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Ultrastructural alterations induced by fenitrothion on fat body cells and midgut cells of *Tenebrio molitor* L. (Insecta, Coleoptera) larvae

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Exposure of *Tenebrio molitor* larvae to the insecticide fenitrothion affected their growth, moulting and survival and caused alterations in the ultrastructure of the fat body cells and midgut cells. The insecticidal stress altered the appearance of the biological membranes. Even though the mortality was low, the nuclei, ground plasm, endoplasmic reticulum and mitochondria revealed numerous prominent malformations: invaginations and swelling of the nuclear envelope, high ratio of condensed chromatin, swollen endoplasmic reticulum elements, heterogeneous fatty acid storing vesicles and fewer cristae in the mitochondria of the fat body cells.

Key words: *Tenebrio molitor*, fat body, midgut, ultrastructure, insecticide.

INTRODUCTION

Agrochemicals, and particularly insecticides, in addition to the intended effect, may have many side effects, such as intracellular malformations (Mehlhorn *et al*., 1999; Matsuda *et al*., 2001; Mehlhorn *et al*., 2001) or impaired reproduction and reduced productivity (Mathew *et al*., 1992; Debnath & Mandal, 2000; Fila *et al*., 2002; Miyo & Oguma, 2002). These effects may lead to abnormalities at the levels of populations or communities. Insects have developed various mechanisms of resistance, including behavioural, physiological and biochemical ones. Larval, pupal and imaginal stages may differ in resistance (Medina *et al*., 2001; Bouvier *et al*., 2002). Accurate and precise knowledge of these processes is helpful for a proper application of insecticides, resulting in a lower contamination of the environment and protection of the non-target animals.

The biological action of insecticides greatly depends on concentration. When a low dose of a chemical is used, effects are observed that are not easy to notice compared with a higher dose which may result in acute tissue toxification. Hence, we checked the effect of a low concentration of a popular insecticide – fenitrothion – on the growth, development and ultrastructure of *T. molitor* larvae. We chose a low concentration, causing 5% mortality within the first four days of exposure in order to avoid massive lethal effects.

Ultrastructural changes may explain some of the effects visible to the naked eye. We chose two organs: the fat body and the midgut. Fat body, because it is responsible for tissue detoxification (organophosphorous insecticides are highly soluble in fats). Gut cells, because they are the first ones which receive insecticides and thus are "a gate" for these chemicals. We decided to test a long-term exposure to fenitrothion in order to monitor the effects of accumulation of the insecticide, which may lead to serious malfunctions and malformations, even when the applied concentration is low.

MATERIALS AND METHODS

Insect

The experiments were carried out on *Tenebrio molitor* L. (Insecta: Coleoptera) larvae. Insects were ob-

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tained from a colony, permanently maintained at the Department of Animal Physiology and Developmental Biology, UAM, Poznań. The conditions of maintenance of the colony were described by Rosiński *et al*. (1979). The imagoes were fed on vegetables (carrot, lettuce) and the eggs are collected every two days. Developing larvae are kept on cornflakes, with the addition of vegetables as a meal. Pupae are separated and transferred onto flour, where they finish their development.

In our experiments, larvae developed from eggs which were laid during the same day. The larvae were subjected to a semi-synthetic diet, prepared according to David *et al*. (1975). They were maintained in chambers at 26°C with a 16:8 h (L:D) photoperiod.

Chemicals

Fenitrothion (technical grade samples, 99% purity) was purchased from Promochem, Warsaw, Poland. All other chemicals were of analytical quality and purchased from commercial suppliers.

Calculation of lethal concentrations

Concentrations of the insecticide were established during a series of preliminary experiments. Larvae weighing 80 ± 5 mg (*T. molitor* does not have a permanent number of instars and duration of larvae development may vary significantly), were divided into six groups of 30 individuals. Each insect, kept separately in a phial, received a portion of a nutrient in the form of a pill cut off from the nourishment (0.032 ± 0.007 g) with 10 µl of the insecticide in solution (50% ethanol). The nutrient was made on agar which was cut into fine ribbons. Small discs were then obtained with a cork borer out of the ribbons, soaked with fenitrothion, left for an hour to let the alcohol evaporate and presented to the insects. Each of the six groups differed in the concentration of fenitrothion added to the nutrient. Larvae were exposed to the insecticide for four days. A shorter period might result in starvation as a behavioural method of avoiding stress. Besides, the ultrastructural response can be seen more clearly after a few days. After four days, the number of dead larvae was scored. After four repetitions of the experiment, with gradually narrower ranges of concentrations of fenitrothion, the mortality-concentration curves and the LC_{50} values were determined using Probit analyses (Finney, 1971):

Probit of lethality (logY) = logA + B × logX (log of concentration)

Values of LC_5 were determined using the same citation. Control groups received nutrient with addition of ethanol. Experiments were repeated 3 times.

