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【Research report】

幾丁質合成抑制劑二福隆對白線斑紋幼蟲之生長與發育之影響【研究報告】

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Abstract

摘要

本研究係根據實驗室內以二福隆處理白線斑蚊幼蟲後之蟲體發生異常的時期及比例評估此藥劑對蚊之效應。同時以石蠟切片，掃描及穿透式電子顯微技術觀察此藥劑對幼蟲之表皮及其他組織之作用。 試驗證實以二福隆處理各齡的幼蟲一天，即可使幼蟲或其蛻變而成之蛹或成蟲之發育異常。愈幼齡之幼蟲感受性愈大。當第二、三、四各齡的幼蟲經中間致死濃度之二福隆處理後，前二者多於幼蟲蛻皮時死亡，第四齡的幼蟲之死亡時間則延至幼蟲─蛹之中間期，甚至到成蟲羽化之時。將幼蟲自第四齡開鉛至蛹生成之期間均置於濃度為 12.5ppb 之二福隆的水中飼養，取樣作成石蠟切片，顯示此批幼蟲的內部器官及組織均未損傷，僅看到發育遲滯的幼蟲之腹部在蛻皮時有表皮增厚、表皮與真皮細胞未順利分離的現象。以 0.125ppb之二福隆處理第三齡之幼蟲一天，取倖存至第四齡的幼蟲以掃描電子顯微鏡觀察，發現部分幼蟲之胸部皮層破裂，或尾鰓腫脹。經此種處理的幼蟲之超薄切片呈現表皮紋理較為模糊之情形。 以上結果雖然尚不足以證明二福隆能直接抑制幾丁質的合成，但已顯示舊表皮與真皮細胞之延遲分離，與新表皮的改變可能是此藥劑阻礙蚊蟲蛻皮或發育之重要原因。有關此藥劑之作用機制，文末亦略加討論。

Key words:
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EFFECT OF DIMILIN, A CHITIN SYNTHESIS INHIBITOR, ON THE GROWTH AND DEVELOPMENT OF LARVAE OF *AEDES ALBOPICTUS* SKUSE

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Abstract

The growth inhibiting effects of Dimilin on larvae of *Aedes albopictus* Skuse were evaluated morphologically and histologically with light, scanning and transmission electron microscopy in a laboratory setting. We observed various aberrations in the larvae, pupae and adults after the larvae had been exposed to Dimilin for 24 hours. These aberrations were dose-dependent, and younger instars being more susceptible than the older ones. When treated with Dimilin at 50% lethal concentration (LC 50), most 2nd- and 3rd-inster larvae died during molting, while most 4th-instar larvae developed into larval-pupal intermediates or abnormal adults. Daily histological inspections of the 4th-instar larvae treated with 12.5 ppb of Dimilin revealed no noticeable damage in the internal organs and tissues of larvae. However, when the development of the larvae was delayed by Dimilin treatments, their abdominal cuticle became thicker and could not apolyze from the epidermis at molting. Exposing 3rd-instar to 0.125 ppb of Dimilin caused the splitting of cuticle and swelling of anal gills in some surviving 4th-instar larvae, as observed under a scanning electron microscope. Ultrathin sections of the integument of these larvae showed that the endocuticular laminae became obscured.

Key words: *Aedes albopictus*, Dimilin, larvicidal action, morphological abnormalities, histological examination, electron microscopy.

Introduction

Lack of specificity is one reason for the environmental damages produced by broad spectrum insecticides developed during the past three decades. Not only do these chemicals kill beneficial insects as well as harmful ones, they also cause toxic effects on other kinds of animals (Marx, 1977). For safer chemical control, it would seem reasonable to choose substances that damage vital systems which are specific to the arthropods (Busvine *et al.*, 1976). Chitin, in this regard, is the most abundant organic skeleton component of insects, other invertebrates and fungi, but is absent in vertebrates and higher plants (Muzzerelli, 1977; Richard, 1951). Insecticides that disrupt chitin deposition may therefore have selective advantages over earlier types that alter nervous action or

bioenergetic reaction indiscriminatively in insects and mammals (Hajjar and Casida, 1978).

