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Histopathological effects of chlorpyrifos on the midgut of 3rd larval instar of oriental latrine fly, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae)

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Abstract
Chrysomya megacephala, is a common blow fly species of medical importance in many parts of the world. Larvae of this species are known to cause myiasis in several mammal species, including humans. The gut of *C. megacephala* larva is a single, long tubular organ system, consisting of the stomodaeum, mesenteron, proctodaeum and accessory organs projecting from the main digestive tube. This study aimed to investigate the midgut of 3rd instar larvae of *C. megacephala* at the ultrastructural level using light microscopy. Sublethal doses of insecticide chlorpyrifos (0.02%, 0.04% and 0.2%) were provided in food to 3rd instar larvae at two different time periods (24 hours and 48 hours), to examine the alteration from normal histology of midgut. This study indicated that midgut was highly affected after 24 and 48h post-treatment and the intensity of the histopathological effects was dependent on time period and the concentration used. In midgut, treated larvae showed loosely attached epithelial layer in sections which became filled with vacuoles at higher concentrations. Epithelial cells became more or less circular in shape and highly separated from each other but on increasing the concentration, significant elongation in epithelium cells and decrease in lumen of the midgut were seen. The number of epithelial cells has been reduced. The peritrophic membrane was clustered in the centre of the lumen. Outer muscular layer and basement membrane were also affected. The results suggest that chlorpyrifos could be used as an effective insecticide for the control of *C. megacephala.*

Keywords: *Chrysomya megacephala*, midgut, chlorpyrifos, histopathology

Introduction
Over 1,450 blow fly species are distributed over all continents in the world, except Antarctica [1]. *C. megacephala* is most commonly distributed in oriental region and Australasia [2]. *C. megacephala* is one of the most common members of the genus *Chrysomya*. It is a large species, often exceeding 10 mm in length and is of tropical origin. Larvae vary in size according to instar and are shaped more thickly towards the rear [3]. Larval development of *C. megacephala* has been investigated by Wijesundara [4], O'Flynn [5], Wells and Kurahashi [6] and Sukontason *et al.* [7]. The larvae of this species is not only known for causing myiasis [8, 9, 10], but more recently identified as playing an important role in forensic cases [11, 12, 13, 14].

Chlorpyrifos [O, O- diethyl-O-(3, 5, 6-trichloro-2-pyridyl)-phosphorothioate], is one of the broad range organophosphate insecticides utilized both in farming and household pest control agents because of its non- carcinogenic and non-teratogenic nature [15]. It acts as acetylcholine esterase inhibitor and targets on the nervous system of the insects [16, 17]. It is considered moderately hazardous to humans by the World Health Organization. As a consequence, Chlorpyrifos has been accounted for as one of the commonly utilized organophosphate pesticide [18, 19].

With respect to the various internal systems of flies, the purpose of the gut is to carry food through the body that will absorb the nutrients and expel the waste; therefore, ultrastructure of the gut has been investigated intensively in several species of insects and flies. These include fruit fly, *Bactrocera dorsalis* [20, 21]; mosquitoes, *Aedes (Stegomyia) aegypti* [22, 23] and *Culex quinquefasciatus* [24]; sand flies, *Lutzomyia intermedia* [25], *Lutzomyia longipalpis* [26]; bot fly, *Dermatobia hominis* [27]; black fly, *Simulium pertinax* [28]. Effects of various insecticides on the gut histology have also been performed by various investigators in the southern armyworm larva treated with lead arsenate [29], in *Pieris brassicae* induced by the 5-endotoxin of *Bacillus thuringiensis* [30], in the larvae of *Chironomus decorus* Johannsen and *Tanypus grodhausi* Sublette treated with diflubenzuron and Bay SIR-8514 [31], in *Culex pipiens* treated with *Artemisia judaica* and *Anagallis arvensis* [32], in *Spodoptera exigua* with action of diflubenzuron, malathion and Cypermethrin [33], in *Helicoverpa armigera* fed with leaf extract of plant *Lantana camara* [34], in *Rhynchophorus ferrugineus* larvae treated with zinc sulfate [35], on *Cephalopina titillator* larvae treated with pyriproxyfen and chlorfluazuron [36], on *Synthesiomyia nudiseta* larvae treated with the volatile oils of *Cupressus macrocarpa* and *Alpinia officinarum* [37], in the larvae of *Mythima seperata* treated with fraxinellone [38]. In regard to *C. megacephala* larvae, the morphology of the alimentary canal has been examined [39] and the dissection and morphometric analysis has been described [40]. However, very little information is available regarding toxicology of insecticides with reference to larvae of *C. megacephala*, therefore, the objective of this study is to investigate the midgut of 3rd larval instar of C. *megacephala* at ultrastructural level and to illustrate the histological effects of chlorpyrifos on midgut.