Exposure to LC_5

When new larvae reached the same weight of 80 ± 5 mg, their population was divided into four groups of 40 insects each. They were reared on the same nutrient with addition of a calculated concentration of fenitrothion. After six and twelve days of exposure (i.e. on the seventh and thirteenth day of the experiment, respectively) some individuals were prepared for Transmission Electron Microscopy (TEM) observation.

TEM preparation

Samples were fixed with 2% glutaraldehyde in 0.175 M cacodylate buffer, postfixed with 1% osmium tetroxide, dehydrated and finally embedded in LR White soft resin. Ultrathin sections were obtained using a Leica ultramicrotome, stained with uranyl acetate and lead citrate and observed under a JEOL 1200EX II JEM transmission electron microscope.

Statistical analyses and randomness of samples

There were four replications for each treatment. The larvae were weighed every day and the number of insects, which underwent molting, was recorded. On the seventh and thirteenth day of the experiment, six to eight individuals, chosen at random (but not prior to, during or just after molting), were used for ultrastructural examination. Numbers of molting and dead animals were determined. The means ± SD were calculated for each day of exposure. Results were analyzed by Student's t test and p values at 0.05 were considered statistically significant.

RESULTS

Calculated concentrations

The LC_5 concentration of fenitrothion for the examined population was 0.35×10^{-5} mg per insect per day. Concentrations of the insecticide, causing mortality are shown in Table 1.

Survival during the exposure

Survival of insects is shown in Fig. 1. After few days of relatively low mortality, a rapid loss of surviving fraction was observed in the exposed group. Mean-

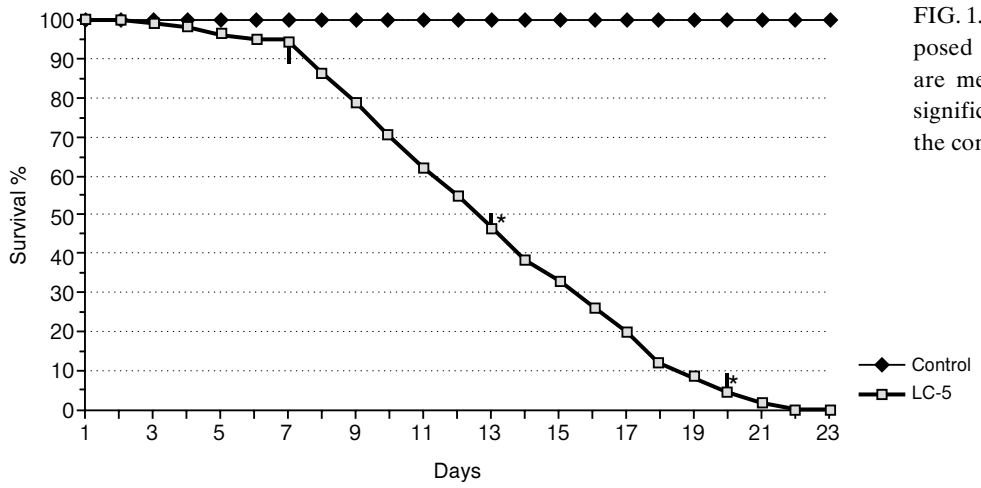

FIG. 1. Survival of animals exposed to fenitrothion. Data are means ± SD. * = values significantly different from the control, $p < 0.05$.

TABLE 1. Values of fenitrothion lethal concentrations for different percentages of the tested population. Data are means of the three experiments

LC	Concentration of fenitrothion (mg per insect per day)
5	0.350×10^{-5}
10	0.451×10^{-2}
30	0.765×10^{-2}
50	0.110×10^{-1}
95	0.346×10^{-1}

while, the control population remained stable, with no mortality at all.

Molting

A substantial inhibition of molting was observed when larvae were exposed to LC_5 of fenitrothion (Table 2).

