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Chlorpyrifos Affects Testis and Vas Deferens in the Mangrove Crab, *Episesarma tetragonum* (Fabricius)

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Abstract: Accumulation of pesticides in the aquatic system has a high impact on the reproductive physiology of crustaceans. The objective of the present study was to assess the possible ultrastructural effects of chlorpyrifos exposure on testis and Vas deferens in Mangrove crab, *Episesarma tetragonum* using electron microscopy. The testis of experimental crabs showed atrophied seminiferous epithelia, germinal layer in the seminiferous tubules exhibited severe necrosis, associated with impaired spermatogenesis as well as edema in the interstitial space, and there were less number of spermatozoids in the lumen of the many tubules. The treated vas deferens exhibited destruction in the seminal plasma. Large number of spermatophores got splitted and spermatozoa are scattered, vassal epithelium nucleus got distorted. These clinical manifestations expressed in crabs following the exposure of chlorpyrifos significantly reduce the testicular activity and substantially inhibits the seminal secretions, which ultimately lead to impairment of reproduction.

Keywords: Chlorpyrifos, Testis, Vas deferens, *Episesarma tetragonum*

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Introduction

Crabs are particularly useful in aquatic environmental research for a variety of reasons, including their importance in ecosystem processes and their ability to detect contaminated environments. The terrestrial crab is an important and helpful model for population studies, as well as advantageous to human activities. It also contains concentrated forms of proteins and oil, both of which help to prevent heart disease. Since crabs are abundant locally and are used in food as a diet by some people due to their high nutritional content, it has the potential to be marketed as a poor man's protein. The widespread use of chlorinated insecticides to manage pest species causes ecological disruptions, affecting non target organisms. There is several information available

on the toxicities of pesticide contamination on aquatic creatures (Deka and Mahanta, 2012; Dode *et al*., 2012; Das *et al*., 2013).

Pesticide usage in agriculture has consequences not only for public health but also for the natural environment and non-target aquatic creatures via surface runoff from treated areas (Maharajan *et al*., 2015). Histopathological examinations can help determine the risk for contamination. Furthermore, ultrastructural analysis has been shown to be a useful method for assessing the impacts of pollutants on key organs, which in turn affect numerous other metabolic processes such as growth and reproduction. This method is also useful for detecting early toxicant effects on cells (Hinton *et al*., 1992). Male-related reproductive problems have been documented in a variety of species. Invertebrates, on the other hand, have seen significantly fewer examples from both laboratory and field studies. Organo-phosphate insecticides are known to cause damage to several crustaceans *Barytelphusa guerini* (Patil *et al*., 2008), *Palaemonetes argentines* and *Trichodactylus borellianus* (Montagna and Collins, 2008) and *Paratelphusa jacquemontii* (Maharajan *et al*., 2015). An important component of chemical contamination of the aquatic environment is the study of the influence of pesticides on aquatic species. Many insecticides are now widely used and used in agricultural operations. These pesticides have a variety of physiological impacts, including enzyme inhibition, growth, food intake, metabolism, and overall animal development. According to the documentation provided above, a natural mangrove crab, *Episesarma tetragonum* was used in this study as a test animal to better understand the hazardous effects of chlorpyrifos.

Materials and Methods

Chemicals:

Chlorpyrifos (CAS: 2921-88-2) was purchased from Jeyban, Sabari Crop Care Sciences (P) (Chennai, India). The pesticide volume of 1 ml was diluted with 1 L Milli-Q deionised water for stock solution preparation.

Experimental animal:

Mangrove crabs, *Episesarma tetragonum*, were collected from the Muthupettai mangrove in Thiruvarur Dist, Tamil Nadu, India, with carapace sizes 35.5 ± 2.5 cm and weight 50.5 ± 1.2 g and transferred to the laboratory at Khadir Mohideen College, Adirampattinam. One-week adaptation to laboratory conditions was maintained in aquaria with 200 L of well-aerated filtered estuary water at a temperature of 27 ± 2°C before the experiment. The studies were carried out in compliance with local and national guidelines for animal experimentation, and every precaution was made to avoid any cruelty.