As one of the first chitin synthesis inhibitors, diflubenzuron (Dimilin, N-[(4-chlorophenyl) amino] carbonyl-2, 6-difluorobenzamide) has received great attention (Deul *et al.*, 1978; Post *et al.*, 1974). Although there is general agreement that diflubenzuron disrupts the formation of insect cuticle, disagreement exists as to the molecular basis for this action (Mayer *et al.*, 1981). It is effective against mosquitoes (Busvine *et al.*, 1976; Grosscurt, 1978; Kottkamp and Meisch, 1985; Mulla *et al.*, 1974; Nelson *et al.*, 1985; Schaefer *et al.*, 1975; Sharma *et al.*, 1979), but has low, short term toxicity to many plant species and aquatic organisms (Miura and Takahashi, 1974; Mulder and Gijswijt, 1973).

Aedes albopictus, a potential vector of dengue and encephalites, is a very common species of mosquito around human habitats in Taiwan. Laboratory evaluation of the effects of Dimilin on this species of mosquito may therefore not only be a model to study its usefulness in controlling the household mosquitoes, but with local public health significance. Since contradictory effects on the unmoulted insects after treatment with Dimilin have been reported (Awad and Mulla, 1984; Gijswijt *et al.*, 1979; Soltani, 1984), the present study examines whether Dimilin prevent the molting of treated insects by interfering with the formation of new cuticles. We evaluated the sensitivity of various stages of immature *A. albopictus* to Dimilin treatment, based on morphological observations as well as light, scanning and transmission microscopy.

Materials and Methods

Larvae of mosquito *Aedes albopictus* were reared in an enamel pan at 28+3°C and fed with a mixture of two parts of rabbit chow and 1 part of yeast. Adults were fed with 5% sucrose sap absorbed in the cotton plug. Various dilutions of the test chemical, Dimilin, produced by Thompson Hayward Chemical Company and generously provided by Jehng-Ann Chemical Company, were made in distilled water from a 25% wettable powder. To establish the effective dosage (ED) for the compound, 4 groups (25 each group) of 2nd-, 3rd-, and 4th-instar larvae were exposed for 24 hours in 100 ml of a particular concentration of Dimilin in a paper cup. They were subsequently washed three times and reared under normal condition as described above. The mortality of the larvae and pupae was recorded daily. Log-dose/probit regression analysis (Finney, 1971) was used to estimate the quantal response of the larvae to Dimilin. Representative specimens with various degrees of morphological aberrations were photographed for symptomatic and qualitative assessments.

Specimens for histological study included treated larvae, pupae and with untreated ones. The 4th-instar larvae, treated with 12.5 ppb of Dimilin, were killed daily and prepared according to procedures of Awad and Mulla (1984). They were then embedded in paraplast and 6 μm sections were cut by an American Optical (AO) 820 Spencer mictrotome and stained with Delafield haematoxylin and eosin (Humason, 1967).

For electron microscopic studies, the 4th-instar larvae or pupae derived from larvae or pupae derived from larvae treated with 0.125 ppb of Dimilin for 24 hours at the 3rd-instar larval stage were taken for both SEM an TEM evaluations:

(1) SEM study—The whole larvae were fixed in 5% gl ıtaraldehyde and dehydrated in an alcoholic series, then dried in critical point apparatus. Dried specimens were coated with carbon and gold in an evaporator and then examined by a Jeol electon microscope at 15 KV.

(2) TEM study—The larva was cut into small pieces and then fixed in 3% glutaraldehyde in pH 7.2, 0.1 M phosphate buffer at 4°C for 2 hours and post-fixed in 1% osmic acid in pH 7.2, 0.1 M phosphate buffer at 4°C for 2 hours. After dehydration in

the alcoholic series, the specimens were embedded in spur epon. Ultrathin sections were cut by a LKB microtome with glass knife. The sections were stained with 2% uranyl acetate and 0.05% lead citrate. The stained sections were examined by Hitachi electron microscope at 60 KV.