Body mass

Larval weight was substantially decreased after exposure to the insecticide. Two days were enough to observe a decrease of the body mass in the group exposed to the insecticide. A continuous loss of body mass was observed in the insects which were fed with the addition of insecticide, whereas for the whole duration of the experiment control animals weighed steadily in the range 70-85 mg. After ten days, the body mass of the insects in the groups was approximately 50% lower than that of the non-treated animals (Fig. 2).

TABLE 2. Cumulated number of molting observed during the experiment. Data are means ± SD of the three experiments

After *n* days	LC_5	Control
1	3.7 ± 0.6	0.7 ± 0.6
3	8.1 ± 0.0	3.4 ± 0.6
6	8.8 ± 0.6	4.0 ± 1.00
8	8.8 ± 0.6	8.4 ± 1.7
10	8.8 ± 0.6	12.7 ± 1.1
13	8.8 ± 0.6	17.3 ± 0.6
15	8.8 ± 0.6	19.0 ± 1.7
17	8.8 ± 0.6	23.7 ± 2.1
20	8.8 ± 0.6	28.4 ± 2.5

Ultrastructure

From the first days of exposure prominent changes in the ultrastructure of the fat body were observed. Mitochondria of the exposed fat body cells were swollen and had fewer cristae than those of the control cells (compare Figs 3 and 5). The nucleus had lost its oval shape, the nuclear envelope was folded and the patches of dense chromatin became bigger (Figs 4, 5). Dense vesicles and myelin structures were also present. These changes became greater as the time of exposure became longer. Changes in the electron density of fatty granules stored in the fat body cells were further observed. Mitochondria were often grouped together, with rough endoplasmic reticulum elements penetrating the cytoplasm among them.

Midgut cells of the unexposed insects revealed

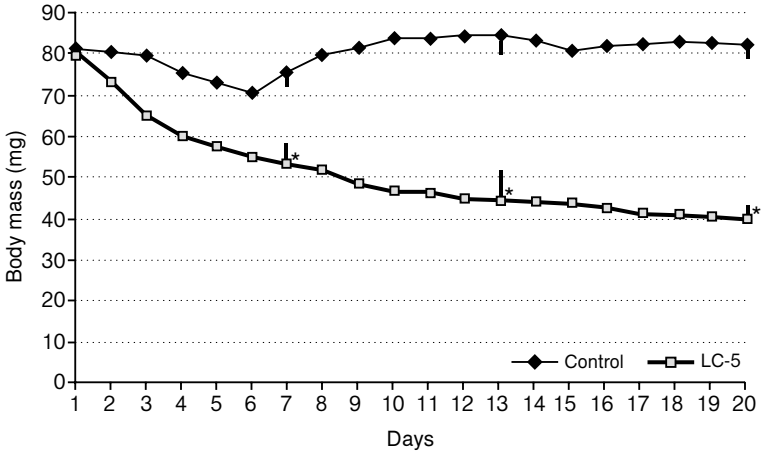

FIG. 2. Changes of body mass of animals exposed to fenitrothion. Data are means ± SD. * = values significantly different from the control, $p < 0.05$.

