

Ultrastructural alteration of larvae and puparia of blow fly *Chrysomya megacephala* (F.) (Diptera: Calliphoridae) and house fly *Musca domestica* L. (Diptera: Muscidae) exposed to neem extract

Sirisuda Siriwattananurungsee¹, Kabkaew L. Sukontason¹, Budsabong Kuntalue² and Kom Sukontason¹

¹Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.

²Electron Microscopy Research and Service Center (EMRSC), Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.

Corresponding author E-mail: ksukonta@mail.med.cmu.ac.th

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Abstract. The effect of neem, *Azadirachta indica* A. Juss, on ultrastructural alteration of larvae and puparia of the blow fly, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and house fly, *Musca domestica* (Linnaeus) (Diptera: Muscidae), was investigated in the laboratory by using neem extract containing 0.24% azadirachtin A. Larvae of both species exposed to single dipping with the neem product exhibited swelling of the integument in relation to the control as determined by scanning electron microscopy (SEM). Transmission electron microscopy (TEM) analysis revealed slight thickness of epicuticle, but intense thickness of procuticle. Multiple treatment of the larvae displayed noticeably swelling integument and bleb formation on the integument, indicating a dose-dependent relationship. Puparia of both fly species treated with neem product showed similar appearance under SEM and TEM analyses.

INTRODUCTION

The blowfly, *Chrysomya megacephala* (F), and housefly, *Musca domestica* L., are the two most prevalent fly pests in Thailand, occupying ≈90% of all specimens collected in several areas of the country. Adults have been implicated as mechanical carriers of numerous pathogens (viruses, bacteria, parasites) to humans that may lead to disease (Greenberg, 1971; Monzon *et al.*, 1991; Sulaiman *et al.*, 2000; Sukontason *et al.*, 2007). In Thailand, Sukontason *et al.* (2007) lists 42 bacterial species harbored by these flies. The larvae (maggots) of these flies also cause myiasis in humans and animals (Zumpt, 1965; Kumarasinghe *et al.*, 2000; Sehgal *et al.*, 2002).

Several methods are being employed for the control of blowfly and housefly populations, including sanitation management and chemical insecticides. Although

chemical insecticides are the most common and effective control strategy, continuous application could lead to resistance and contamination of the environment. The use of phytochemicals derived from plant resources is an alternative to synthetic insecticides. The phytochemicals, bio-pesticide derived from the neem tree, *Azadirachta indica* A. Juss (Meliaceae) have been demonstrated to have insecticidal properties (Schmutterer, 1990), such as reduction in fecundity and post-embryonic development (Singh, 2003), reduction of adult longevity (Steffens & Schmutterer, 1982), antifeedancy (Su & Mulla, 1998), larvicidal properties (Miller & Chamberlain, 1989) and repellency (Sharma *et al.*, 1993). Our study aimed to investigate the efficacy of neem against *C. megacephala* and *M. domestica* by monitoring the morphological changes of larvae and puparia after direct contact with neem extract using scanning

electron microscopy (SEM) and transmission electron microscope (TEM).

MATERIALS AND METHODS

Fly colony

Chrysomya megacephala and *M. domestica* used in this study were obtained from a laboratory colony maintained for about 3 years in the fly rearing room at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand. Laboratory colonies were maintained at an ambient temperature of 24-28°C with a light/dark photoperiod of ≈12:12 hours. Larvae were fed a fresh pork liver diet. Adults were reared on two kinds of food: (1) a mixture of 10% (w/v) sugar solution and multivitamin syrup solution and (2) fresh pork liver (used as both a food source and oviposition site). The detailed procedures of rearing has been described previously (Sukontason *et al.*, 2004a).

Neem extract

The commercial neem or Sadao Thai 111 (THAI NEEM PRODUCTS CO., LTD, Thailand) was obtained from the neem seed, *Azadirachta indica* var. *siamensis* and extracted with ethanol. This neem extract was investigated for the content of azadirachtins using High Pressure Liquid Chromatography (HPLC) by the Department of Agriculture, Ministry of Agriculture and Cooperatives, with 0.24% azadirachtin A being found. For administration, the neem extract was diluted in distilled water at the final volume of 0.2% concentration of neem extract solution. The assessment of neem toxicity against *C. megacephala* and *M. domestica* was performed using the dipping bioassay described by Mitchell *et al.* (2004).