Materials and methods

Rearing and Maintenance of *C. megacephala*

C. megacephala were collected from the campus of Aligarh Muslim University, Aligarh and reared in the insectary according to the preceding research of the author (Yasmeen and Amir) [41].

Preparation of insecticidal concentrates

0.2% stock solution of chloropyrifos was prepared in distilled water. Then this concentration was diluted 5 times and 10 times to get desired concentration of 0.04% and 0.02% respectively.

Sampling of Experimental Insects

3rd instar larvae were treated with 2 ml of each insecticide concentrations (0.2%, 0.04% and 0.02%) by ingestion method. Parallel to these, a control set up was also maintained.

Histological preparation of midgut for light microscopy

After 24 and 48 hours of treatment, approximately 10 specimens of each concentrations and control larvae were individually dissected in petridish containing Ringer's solution to obtain midgut by using fine entomological needles under a binocular dissecting microscope at 40X magnification and fixed immediadiately in Bouin's solution for 18 hours. After that tissues were washed 2-3 times in tap water to remove excess picric acid and dehydration proceeded in ascending grades of alcohol i.e. 30%, 50%, 70%, 80%, 90% for 15 minutes each while in 96% and 100% alcohol for half an hour each followed by mixture of 100% alcohol and xylene solution (1:1) for 10 minutes. Incubation was done at 60ºC in xylene and paraffin wax (1:1) for 15 minutes then in pure wax for 2 hours. Midgut was then embedded in parrafin wax whose 5 µm sections were cut into ribbon. Then the ribbons were placed on glass slide having albumen and few drops of glycerin. Slides were then stretched on warming table to remove creases. Slides were processed in 2 changes of xylene and then descending grades of alcohol series 100%, 96%, 90%, 80%, 70%, 50%, 30% for 5 minutes each and then in distilled water for 5 minutes each. Slides were stained in hematoxylin for 10 seconds, then washed in tap water and counter stained with Eosin for 20 minutes followed by upgrade dehydration in alcohol for 5 minutes each and then 2 changes of xylene for 10 minutes each. After air drying slides were mounted using D.P.X. to observe under compound microscope. Photographs were taken using Nikon Eclipse compound microscope at 10x and 40x magnification.

Results and discussion

Histological study of the midgut of *C. megacephala* larvae (Figs. 1&2)

The gut of *C. megacephala* larva is made up of foregut, midgut and hindgut with sphincters controlling food movement between these regions. A bulb-like cardia comprises the junction of foregut and midgut. This structure is composed of two parts: the anterior foregut tissue and posterior midgut tissue. At the junction of midgut and hindgut malpighian tubules are present.

The midgut of 3rd instar larvae is the longest portion of the alimentary canal lying convoluted and twisted within the body cavity. Midgut is further subdivided into the anterior, middle and posterior midgut. The anterior midgut emerges from the cardia and junction of the gastric caeca. Posterior to the anterior midgut is the more dilated area, the middle midgut. The anterior and middle segments are comparatively thick and the posterior segment becoming narrow and convoluted at the hind end. The external surface of midgut is very smooth with inner circular and outer longitudinal muscles. Insertion of tracheal tubules into the midgut surface is also quite prevalent. Midgut tissue contains peritrophic membrane within its central lumen and is preceded by a single layer of cuboidal epithelial cells resting on the basement membrane. The peritrophic membrane present as a thin, thread like, transparent membrane. Epithelial cells project inward from their basement membranes (Fig. 1&2). The thickness of the basement membrane in *C. megacephala* depends on nutrition throughout the larval stages that facilitates the transport of products between the digestive tract and the haemolymph. Cuticular lining is absent in midgut.