Results

I. *Larvicidal action*

Various toxic effects of Dimilin on the development of *Aedes albopictus* were observed after 24-hour of exposure. Dimilin showed a dose-dependent (0.00025 ppb to 25 ppm) reduction in normal pupation and adult emergence (Table 1). The effective doses that

Table 1. Effect of Dimilin on survial, pupation and emergence of various stages of Larvae of *A. albopictus*

Stage treated	Concentration of dimilin (ppb)	Larval mortality (%)	Pupal mortality (%)	Emerged adult	
				Abnormal (%)	Normal (%)
2nd-instar	0	0	1	0	99
	0.00025	30	3	1	66
	0.0025	44	1	0	55
	0.025	67	2	0	31
	0.25	82	2	0	16
	2.5	88	1	0	11
	12.5	89	3	0	8
	25	94	1	0	5
3rd-instar	0	0	0	0	100
	0.025	12	7	1	80
	0.25	60	6	1	33
	2.5	64	8	0	28
	25	92	0	0	8
	250	100	0	0	0
4th-instar	0	0	0	0	100
	0.025	0	1	0	99
	0.25	2	1	1	96
	2.5	3	2	2	93
	25	16	17	0	67
	250	30	56	3	11
	2500	95	4	0	1

Table 2. Log-dose/probit regression analysis of emergence inhibition of various stages of larvae treated with Dimilin

Stage treated	Intercept	Slope	EC 50 (ppb)	95% Fiducial limit	Heterogeneity factor
2nd instar	6.0771	0.4210	0.0028	0.0012-0.0055	—
3rd instar	5.5012	0.7392	0.2099	0.0143-1.0047	4.77*(DF=3)
4th instar	3.2347	1.1033	39.5816	6.54 -254.54	9.47*(DF=4)

* Possible heterogeneity.

inhibited 50% of adult emerged, as determined by log/dose probit regression analysis (Table 2), were 0.028 ppb, 0.21 ppb and 39.6 ppb for 2nd-, 3rd- and 4th-instar repectively. Thus 2nd-instar was identified to be the most sensitive stage. Many 2nd-instar larvae died at the end of 24-hour exposure if the treated dose was above 0.25 ppb. When treated at 3rd-instar, larval death occurred either at 3rd- or 4th-instar only if the doses of Dimilin were above 2.5 ppb. Treated with 250 ppb the 4th-instar larva were affected during the period of pupal metamorphosis.

II. *Gross morphological aberrations*

Dimilin induced various degrees of developmental aberrations in immature stages as well as adult. The most common symptoms of the died larvae were partial exuviation (Fig. 1-A). Dorsal splitting (arrow) of thoracic cuticle had also been observed in the dying larva (Fig. 1-B). The most characteristic effect resulted from the treatment was formation of various types of larval-pupal intermediates: some individuals had larval cuticle with pupa inside, the respiratory trumpets (arrows) were visible in the thorax (Fig. 1-C). Some pupae liberated themselves in part from the larval cuticle but retained the larval head capsule (Fig. 1-D). Incomplete adult eclosion was observed occasionally. These individuals had distinguishable adult structures which appeared to be normally pigmented (Fig. 1-E), but failed to free themselves from the pupal exuvia. The abdomen of some morbid pupae was held recurved dorsally (Fig. 1-F). Some adults remained within the pupal skin by their suck or curved tarsi of legs (Fig. 1-G, H).

III. *Histological examinations*

The integument of the 4th-instar larvae subjected to a continuous Dimilin treatments through larvahood was normal (Fig. 2-A). It consisted of the outer noncellular cuticle (C) and the inner cellular epidermis (E), both of these were impacted to each other. Cells of the latter were usually squamous and arranged as a single layer. Fat body (FB) of this larva still had globules that are known to be fat, protein and glycogen (Fig. 2-B). Other internal organs and tissues such as caeca (Ce), intestine (It), peritrophic membrane (Pm), nerve ganglion (G) and muscle (M) had not been damaged by Dimilin treatments (Fig. 2-C). At molting the epidermis separated from the old cuticle, while tracheal epidermis similarily retracted from the linings. But if the abdominal integumen (Im) in this larva would fail to apolyze and become thicker (Fig. 2-D).