Bioassay for SEM and TEM

Larvae and puparia of *C. megacephala* were collected randomly from the maintenance boxes. These specimens were divided into 3 groups per stage; 30 specimens in each group. The third larval instars were dipped once on the first day (single dose) and every day until the beginning of pupariation

(multiple doses). For the puparia, dipping was carried out once on 3-day-old pupae puparia (single dose) and every day of pupariation until emergence (multiple doses). When dipping, the specimens were wrapped with voile cloth, and dipped for exactly 1 second into the 0.2% diluted commercial neem extract previously described. Treated larvae were transferred into a rearing box containing fresh pork liver. After 24 hours, they were collected from the rearing box and processed for the SEM and TEM. Control specimens were dipped in distilled water. When multiple dipping, the neem extract was changed daily.

Regarding *M. domestica*, the larvae and puparia were also examined by SEM and TEM analyses, with the methodology being the same as previously described in *C. megacephala*.

For SEM procedure, some larvae and puparia of *C. megacephala* and *M. domestica* from both the control and treated groups were randomly selected at 24 hours after exposure in each treatment. These specimens were killed in hot water (≈90°C) for 3 minutes and then attached by double-stick tape to aluminum stubs. The larvae were dipped with liquid nitrogen for 15-20 seconds. They were viewed with a JEOL JSM-5910LV scanning electron microscope (Japan). The puparia were coated with gold (Au) in the sputter-coating apparatus (SPI Supplies Division of Structure Probe, Inc., USA) before being viewed with SEM.

The procedure for TEM was performed after specimens were killed in hot water (≈90°C) for 3 minutes. The dead larvae were then prefixed with a 2.5% glutaraldehyde mixture in phosphate-buffered saline (PBS), pH 7.4 at 4°C for 24 hours. In addition, larvae were rinsed twice with PBS at 10-minute intervals, and postfixed with 1% osmium tetroxide at room temperature for 3-4 days. Specimens were then rinsed twice with PBS and dehydrated with alcohol. The dehydration process involved the larvae being sequentially subjected to the following increased alcohol concentrations: 30%, 50%, 70%, 80% and 90%. Larvae remained in each concentration of alcohol for 12 hours during each step of the dehydration process. After

treatment in the alcohol concentrations, they were placed in absolute alcohol for a further two 12-hour periods. After which they were placed in acetone for 2 hours before being transferred to a ratio of resin:acetone of 1:3 for 24 hours, 1:1 for 24 hours and subsequently 3:1 for 24 hours. Finally they were submerged twice in a bath containing resin for 3 hours. Specimens were embedded in Spurr's resin by placing them into a plastic block, and incubating them at 70°C for 24 hours. A thick section (0.5 μ m) of the larvae or pupae was cut with a glass knife on an Ultramicrotome (Becthai, USA). Ultrathin sections (90 nm) were prepared from these re-embedded blocks, with serial sections collected from copper slot grids. Sections were post-stained with uranyl acetate and lead citrate before examination by a ZEISS EM-10A transmission electron microscope (ZEISS: West Germany).

RESULTS

The surface topography of *C. megacephala* larva as studied by SEM has been described in great detail in a previous study (Sukontason *et al.*, 2003). In brief, the third instar is muscoid-shaped and composed of 12 segments. The cephalic segment possesses a pair of dorsal organs, terminal organs, ventral organs and a pair of mouthhooks. The prothoracic anterior spiracles contain 10-13 papillae in a single row. The posterior spiracular discs are located in a shallow cavity. The integument of larva is smooth, except the intersegmental spines. As for *M. domestica*, the morphology of the third instar is similar to that of *C. megacephala*, except the anterior spiracle contains 5-7 papillae.

For puparia, the surface topography of *C. megacephala* and *M. domestica* have been previously described (Siriwattananarungsee *et al.*, 2005). The puparia of these flies were typically cylindrical-shaped and coarctate. Immediate after molting, the puparium is creamy white, but it steadily darkens until the mature puparium is mahogany brown. The surface between the intersegmental spines of the puparium was smooth. By

observing under TEM, the arrangement of integumental layers is regular in pattern. The cross-sections of intersegmental spines displayed a normal appearance.