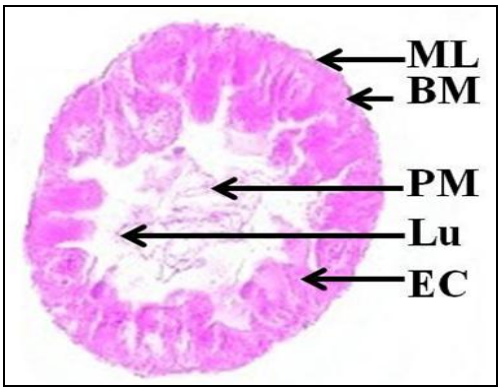

Fig 1: T.S. of midgut of 3rd instar larvae of *C. megacephala* (10x)

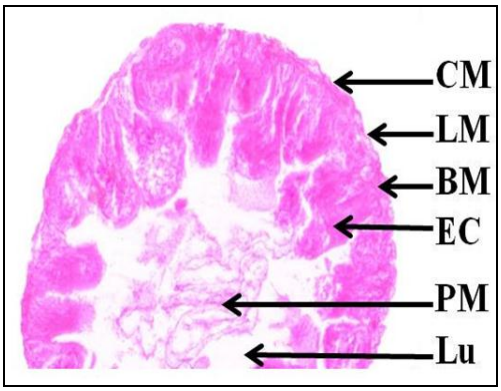

Fig 2: T.S. of midgut of 3rd instar larvae of *C. megacephala* (40x)

C. megacephala larvae exposed to sublethal doses of chlorpyrifos (Figs. 3-14)

The most characteristic effects were midgut epithelial cell vacuolization, alterations in cell size and shape, which were quite evident at all concentrations (0.02%, 0.04%, 0.2%) of chlorpyrifos. After 24 hours treatment with 0.02% concentration, few epithelial cells were seen showing vacuolization, slight increase in size and were circular in shape (Fig. 3&4). In sections of 48 hours post-treatment larvae, vacuolization was more pronounced and epithelial

cells get detached from muscular layer, have reduced cytoplasm and were distantly located from one another. Size and number of epithelial cells were decreased (Fig. 5&6). In comparison to this, under the influence of higher dose i.e. 0.04% concentration, the structural disorganization of the epithelial cells were more evident, showing cells without the characteristic morphology. Cells have shown increased distortion, vacuolization, epithelium cell contents passing into the midgut lumen. Epithelial cells were detached from basement membrane and were randomly located, decreasing the size of the gut lumen at 24 hours (Fig. 7&8). These effects became enhanced after prolonging the time period (48 hours), a rapid swelling of the epithelial cells occurred which was accompanied by the development of protrusions growing out towards the gut lumen (Fig. 9&10). As we further increase the dose to 0.2% concentration, the epithelial cells were excessively vacuolated, only epithelial linings were visible and extremely elongated covering the lumen (Fig. 11&12). These effects further showed prominence after 48 hours of treatment, epithelial cells revealed maximum degeneration, almost lost their identity, cells were devoid of cytoplasm (Fig. 13&14). Similar observations were reported by Woke [29] in the southern armyworm larva treated with lead arsenate, by Ebersold *et al*. [30] in *Pieris brassicae* induced by the 5-endotoxin of *Bacillus thuringiensis*, by Pelsue [31] in the larvae of *Chironomus decorus* Johannsen and *Tanypus grodhausi* Sublette treated with the insect growth regulators diflubenzuron and Bay SIR-8514, by Hamouda *et al.* [32] in 3rd larval instar of *Culex pipiens* treated with various fractions of *Artemisia judaica* and *Anagallis arvensis* mixed with water, by Younes *et al.* [33] in the larval midgut of *Spodoptera exigua* with action of diflubenzuron, malathion and cypermethrin, by Cavados *et al.* [28] in *Simulium pertinax* larvae treated with delta-endotoxins of *B. thuringiensis* Serovar *israelensis*, by Knaak and Fiuza [42] in *Anticarsia gemmatalis* treated with nucleopolyhedrovirus and *B. thuringiensis* Serovar *kurstaki*, by Prasad [34] in the midgut tissues of fourth instar larvae of *Helicoverpa armigera* fed with leaf extract of plant *Lantana camara*, by Al-Dhafar and Sharaby [35] in *Rhynchophorus ferrugineus* larvae treated with zinc sulfate.