IV. *Ultrastructures of Dimilin-treated and untreated larvae*

When examined by a scanning electron microscope, the untreated larva had a rigid and wrinke integument on the thorax (pT, mT and tT) (Fig. 3-A), but splitting in the thoracic integument (It) was found in some treated larvae (Fig. 3-B). The untreated larvae usually had porous anal gills (G) with very delicate net-work structure (Fig. 3-C). Swollen anal gills without net-work structure had been observed in some of the treated larvae (Fig. 3-D).

The ultrathin section of the thorax of the normal larvae showed that cuticles of the thoracic integument consisted of a number of layers (Fig. 4-A). There was the outermost electron dense epicuticle (arrow), followed consecutively by the homogenous exocuticle (EX) and the laminar endocuticle (EN). The thickness of the former two layers was from 20 to 25 nm, and the latter one was about 65 nm. In general, the laminar endocuticle appeared as alternating parallel light and dark bands. When sections were cut at oblique angles to the cuticle surface, acute pattern appeared to be superimposed on these laminae (Fig. 4-B). But in the Dimilin-treated larvae, the laminar layers became obscured (Fig. 4-C).

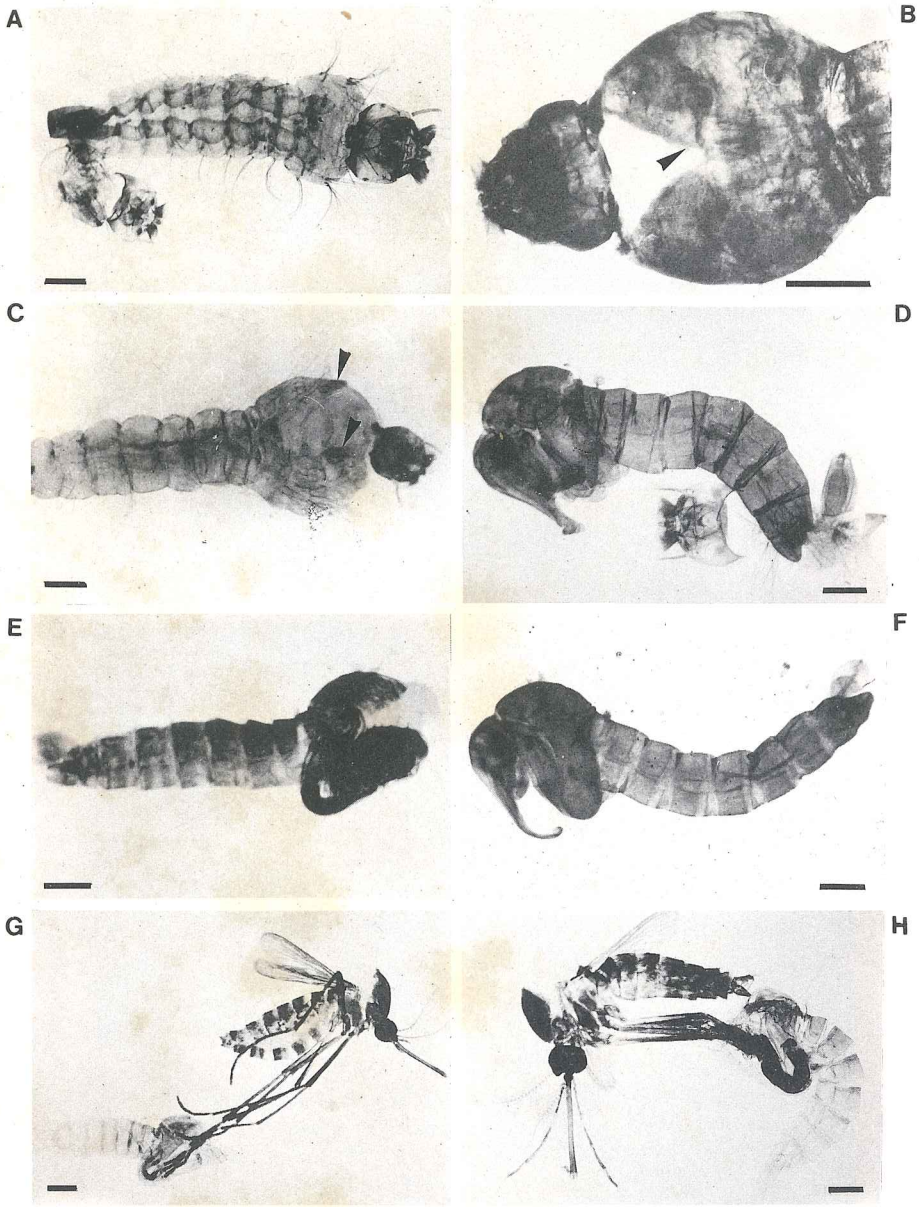

Fig. 1. A-H. Morphological aberrations of larvae and pupae of *A. albopictus* after treatment with Dimilin (Bar=500 μm).