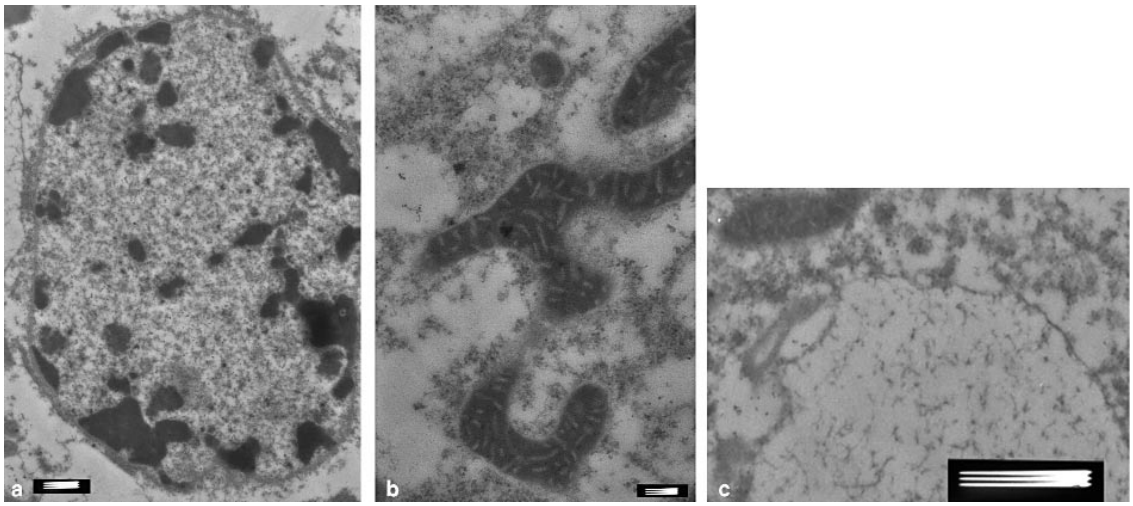

FIG. 3. Fat body cells, controls. Nuclei are oval, with condensed chromatin grouped in small patches (a, scale bar = 1 μm). Variously-shaped mitochondria are electron dense (b, scale bar = 200 nm). Fat granules are electron lucent but not homogenous (c, scale bar = 500 nm).

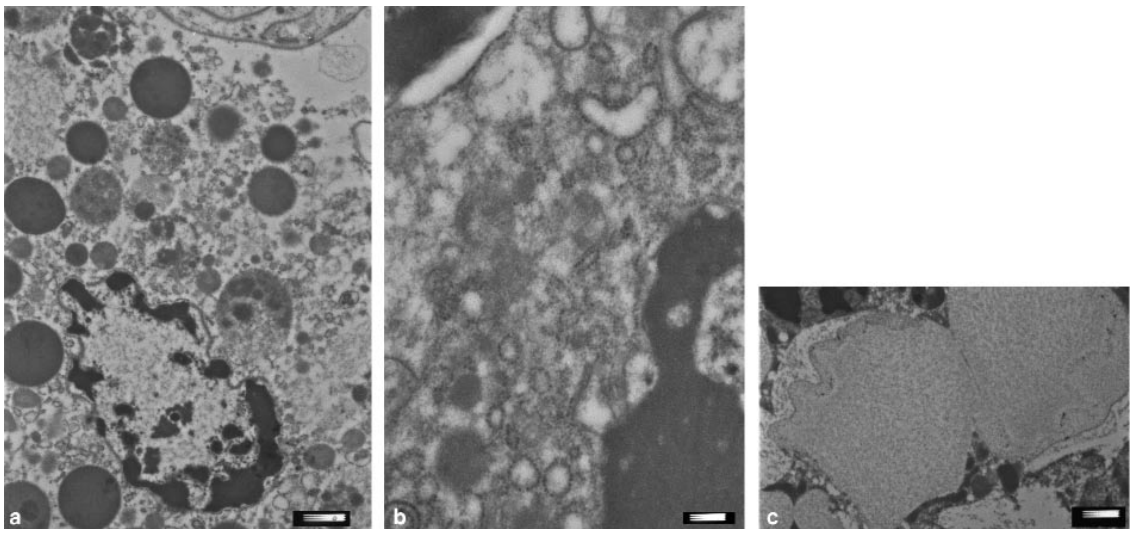

FIG. 4. Fat body cells after 6 days of exposure to fenitrothion. Note the invaginations of nuclear envelope, the high ratio of condensed chromatin (a, scale bar = 1 μm), the swollen endoplasmic reticulum elements (b, scale bar = 200 nm) and the alterations in electron density of fatty acid storing vesicles (c, scale bar = 1 μm).