Besides epithelial cells, effects in basement membrane and muscle layer were also distinctly evident. In the treatments with the insecticide, basement membrane and muscle layer still existed at all concentrations except after 48 hours of treatment with highest concentration, i.e. 0.2%. At lower concentration (0.02%), slight degeneration was noticeable in basement membrane and muscle layer at 24 hours (Fig. 3&4), but these degenerations were more pronounced after extending the time and on increasing the dose (Fig. 5-8). At 0.04% concentration, basement membrane get detached from the muscle layer after 48 hours of treatment (Fig. 9&10). At highest concentration (0.2%), basement membrane disappeared completely and muscle layer became narrower and fragile after 48 hours (Fig. 13&14). Similar findings were previously mentioned on *S. exi*gua larvae treated with diflubenzuron, malathion and Cypermethrin by Younes *et al.* [33], on camel nasal bot fly, *Cephalopina titillator* larvae treated with two insect growth regulators, pyriproxyfen and chlorfluazuron by Bassiony and Nady [36], on *Synthesiomyia nudiseta* larvae treated with the volatile oils of *Cupressus macrocarpa* and *Alpinia officinarum* by Khalaf *et al.* [37], on *R. ferrugineus* larvae treated with zinc sulfate by Al-Dhafar and Sharaby [35].

On applying insecticide, peritrophic membrane started disintegrating at lowest concentration and this degeneration was seen increasing during the treatment on increasing the dose and prolonging the time period. At 0.04% concentration, peritrophic membrane gets clumped after 48 hours (Fig. 9&10). On increasing the concentration (0.2%), clumping in the peritrophic membrane get more conspicuous after 24 hours of treatment (Fig. 11&12) and it disappeared completely after 48 hours (Fig. 13&14). Similar changes were observed in the midgut of *C. pipiens* larvae treated with oil extract of chamomile plant by Ahmad [43], in *C. pipiens* larvae treated with *A. judaica* and *A. arvensis* extracts by Hamouda *et al.* [32], in *H. armigera* larvae treated with protein toxin from *Xenorhabdus nematophilus* by Nangong *et al.* [44], in the larvae of *Mythima seperata* treated with fraxinellone by Lu *et al.* [38], in the larvae of *Heteracris littoralis* treated with natural plant essential oils by Sharaby *et al.* [45], in *R. ferrugineus* larvae treated with zinc sulfate by Al-Dhafar and Sharaby [35].

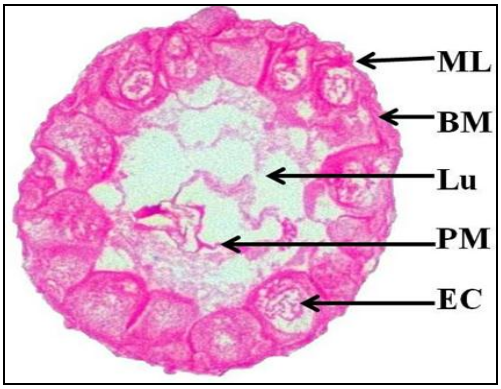

Fig 3: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 24 hours exposure of 0.02% concentration of chlorpyrifos (10X)