Untreated *C. megacephala* and *M. domestica* larvae displayed a normal appearance with a smooth integument and well-shaped intersegmental spines (Figures 1A and 2A). The surface ultrastructure remained unchanged as previously described. Investigation by TEM revealed that the average thickness of the epicuticles of *C. megacephala* and *M. domestica* were 1.95 μ m (range 1.50-2.60; Figure 1D) and 1.20 μ m (range 1.00-1.40; Figure 2D), respectively; whereas procuticles (composed of exocuticle and endocuticle) were 23.83 μ m (range 22.25-26.75) and 27.50 μ m (range 26.00-29.00), respectively (Table 1). Larvae of both *C. megacephala* (Figure 1B) and *M. domestica* dipped singly in the neem extract revealed limited damage expressed by a slightly swollen integument (Figure 2B). Ultrathin section showed slight thickness of epicuticle [average 2.19 μ m in *C. megacephala* (Figure 1E) and 1.35 μ m in *M. domestica*], but intense thickness of procuticle [average 33.88 μ m in *C. megacephala* and 53.75 μ m in *M. domestica*] (Table 1). In multiple dipping both *C. megacephala* (Figure 1C) and *M. domestica* larvae (Figure 2C) exhibited noticeable swelling in both integument and intersegmental spines, corresponding with the thickness in epicuticle and procuticle shown in Table 1. Blebbing was observed in the integument of *M. domestica* larvae subjected to multiple treatments of azadirachtins (Figure 2E).

The puparia of control *C. megacephala* and *M. domestica* under SEM did not show any signs of damage, having integuments of smooth appearance after scanning electron microscopic analysis (Figures 3A and 4A, respectively). By contrast, the neem extract treatment even in single dipping was characterized by blebbing in the surface ultrastructure of *C. megacephala* and *M. domestica* puparia (Figures 3B and 4B). The intensity of the damage increased significantly with multiple applications, showing more blebbing as seen in Figures 3C and 4C. Cracking of the integument was