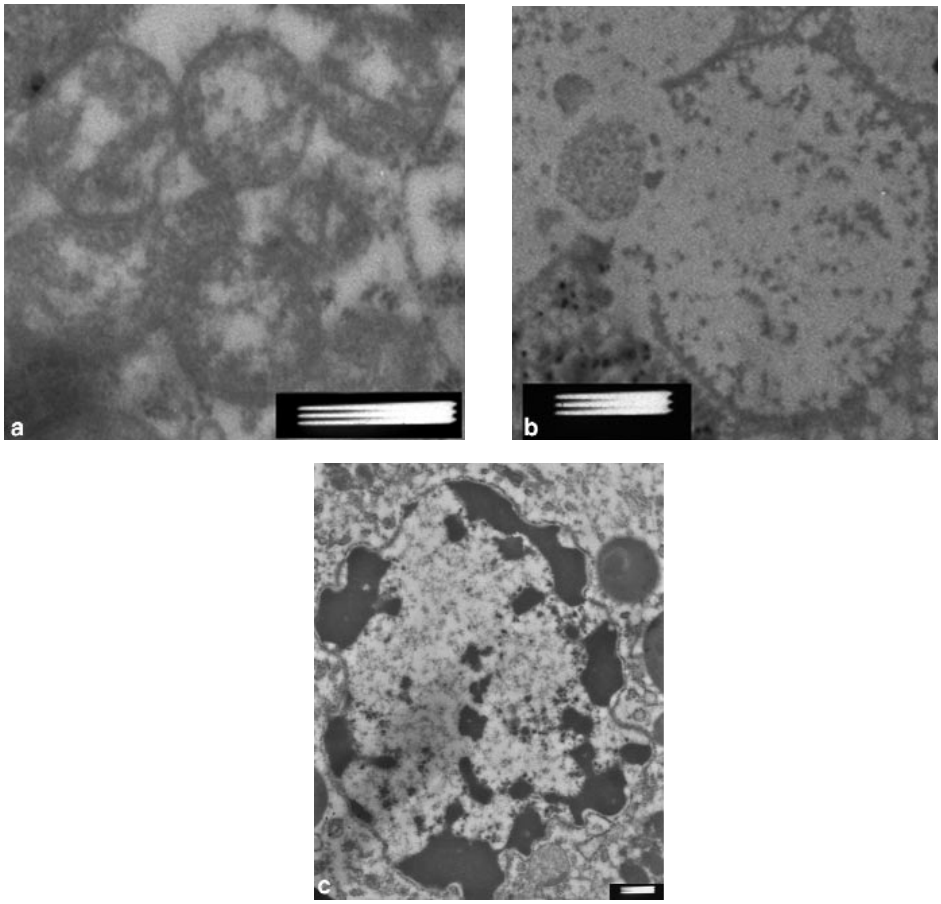

FIG. 5. Fat body cells after 12 days of exposure to fenitrothion. Note the electron lucent mitochondria with few cristae only (a, scale bar = 500 nm) and the changes in electron density and shape of fatty granules (b, scale bar = 200 nm). The nuclear envelope is swollen and invaginations appear (c, scale bar = 500 nm).

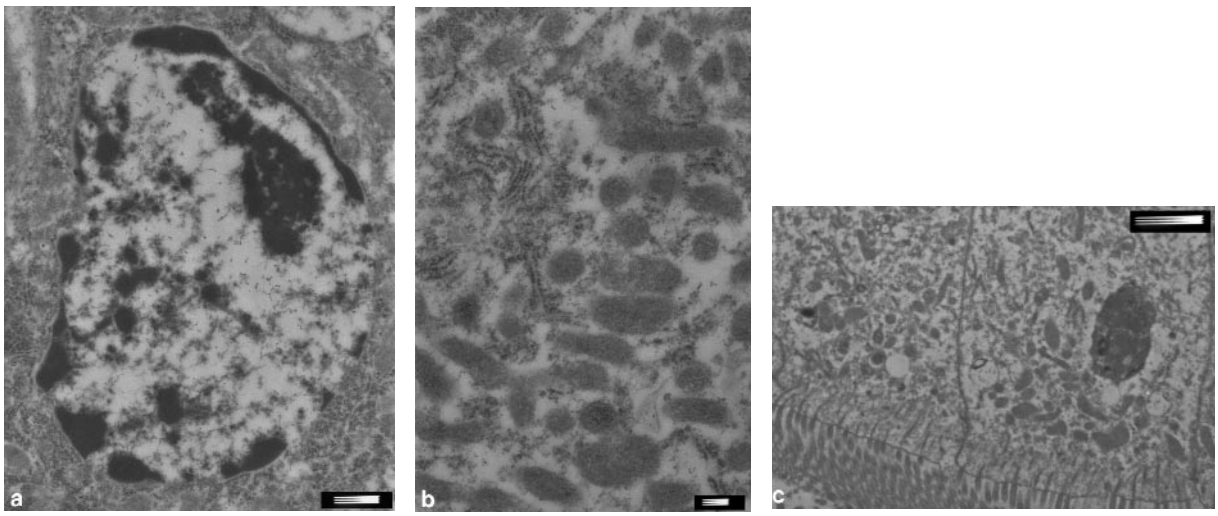

FIG. 6. Midgut cells, controls. Nuclei are oval, with a rim of heterochromatin attached to the nuclear envelope and some clusters within the nucleoplasm (a, scale bar = 500 nm). Electron dense mitochondria (b, scale bar = 200 nm) are grouped closely to microvilli (c, scale bar = 2 μm). Rough endoplasmic reticulum forms cytoplasm-penetrating ribbons (b).