Experiment:

Experimental crabs were randomly divided into three groups with triplicates (10 crabs per group) and exposed to sub-lethal concentrations of insecticide chlorpyrifos at levels 0.0294 and 0.0588 ppm for 28 days in 100 L aquaria. Crabs were fed commercial pellets from clams twice a day (10:00 and 16:00 h), and the system immediately eliminated non-consumed food. Mortality and behaviour were checked daily. Replenishing of media was carried out every second day with re-dosing of chlorpyrifos solution. Concentrations did not differ more than 10%. For experiment evaluation, two crabs were taken at 0, 7 and 28 days of exposure from each experimental group.

Ultrastructural study:

Experimental tissues, after being fixed in 3% buffered glutaraldehyde for 24 h, was washed thoroughly with 0.1 M phosphate buffer and post-fixed in osmium tetroxide for 1–2 h at 4°C. After a brief wash in 0.1 M phosphate buffer, AG tissue was dehydrated in a graded series of ethanol (70–90%). Following dehydration in 90% ethanol, the sample was incubated with 2% ethanolic uranyl acetate (en bloc staining) and subsequently dehydrated with 100% ethanol. Propylene oxide was used as the clearing agent. The tissue was infiltrated for at least 6 h in a 1:1 combination of

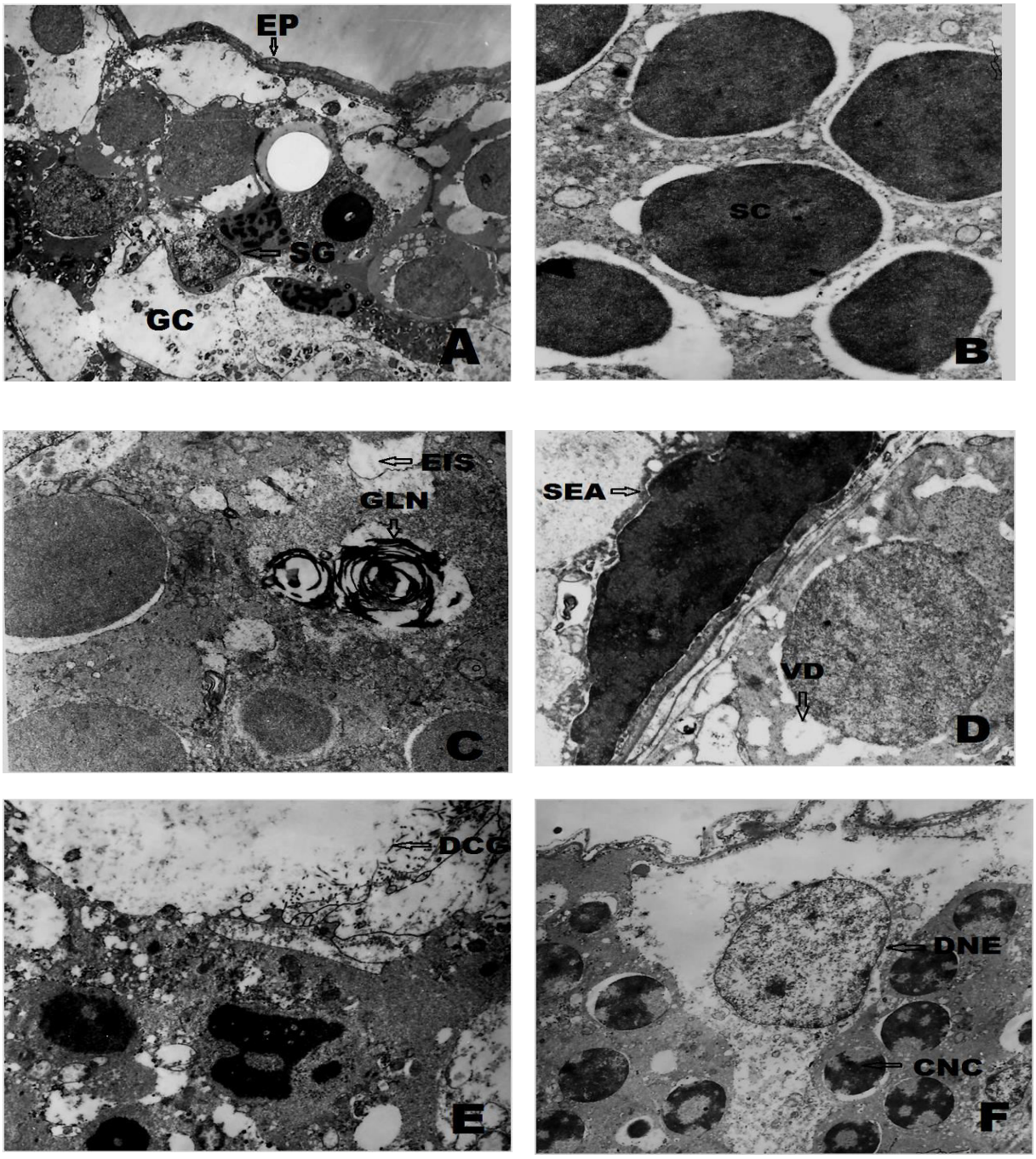

Fig. 1: Ultrastructural micrograph of testis in *E. tetragonum.* A and B Control; C-After 7 days of exposure to 0.0294 ppm concentration of Chlorpyrifos; D -After 28 days of exposure to 0.0294 ppm concentration of Chlorpyrifos; E and F-After 28 days of exposure to 0.0588 ppm concentration of Chlorpyrifos. EP- Epithelium, SG - Spermatogonium, SC -Spermatocyte, GC - Gonadal cell, VD - Vascular degeneration, EIS - Edema in interstitial space, GLN -Germinal layer necrosis, SEA -Seminiferous epithelia atrophied, CNC-Concentration of nuclear chromatin, DC - Degeneration of cytoplasm, DNE - Damaged nuclear envelope.

propylene oxide and araldite, followed by 8 hours in pure araldite. The tissue was then implanted in araldite and placed in an oven at 60°C for two days to polymerize. To achieve the precise placement of the tissue, semi-thin (1 µm) slices stained with 1% toluidine blue were utilised. Ultra-thin (600 A) slices contrasted with uranyl acetate and lead citrate were taken and scanned using a Jeol (USA) transmission electron microscope.

Results

Ultrastructural study of Testis:

The ultrastructural examination of the testis of the control crabs showed normal architecture as evidenced by well-organized sex cells in the seminiferous epithelium (Figs. 1A, B). The cells in each testicular lobe seem to be in a single stage of spermatogenesis however, cells in different lobe may be in different stages of spermatogenesis. In control group cytoplasm organelles and nucleus of spermatogonium and primary spermatocytes were normal and mitochondria were normal with cristae. In the beginning there were not many differences in the fine structure of germinal cells in testes taken from control and treated crabs. At lower concentration after 7 days large number of the developing spermatocytes was observed within the seminiferous epithelium, similar to the control, but vacuolar degeneration appeared in the epithelial cells. The seminiferous epithelia were atrophied in later stages (Figs.1C, D). In higher concentrations the germinal layer in the seminiferous tubules exhibited severe necrosis, associated with impaired spermatogenesis as well as edema in the interstitial space, and there were less number of spermatozoids in the lumen of the many tubules. At 28 days, apoptotic cells were identified by electron microscopic analysis. The apoptotic germ cells showed condensation of nuclear chromatin and degeneration of cytoplasmic organelles (Figs. 1E, F). The structure of the nuclear envelope partially departed in the spermatogonium and mitochondrial cristae collapse in spermatocytes. At higher concentration the nuclear envelope dispersed and accompanied by chromatin condensation and marginalization of spermatocytes.

Ultrastructural study of Vas deferens:

The fine structural analysis of the vas deferens of the control crabs exhibited normal structure of vassal epithelium and spermatophores with spermatozoa (Fig. 2A). The vas deferens of control group showed squamous epithelium with nuclear arrangement. The lumen of vas deferens was filled with homogenous mass of ductal seminal secretions in which spermatozoa are bounded within the double walled capsule clled spermatophore (Fig. 2B). Within the double walled spermatophores, spermatozoa were embedded in the spermatophoric matrix. At lower concentrations the vas deferens showed some epithelial damage (Fig. 2C) and vacuolar degeneration. The lumen showed highly uneven manifestation of spermatophores. As the days of exposure increased there was high level destruction in the seminal plasma. Large number of spermatophores got splitted and spermatozoa are scattered (Fig. 2D). At higher concentration after 28 days of exposure, spermatophore matrix got degenerated and exhibited extensive necrosis. In the vassal epithelium the nucleus got distorted and partial tearing of nuclear membrane (Fig. 2E). The muscle layer of the epithelium showed necrosis and numerous apoptotic cells were seen. Break down of spermatophore wall is noticed. Within the spermatophore the matrix got disoriented and spermatozoa showed more nuclear condensation. The spermatozoa capsule split into fragments at higher concentrations (Fig. 2F).