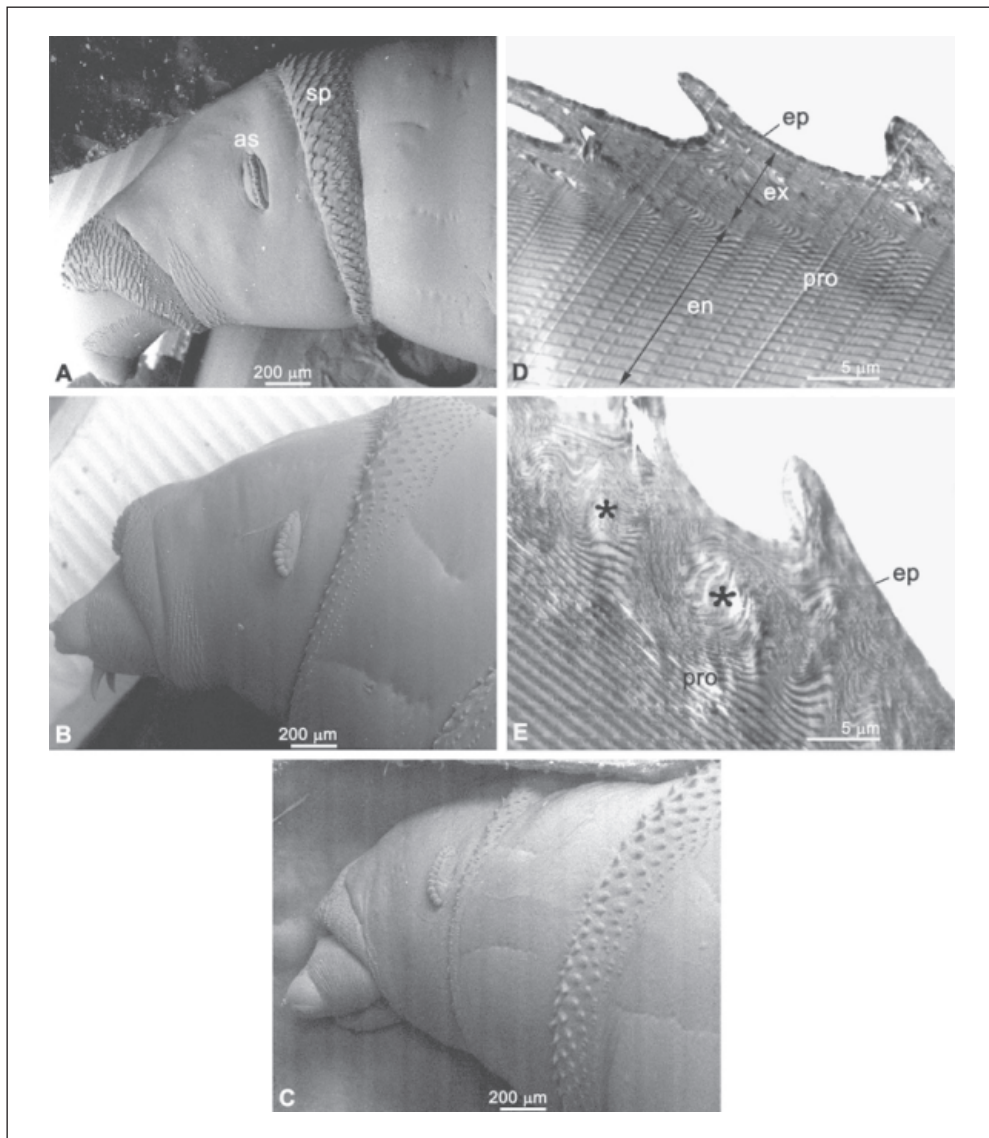


Figure 1. Third instar of *C. megacephala*. (A) SEM micrograph of the anterior end of the control larva showing normal appearance with smooth integument and well-shaped intersegmental spines. (B) SEM micrograph of the anterior end of a larva treated by single dipping in neem extract showing swelling of the integument. (C) SEM micrograph of the anterior end of larva treated by multiple-dipping in neem extract exhibiting noticeable swelling in the integument. (D) TEM micrograph in cross section of integument of the control larva. Integument showed a thin electron-dense epicuticle, followed by lamellated procuticle consisting of exocuticle and endocuticle. (E) TEM micrograph in cross section of integument of larva treated by single dipping. The exocuticle appeared swollen (asterisks). as, anterior spiracle; ex, exocuticle; en, endocuticle; ep, epicuticle; pro, procuticle; sp, spines between prothorax and mesothorax.

also visible in the multiple-dipped puparia of *C. megacephala* (Figure 3C, arrows). TEM examination of neem-treated puparia of both fly species showed that there was minimal increase in the thickness of the puparial

integument, but obvious thickness was observed in multiple-dipped puparia of *M. domestica* (Table 1). Minute swelling of the integument was found in multiple-dipped puparia of *C. megacephala* (Figure 3E,

Table 1. Average thickness of epicuticle and procuticle layer in each experimental group of the larvae and puparia of *C. megacephala* and *M. domestica* being dipped dipped in with neem (0.24% azadirachtin A)

| | <i>C. megacephala</i> | | <i>M. domestica</i> | |
|----------------------|---|---|---|---|
| | Epicuticle (mean and range, μm) | Procuticle (mean and range, μm) | Epicuticle (mean and range, μm) | Procuticle (mean and range, μm) |
| Larva | | | | |
| Control | 1.95 μm [(1.50-2.60), n=3] | 23.83 μm [(22.25-26.75), n=3] | 1.20 μm [(1.00-1.40), n=2] | 27.50 μm [(26.00-29.00), n=2] |
| Treated | | | | |
| <i>Single dose</i> | 2.19 μm [(1.88-2.50), n=2] | 33.88 μm [(33.75-34.00), n=2] | 1.35 μm [(1.25-1.50), n=3] | 53.75 μm [(53.75), n=1] |
| <i>Multiple dose</i> | 3.96 μm [(3.40-4.63), n=5] | 50 μm [(44.69-55.31), n=2] | 3.25 μm [(2.70-3.75), n=4] | 52.03 μm [(51.56-52.50), n=2] |
| Puparia | | | | |
| Control | 2.38 μm [(1.88-3.00), n=3] | 13.38 μm [(12.50-14.25), n=2] | 4.19 μm [(3.00-5.63), n=4] | 23.31 μm [(23.13-23.50), n=2] |
| Treated | | | | |
| <i>Single dose</i> | 2.41 μm [(1.90-3.00), n=4] | 22.17 μm [(20.50-24.25), n=3] | 4.75 μm [(4.00-5.50), n=2] | 21.88 μm [(20.50-23.25), n=2] |
| <i>Multiple dose</i> | 2.50 μm [(2.00-3.00), n=3] | 25.75 μm [(24.75-26.50), n=3] | 4.75 μm [(3.50-5.63), n=4] | 46.25 μm [(34.38-55.00), n=3] |

arrows), when compared with the control (Figure 3D). Moreover, multiple dipping of puparia of *M. domestica* led to obvious blebbing of the integument (Figure 4E), in contrast to the smooth integument of the untreated puparia (Figure 4D).