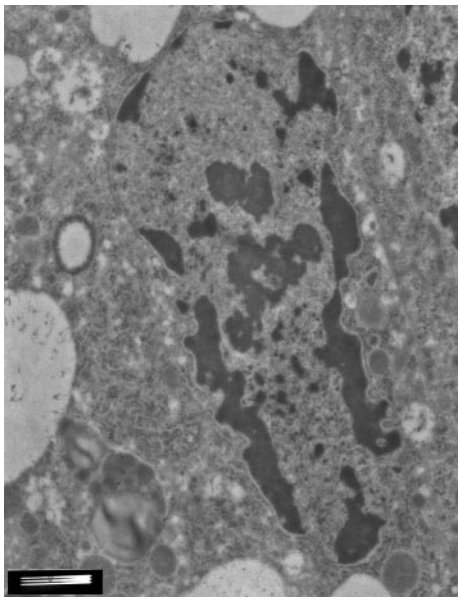

FIG. 7. A midgut cell after 6 days of exposure to fenitrothion. Large patches of condensed chromatin are located closely to the nuclear envelope. Scale bar = 1 μm.

oval nuclei, with a relatively narrow rim of heterochromatin attached to nuclear envelope (Fig. 6). Mitochondria were electron dense and grouped close to microvilli. After six days of exposure, much larger patches of condensed chromatin were observed (Fig. 7) and in the next six days, invaginations of the nuclear envelope were visible, with the perinuclear space being swollen (Fig. 8).

DISCUSSION

Insects are characterised by a broad variety of physiological and biochemical adaptations to their environment (Koval, 1996). Previously, it has been shown that *Spodoptera exigua* (Lepidoptera) develops a resistance to fenitrothion during a long-term exposure to it, whereas *Tenebrio molitor* (Coleoptera) individuals do not (Adamski *et al*., 2003). In the present report, we showed that even a low concentration of the same insecticide, causing only 5% mortality during an exposure of four days, leads to high mortality of the exposed population, if exposure is continuous. Toxicity also caused developmental changes. The process of moulting was stopped. The most probable explanation is that insects had "focused" on detoxification in an effort to minimize the harmful effects of the insecticide. Inhibited moulting may also be the result of disturbed endocrine metabolism. Although there are no numerous data concerning this point, we know that fenitrothion may inhibit antioxidant enzymes and induce oxidative stress in insects (Adamski *et al*., 2003), amphibians (Štajn & Žikic, 1988) and rodents (Naqui & Hasan, 1992). Organophosphorous insecticides may decrease the cytochrome P-450 content (Clos *et al*., 1994) and alter estradiol metabolism by inhibiting certain P-450 isozymes (Berger & Sultatos, 1997).