Discussion

Chlorpyrifos-induced pathological alterations resulted in particular modifications or abnormalities at the tissue level. The general abnormalities found in the testis under lethal and sublethal chlorpyrifos exposure were testicular follicle rupture, collapsed tubules, decreased spermatogenic bulk, and tissue degradation. Injury to androgen secreting cells following copper

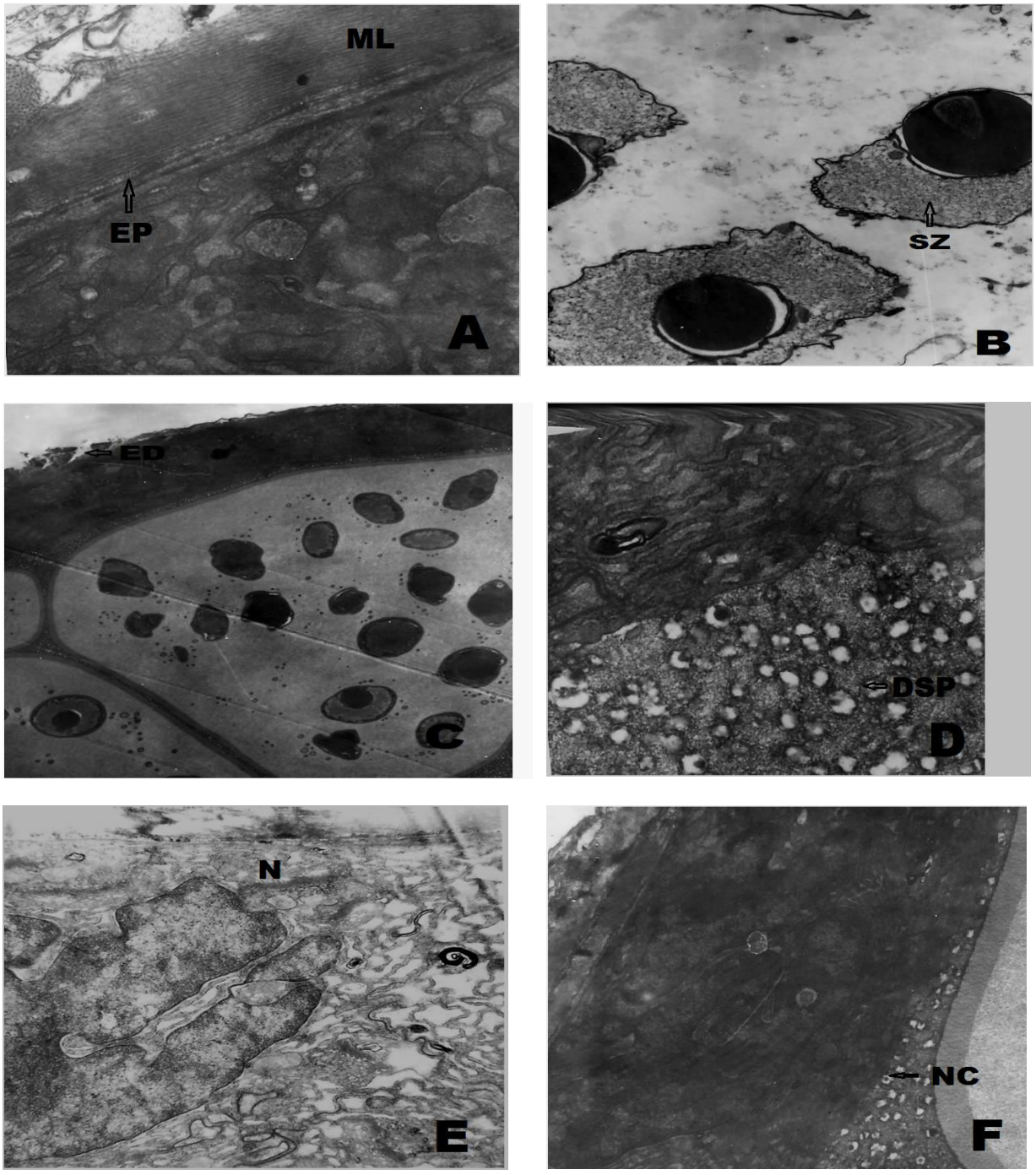

Fig. 2: Ultrastructural micrograph micrograph of vas deferens in *E. tetragonum.* A and B- Control; C and D -After 7 days of exposure to 0.0294 ppm concentration of Chlorpyrifos; E and F -After 28 days of exposure to 0.0588 ppm concentration of Chlorpyrifos. EP - Epithelium, ML - Muscle layer, SZ - Spermatozoa, DSB - Destructions of seminal plasmid, ED - Epithelial damage, N - Necrosis, NC - Nuclear condensation.