DISCUSSION

Azadirachtin, a limnoid from the neem tree, has been known to have insecticidal activity in various groups of arthropods causing disruption of growth, metamorphosis, reproduction, mating, and sexual communication; deterrence of egg laying and feeding; blockage of the ability to swallow; and sterilization of adults (e.g., Schmutterer, 1990; Nogueira *et al.*, 1997; Mulla & Su, 1999; Singh, 2003; Bruce *et al.*, 2004). This study has demonstrated morphological changes in the integument of larvae and puparia of *C. megacephala* and *M. domestica* treated with dipping neem extract. Initially, it was found that dipping once with the neem extract caused integument swelling of larvae of both fly species as determined by SEM. Investigation by TEM confirmed this

phenomena, indicated by the increase in thickness of integument, in particular the procuticle. The evidence of destruction was dose dependent, being progressively more severe with multiple dipping with the neem extract. SEM analysis of multiple-dipped specimens showed they displayed greater signs of damage, including integument swelling, bleb formation, and cracking of the integument. None of the above morphology was observed in the control larvae. Likewise, neem extract-dipped puparia of both species displayed integumental changes (i.e., swollen integument and bleb formation), but not as greatly as in the larvae. This explanation may be due to the hardened integument of puparia, as compared to that of the larvae. Nevertheless, our observations indicate some degree of contact action of the neem extract on integument, which acts as a protective barrier in insects. The effects of neem extract on *C. megacephala* and *M. domestica* seen in our study were consistent with results of previous studies that used azadirachtin, one of the three predominant limnoids of the neem tree (Schmutterer, 1990), on the insect cells of the fall armyworm, *Spodoptera frugiperda* (Reed &