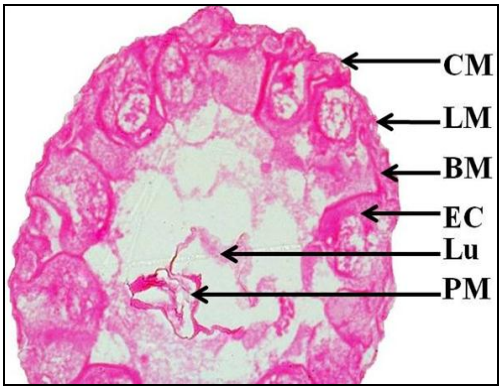

Fig 4: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 24 hours exposure of 0.02% concentration of chlorpyrifos (40X)

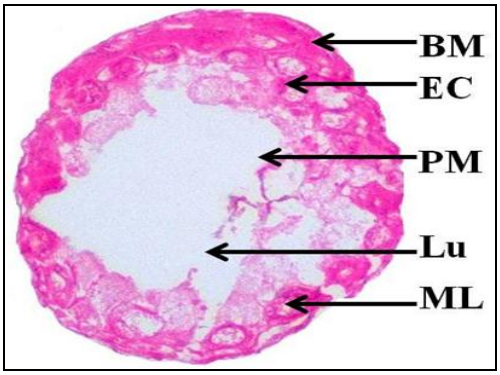

Fig 5: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 48 hours exposure of 0.02% concentration of chlorpyrifos (0X)

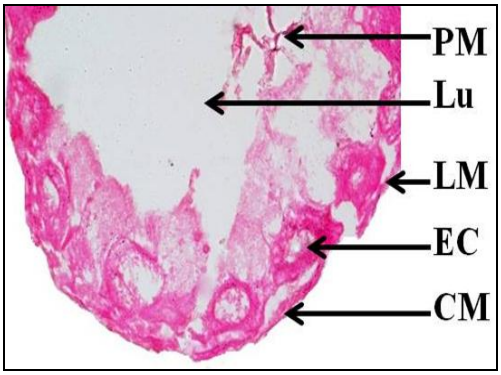

Fig 6: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 48 hours exposure of 0.02% concentration of chlorpyrifos (40X)

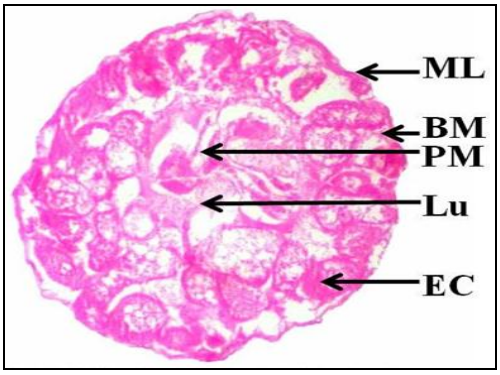

Fig 7: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 24 hours exposure of 0.04% concentration of chlorpyrifos (10X)

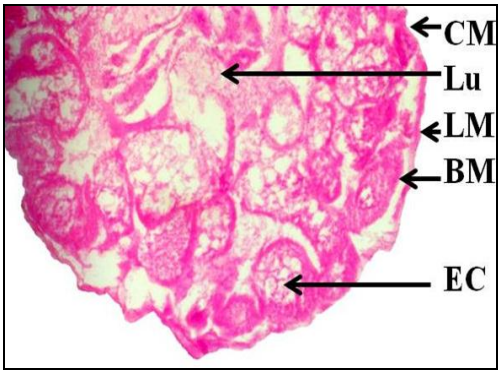

Fig 8: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 24 hours exposure of 0.04% concentration of chlorpyrifos (40X)

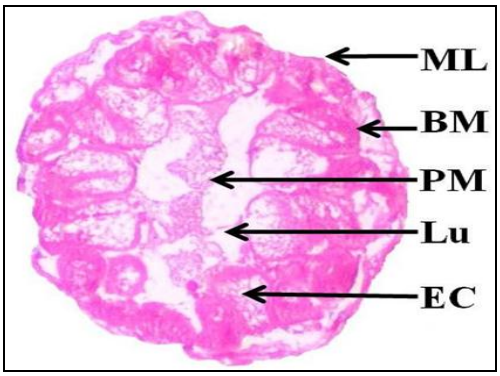

Fig 9: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 48 hours exposure of 0.04% concentration of chlorpyrifos (10X)