exposure could cause abnormal tissue alterations, eventually leading to testicular injury. However, the decrease in spermatozoa could have been caused by a decrease in the circulating testicular level. Reduced spermatogenic material was seen in several tubules, indicating spermatogenesis suppression. Chlorpyrifos impeded the advancement of spermatogenesis in male crabs, particularly from spermatocyte to spermatozoa. These findings suggested that chlorpyrifos inhibits

spermatogenesis in male crustaceans. In contrast to female crustaceans, there have been little investigations on the spermatogenesis regulatory system and the impact of heavy metals on spermatogenesis in male crustaceans.

The vas deferentia are paired structures that transport spermatozoa from the testes to the gonopores at the base of the fifth walking legs. The vas deferens, in addition to acting as a channel for spermatozoa, is also in charge of "packaging" the spermatozoa into a spermatophore. Most brachyuran and anomuran crab spermatophores are single spermatozoa or "sperm balls" (Fig. 1). However, spermatophores in some lower brachyurancrabs (e.g., *Dromidia, Ranilia*) and some anomuran crabs (e.g., *Clibanarius*) are joined to one another in a chain-like form via a thin membrane sheath.

The treated testis of *E. tetragonum* exhibited significant changes when exposed to chlorpyrifos pesticide for different exposure periods. Broad cytotoxic damage, inflammation and other histological abnormalities are quite evident in the treated reproductive tissue. Present study also evident to chlorpyrifos induced cytopatholocal indices in mangrove crab, *E. tetragonum* (Rajesh *et al*., 2013). Testicular inflammation was documented as one of the common responses in aquatic animals exposed to environmental toxicants (Sokal *et al*., 1985). In the treated crab, the progression of spermatogenesis, especially from spermatocyte to spermatozoa, was inhibited by treatment with chlorpyrifos. These results suggested that chlorpyrifos has negative effects on spermatogenesis in male crabs. Gangshettiwar (1986) showed thickening and rupturing of testicular tubules, deformation of tubules affecting proliferation zone, reduced spermatogenic mass, vacuolization and degeneration of tissue of prawn, *Macrobrachium lamerri* after exposure to phenol. Disintegration and deformation of spermatocyte, spermatids and spermatozoa were observed during chlorpyrifos exposure. The light microscopic observations also were supported by the ultrastructural observations in the treated testis.

Similar to our results, Bodkhe (1983) observed an irregular arrangement of spermatozoa in the testicular tubules of the crab, *Barytelphusa cunicularis* following exposure to sevimol. Our findings are consistent with the results of El-Ashmawy and Youssef (1999) who demonstrated that Cd induced disordered arrangement of germ cells, sloughing and a decreased spermatogenic cell layer in the seminiferous tubules, destruction of basement membranes, disintegration of spermatocytes, and a complete absence of sperms. At low concentrations treated crabs exhibited disruption in the tubular architecture, arrangement of epithelial cells, reduction in number of spermatophores, and aggregation of the granular substances in the vas deferens. Similarly, TBT can affect sperm count and male reproductive systems in aquatic organisms (Fent and Hunn., 1995; Zhang *et al*., 2007). In rats the organophosphate pesticide affected the vas deferens with impairment of spermatozoa, cellular necrosis, nuclear pyknosis which supports our present study (Prashanthi *et al*., 2006). At high concentration of chlorpyrifos exposure, severe disruption in the arrangement of spermatophores and epithelial cells and the reduction in tubule membrane thickness are seen. Chlorpyrifos exposure resulted in unique modifications in gonadal ultrastructure in the current investigation.

Conclusion

Chlorpyrifos exposure caused damage to crab reproductive organs, testis, and vas deferens and disrupted an entire metabolic and other physiological functions. As a result, we recommend that pesticide use in the paddy field area of the Muthupettai mangrove ecosystem should be reduced, and alternative methods to safeguard crustacean biodiversity should be strengthened.

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