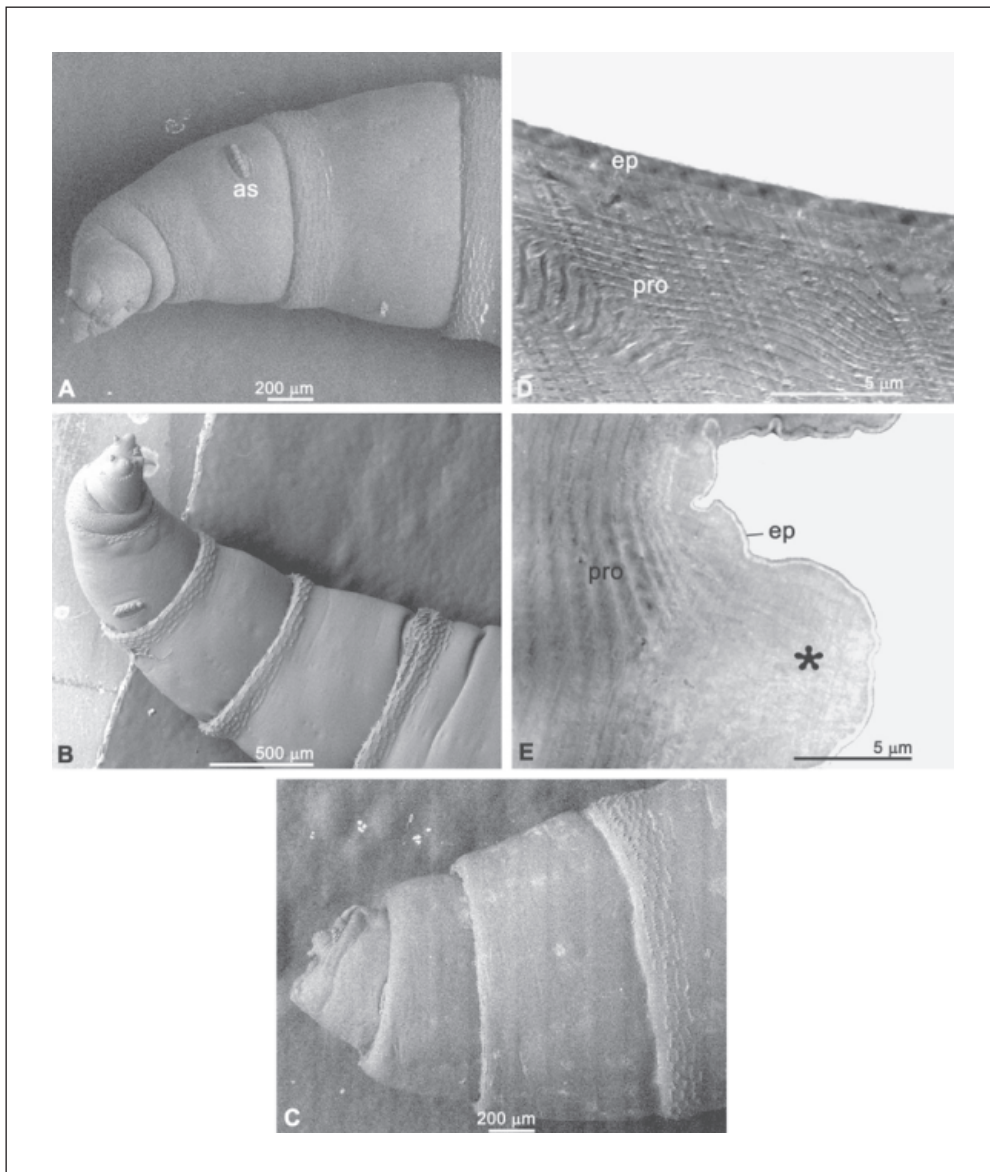


Figure 2. Third instar of *M. domestica*. (A) SEM micrograph of the anterior end of a control larva showing normal appearance with a smooth integument. (B) SEM micrograph of the anterior end of a larva treated by single dipping in neem extract showing a slightly swelling integument. (C) SEM micrograph of the anterior end of a larva treated by multiple dipping in neem extract exhibiting extensive swelling in the integument and a shrunken body. (D) TEM micrograph cross section of a control larva displaying homogenous electron density of epicuticle. (E) TEM micrograph in cross section of integument of larva treated by multiple dipping in neem extract showing a thin electron-dense epicuticle, without homogenous electron density. Bleb formation (asterisk) was observed. as, anterior spiracle; ep, epicuticle; pro, procuticle.

Majumdar, 1998). Insect cells treated with azadirachtin ultrastructurally displayed cell swelling and caused distortions of the cell surface, characterizing by blebbing and holes. Work by Nogueira *et al.* (1997) has

also shown the effect of azadirachtin A in the fine structure of epithelial midgut of bug, *Rhodnius prolixus*. Interestingly, morphological alteration on the integument induced by neem extract in this study, such