Organophosphates may inhibit the activity of

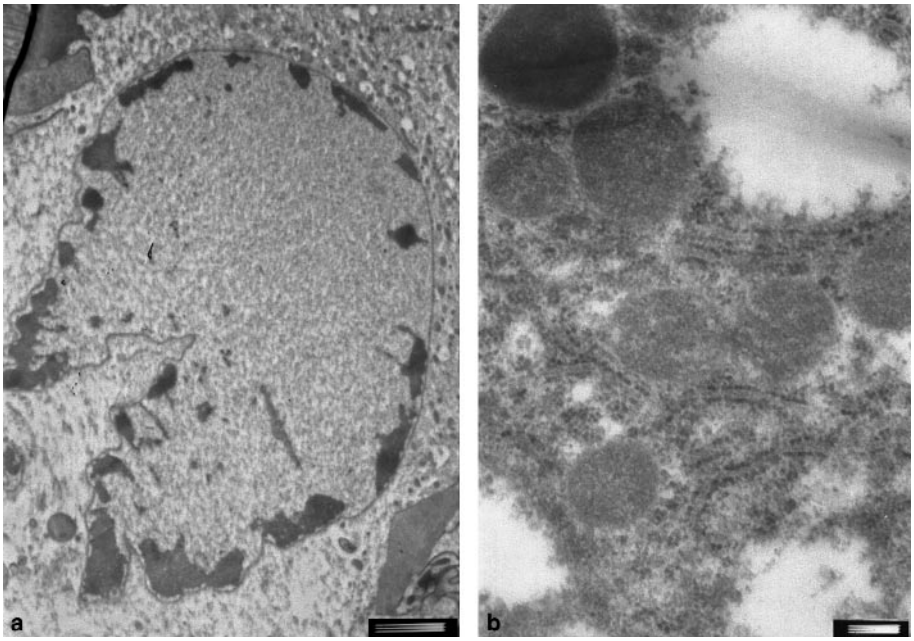

FIG. 8. Midgut cells after 12 days of exposure to fenitrothion. The nuclear envelope is invaginated and swollen (a, scale bar = 2 nm) and mitochondria have an electron dense matrix (b, scale bar = 200 nm).

Na^+/K^+-ATPase (Sancho *et al*., 1997), impair cellular respiration, thus leading to an enhanced level of oxygen free radicals. Then, free radicals react with lipids, increase their peroxidation (Naqui & Hasan, 1992) and change the saturated/unsaturated fatty acid ratio (Domenech *et al*., 1977; Antunes-Madeira & Madeira, 1979). These alterations are likely caused by fenitrothion, too. As an organophosphorous insecticide, fenitrothion is highly soluble in fats. Together with the fact that lipids are the basis for the synthesis of many hormones, it can be concluded, that insecticides may alter the developmental pattern by impairing the synthesis of important hormones in insects. Hence, delayed moulting was observed. Fenitrothion further resulted in malformations of the fat granules within the fat body cells.

There is a constantly growing interest in neonicotinoid insecticides. These substances act on the postsynaptic nicotinic acetylcholine receptors located on the postsynaptic membrane. They have the same physiological effect, i.e. hyperexcitability of the postsynaptic cell (Matsuda *et al*., 2001; Mehlhorn *et al*., 2001). The most prominent changes caused by imidacloprid (neonicotinoid) on *Ctenocephalides felis* fleas are: damage of mitochondria in the nerve and muscle cells, nuclear disintegration, swelling of the perinuclear space and vacuolization within the neurons (Mehlhorn *et al*., 1999). We observed similar features in the fat body cells exposed to fenitrothion. Some of these changes are characteristic for apoptosis, e.g. nuclear fragmentation, chromatin condensation and plasma membrane blebbing (Kerr *et al*., 1972; see also Sarafian & Bredesen, 1994). Myelin structures and dense vesicles, possibly secondary lysosomes and residual bodies, within the cytoplasm may result from the previous process. Hence, at least to some extent, the reported alterations must be of neurological origin, and result from hyperexcitability within the synapses.

The observation of swollen mitochondria is in tune with inhibition of Na^+/K^+-ATPase (Sancho *et al*., 1997), resulting in disturbed respiratory metabolism as well as altered ionic and osmotic conditions within and around these organelles. Our preliminary data (unpublished) on cell cultures revealed ultrastructural changes that resemble those observed in this study. Hence, organelle malformations are likely the result of a direct action of insecticides on the tissue and of an unsatisfactory detoxification process within the exposed cells.

Midgut cells, exposed to fenitrothion, also revealed a similar disintegration of organelles, with the exception of mitochondria, which did not show any structural difference compared with the controls. Presumably, the insecticide passes through the gut cells to the hemolymph and then to the fat body cells, where it resides for a long time displaying its full effect. However, the effects on nucleus are easily visible. Hence, these changes are likely to be of a more general origin – they were caused by the chemical itself, not by the nervous hyperstimulation. On the contrary, the changes in mitochondrial ultrastructure are most likely the result of the activity of fenitrothion within the synapses stimulating the fat body cells. The reported results suggest that even low concentrations of fenitrothion have a complex and broad influence on *T. molitor*.

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REFERENCES

Adamski Z, Ziemnicki K, Fila K, Žikić R, Štajn A, 2003. Effects of long-term exposure to fenitrothion on *Spodoptera exigua* and *Tenebrio molitor* larval development and antioxidant enzyme activity. *Biological letters*, 40: 43-52.

Antunes-Madeira MC, Madeira VR, 1979. Interaction of insecticides with lipid membranes. *Biochimica et biophysica acta*, 550: 384-392.