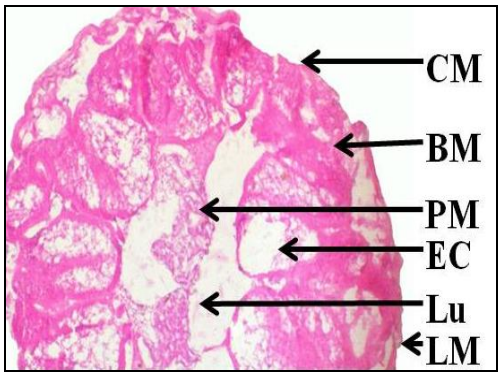

Fig 10: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 48 hours exposure of 0.04% concentration of chlorpyrifos (40X)

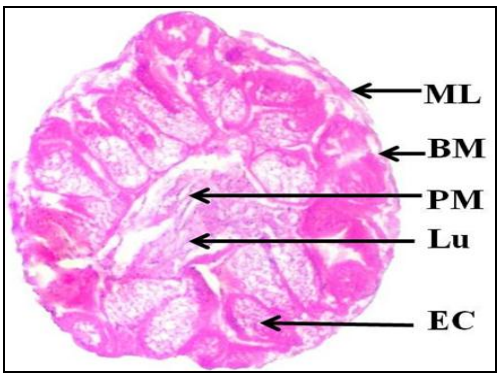

Fig 11: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 24 hours exposure of 0.02% concentration of chlorpyrifos (10X)

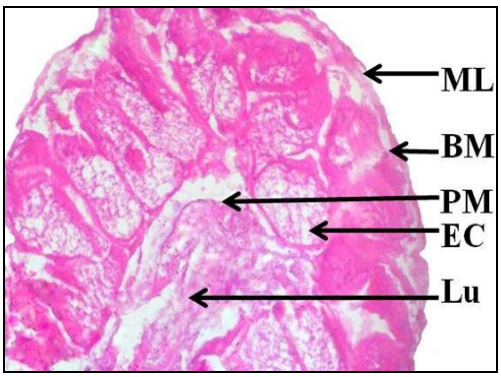

Fig 12: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 24 hours exposure of 0.02% concentration of chlorpyrifos (40X)

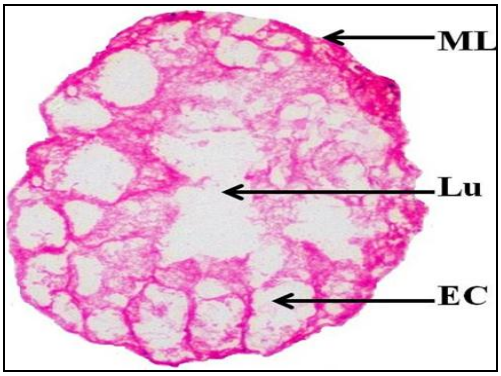

Fig 13: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 48 hours exposure of 0.2% concentration of chlorpyrifos (10X)

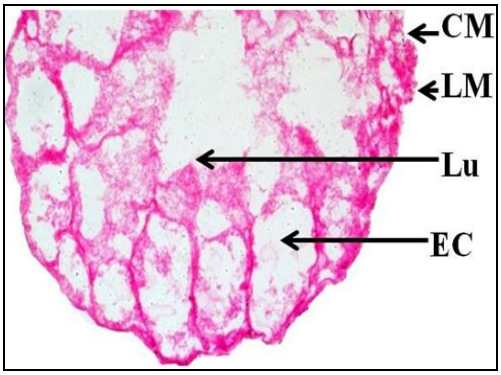

Fig 14: T.S. of midgut of 3rd instar larvae of *C. megacephala* after 48 hours exposure of 0.2% concentration of chlorpyrifos (40X)

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