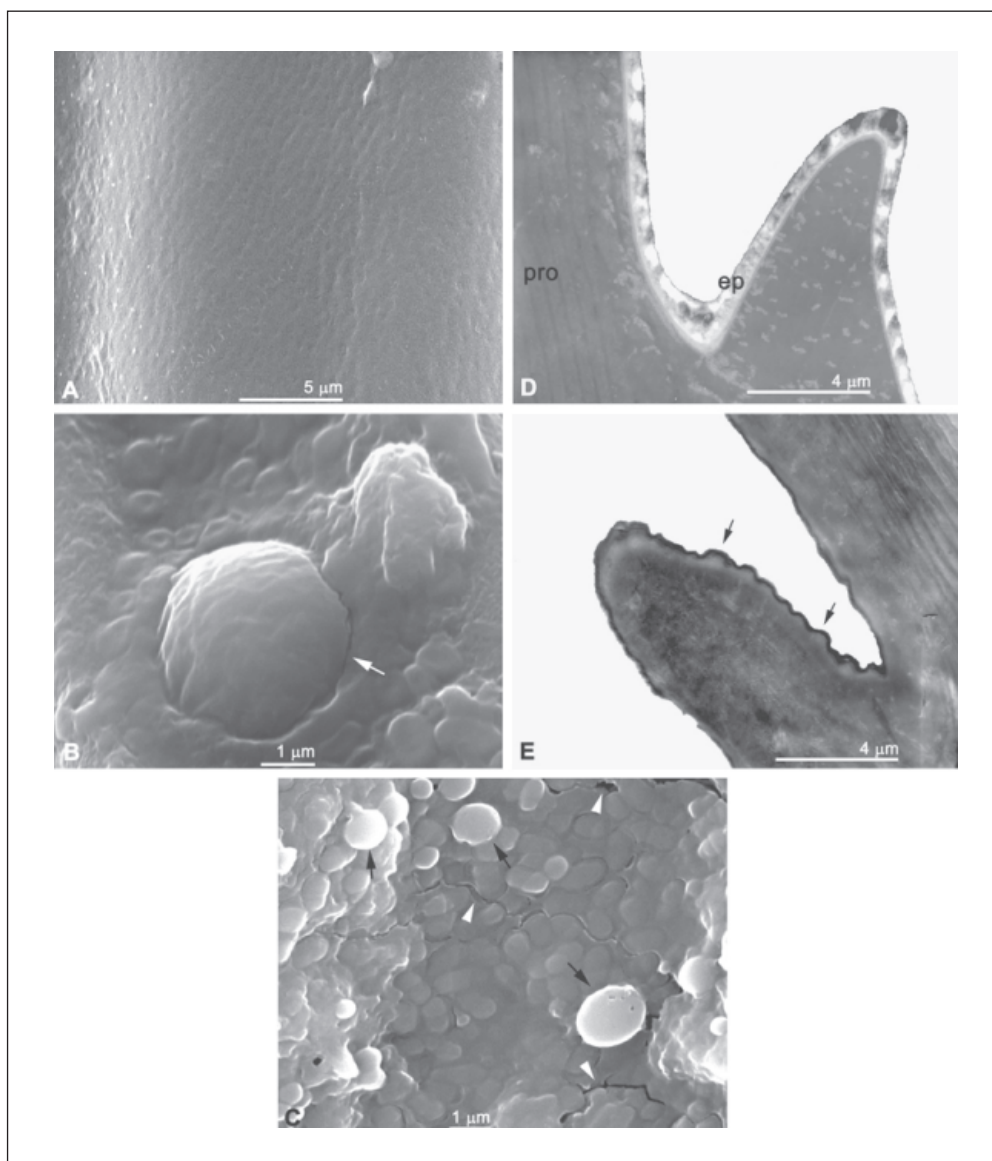


Figure 3. Puparia of *C. megacephala*. (A) SEM micrograph of the control puparia showing normal appearance with a smooth integument. (B) SEM micrograph of the puparia treated by single dipping in neem extract exhibiting blebbing (arrow) in surface ultrastructure. (C) SEM micrograph of the puparia treated by multiple dipping displaying damage in the integument with numerous blebbing (arrows) and crack integument (arrowheads). (D) TEM micrograph cross section of a control puparia displaying a smooth epicuticle. (E) TEM micrograph cross section of the integument of puparia treated by multiple dipping in neem extract. The exocuticle appeared to have an irregular rim (arrows).

as swelling and blebbing, have previously described in a study of *C. megacephala* and *M. domestica* treated by dipping in eucalyptol (Sukontason *et al.*, 2004b). Why these alterations occurred is still unknown. However, the destructive effects on fly larvae

induced by neem extract in this study were in accord with the effects of intestinal parasites administered antiparasitic drugs. In helminths, for example, the surface damages of trematodes such as liver fluke, *Opisthorchis viverrini*, after *in vitro*



