland of amphibian, hence this is the first report. In vertebrates parathyroid gland regulate the low calcium in blood by actions on intestine, bone and kidney [34]. In frogs [13] and rats [29-31] hyperactivity of parathyroid gland has been noticed after toxicant exposure which supports the findings of the present study. Increased levels of parathyroid hormone in blood has been determined in cadmium treated rats by Brzoska and Moniuszko-Jakonink [32]. Koyama and Itazawa [35] have also recorded bone demineralization in cadmium treated carp. They have attributed this to restore plasma calcium levels. In the present study the hyperactivity of parathyroid gland in chlorpyrifos treated *Euphlyctis cyanophlyctis* can be attributed to the observed hypocalcemia which have activated the parathyroid glands to release the hypercalcemic hormone to restore the calcium to normal levels.

5. CONCLUSION

This study could provide further insight into the potential hazards of chlopyrifos contamination and

exposure on the calcium regulating endocrine glands namely ultimobranchial and parathyroid glands of the frog *Euphlyctis cyanophlyctis.*

Authors' Contributions: All authors contributed equally to this work. All authors read and approved the final manuscript.

Conflict of Interest: The authors have no conflict of interest to declare.

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