A. Partially exuvated 4th-instar larva molted from Dimilin-treated 3rd-instar larva.

B. Broken dorsal thoracic integument (arrow) of newly emerged 4th-instar after treatment with Dimilin at 3rd-instar.

C. The trumpets (arrows) of larval-pupal intermediate could be seen after treatment with Dimilin at 4th-instar.

D. Head capsule of 4th-instar larva retained on the newly molted pupa after treatment with Dimilin.

E. Normally pigmented dead late-pupa.

F. The abdomen of morbid pupa recurved dorsally.

G. The female adult, with curved tarsi of mid-leg and hind-leg, remained in the pupal skin.

H. Legs of the male adult retained in the pupal skin.

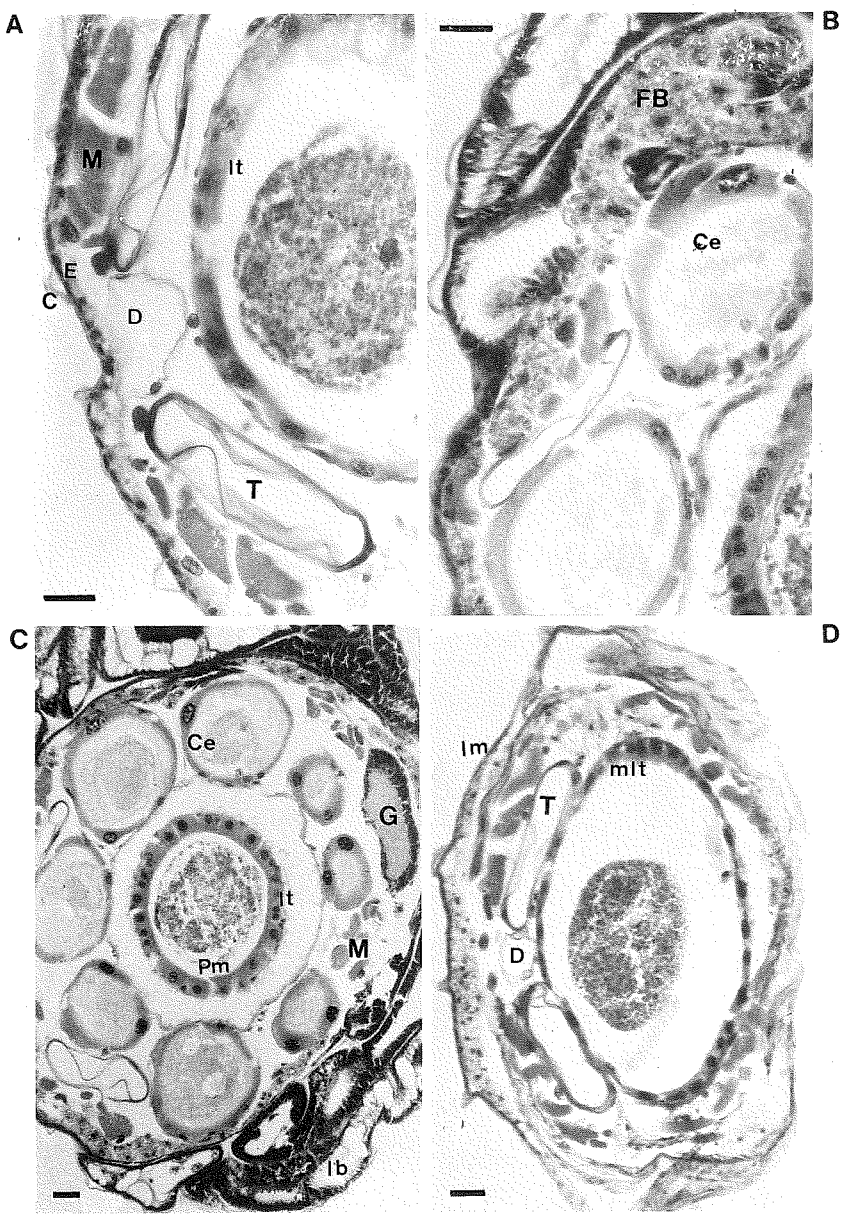

Fig. 2. A-D. Histological normality and aberration in the 4th-instar larva treated with Dimilin through larvahood for 8 days (Bar=30 μm).

A. Larva still had complete cuticle (C) and epidermis (E) in the integument, its muscle (M), dorsal vessel (D) and intestine (It) looked normal, but epidermis of its tracheae (T) became retracted.