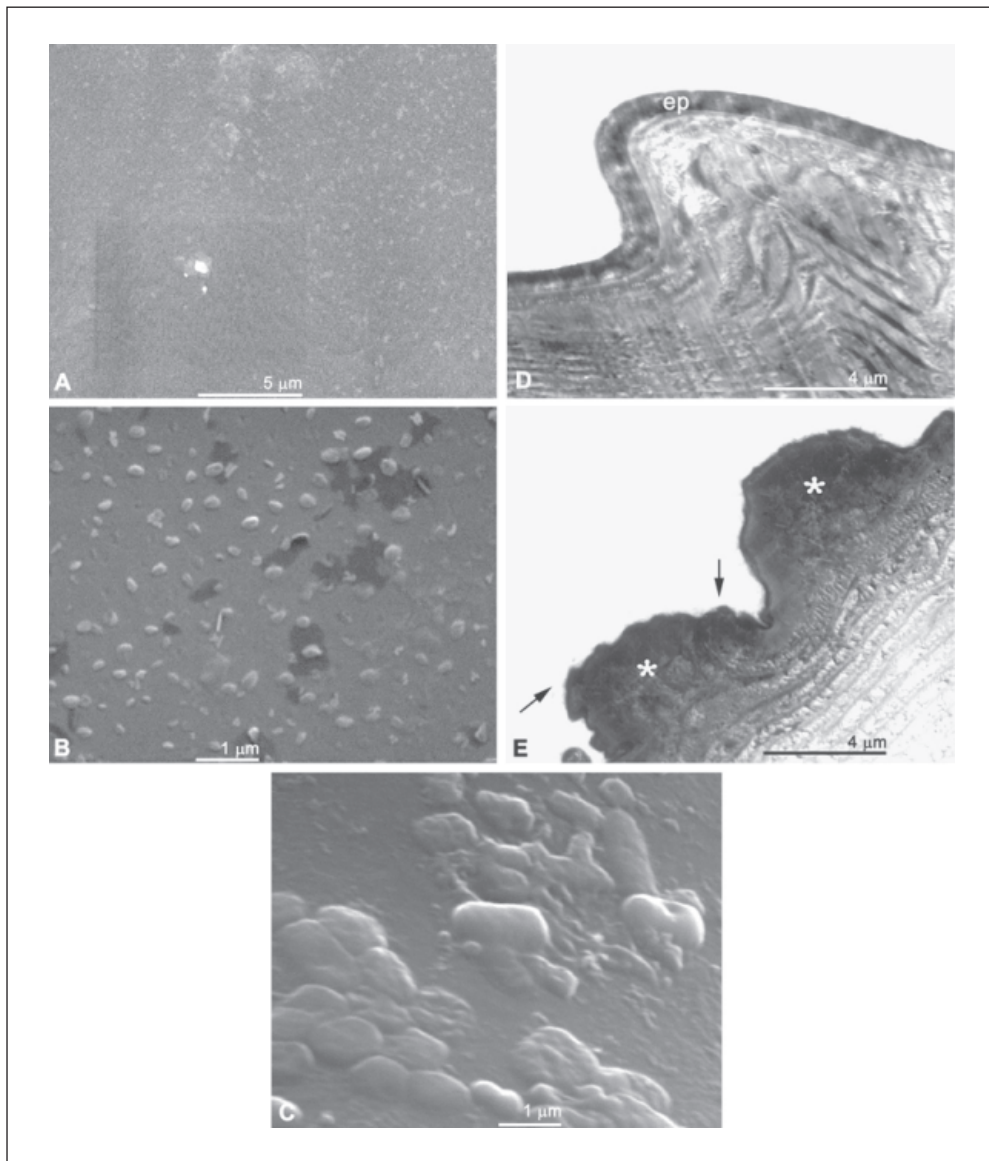


Figure 4. Puparia of *M. domestica*. (A) SEM micrograph of the control puparia showing normal appearance with smooth integument. (B) SEM micrograph of the puparia treated by single dipping in neem extract displaying minute blebbing in surface ultrastructure. (C) SEM micrograph of the puparia treated by multiple dipping displaying damage in integument with striking blebbing. (D) TEM micrograph in cross section of control puparia displaying smooth epicuticle. (E) TEM micrograph in cross section of integument of puparia treated by multiple dipping in neem extract. The exocuticle appeared bleb formation (asterisks) and irregular rim (arrows).

incubation in praziquantel included blebbing, swelling, erosion and disruption of the integument (Apinhasmit & Sobhon, 1996); these corresponded with the effects of artemether, an active compound from *Artemisia annua* L., on the integument of other liver fluke, *Fasciola hepatica* (Keiser

& Morson, 2008). Bleb formation and loss of integument organization might possibly be "stress responses" resulting from any harmful condition (Pérez-Serrano *et al.*, 1994).

In conclusion, the present study utilizing SEM has revealed that neem extract caused

morphological alteration in fly larvae in a dose-dependent manner, leading to swelling and bleb formation in the integument. TEM investigation confirmed such alterations in both larvae and puparia. Application of the neem-based product against immature stages of these flies warrants further investigation.

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