Berger CW Jr, Sultatos LG, 1997. The effects of the phosphorothioate insecticide fenitrothion on mammalian cytochrome P450-dependent metabolism of estradiol. *Fundamental and applied toxicology*, 37: 150-157.

Bouvier JC, Boivin T, Beslay D, Sauphanor B, 2002. Age-dependent response to insecticides and enzymatic variation in susceptible and resistant codling moth larvae. *Archives of insect biochemistry and physiology*, 51: 55-66.

Clos MV, Ramoneda M, Garcia G, 1994. Modification of testicular cytochrome P-450 after fenitrothion administration. *General pharmacology*, 25: 499-503.

David WAL, Ellaby S, Taylor G, 1975. Rearing *Spodoptera exempta* on semi-synthetic diets and on growing

maize. *Entomologia experimentalis et applicata*, 19: 226-236.

Debnath D, Mandal TK, 2000. Study of quainalphos (an environmental oestrogenic insecticide) formulation (Ekalux 25 E. C.) – induced damage of the testicular tissues and antioxidant defence system in Sprague-Dawley albino rats. *Journal of applied toxicology*, 20: 197-204.

Domenech CE, Machado de Domenech EE, Balegno HF, 1977. Pesticide action and membrane fluidity. *FEBS letters*, 74: 243-246.

Fila K, Adamski Z, Ziemnicki K, 2002. Exposure to fenitrothion causes malfunctions of *Spodoptera exigua* Hübn. (Lep., Noctuidae) eggs. *Journal of applied entomology*, 126: 114-118.

Finney DJ, 1971. *Probit analysis*. Cambridge University Press, London/New York.

Kerr J, Wyllie A, Currie A, 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissues kinetics. *Brain journal of cancer*, 26: 239-257.

Koval TM, 1996. Moths: myths and mysteries of stress resistance. *BioEssays*, 18: 149-156.

Mathew G, Vijayalaxmi KK, Rahiman MA, 1992. Methyl parathion-induced sperm shape abnormalities in mouse. *Mutation research journals*, 280: 169-173.

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB, 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in pharmacological sciences*, 22: 573-580.

Medina P, Bidia F, Tirry L, Smagghe G, Viñuela E, 2001. Compatibility of spinosad, tebufenozide and azadirachtin with eggs and pupae of the predator *Chrysoperla carnea* (Stephens) under laboratory conditions. *Biocontrol science and technology*, 11: 597-610.

Mehlhorn H, Mencke N, Hansen O, 1999. Effects of imidacloprid on adult and larval stages of the flea *Ctenocephalides felis* after *in vivo* and *in vitro* application: a light- and electron-microscopy study. *Parasitology research*, 85: 625-637.

Mehlhorn H, Hansen O, Mencke N, 2001. Comparative study on the effects of three insecticides (fipronil, imidacloprid, selamectin) on developmental stages of the cat flea (*Ctenocephalides felis* Bouche 1835): a light and electron microscopic analysis of *in vivo* and *in vitro* experiments. *Parasitology research*, 87: 198-207.

Miyo T, Oguma Y, 2002. Negative correlations between resistance to three organophosphate insecticides and productivity within a natural population of *Drosophila melanogaster* (Diptera: Drosophilidae). *Journal of economical entomology*, 95: 1229-1238.

Naqui SM, Hasan M, 1992. Acetylhomocysteine thiolactonate protection against phosphamidon-induced alteration of regional superoxide dismutase activity in the central nervous system and its correlation with altered lipid peroxidation. *Indian journal of experimental biology*, 30: 850-852.

Rosiński G, Wrzeszcz A, Obuchowicz L, 1979. Differences in trechalase activity in the intestine of fed and starved larvae of *Tenebrio molitor* L. *Insect biochemistry*, 9: 485-488.

Sancho E, Ferrando MD, Andreau E, 1997. Inhibition of gill Na^+/K^+-ATPase activity in the eel, *Anguilla anguilla*. *Ecotoxicology and environmental safety*, 38: 132-136.

Sarafian T, Bredesen D, 1994. Is apoptosis mediated by reactive oxygen species? *Free radicals research*, 21: 1-8.

Štajn A, Žikic R, 1988. The influence of insecticide fenitrothion on the concentration of ascorbic acid and on the activity of SOD and catalase in tissues of the frog *Rana esculenta* L. *Jugoslavica physiologica et pharmacologica acta*, 24: 453-454.