B. Larva had normal caeca (Ce) and fat body (FB) with globules.

C. Larva had normal imaginal buds (Ib), muscle (M), ganglion (G), caeca (Ce), intestine (It) and peritrophic membrane (Pm).

D. The abdominal integument (Im) of the Larva, appeared to have delayed-development, did not perform apolysis at molting. However, the tracheal (T) linings of this larvae had separated from the tracheal cells (D, dorsal vessel; mIt, midgut).

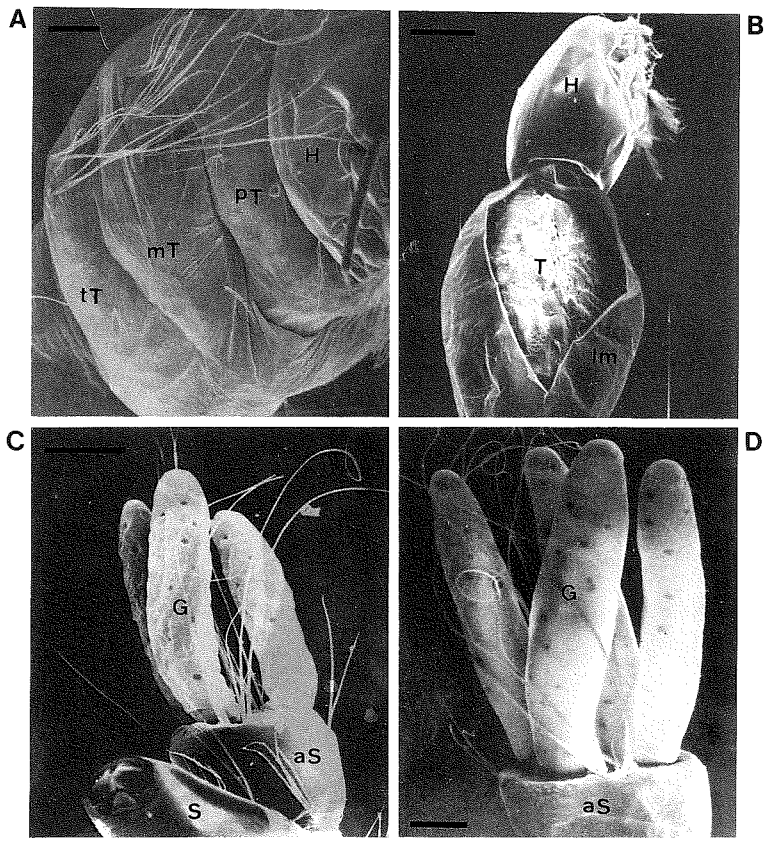

Fig. 3. A-D. Scanning electron micrographs of untreated and Dimilin (0.125 ppb) treated larvae of *A. albopictus* (Bar=100 μm).

A. Thorax (pT, prothorax; mT, mesothorex; tT, metathorax) and head (H) of untreated 4th-instar larva.

B. Splitting of the thoracic integument (Im) of 4th-instar larve molted from Dimilin-treated 3rd-instar larva.

C. Anal gills (G), anal segment (aS) and siphon (S) of untreated 4th-instar larva.

D. Swelling anal gills (G) of Dimilin-treated 4th-instar larva.

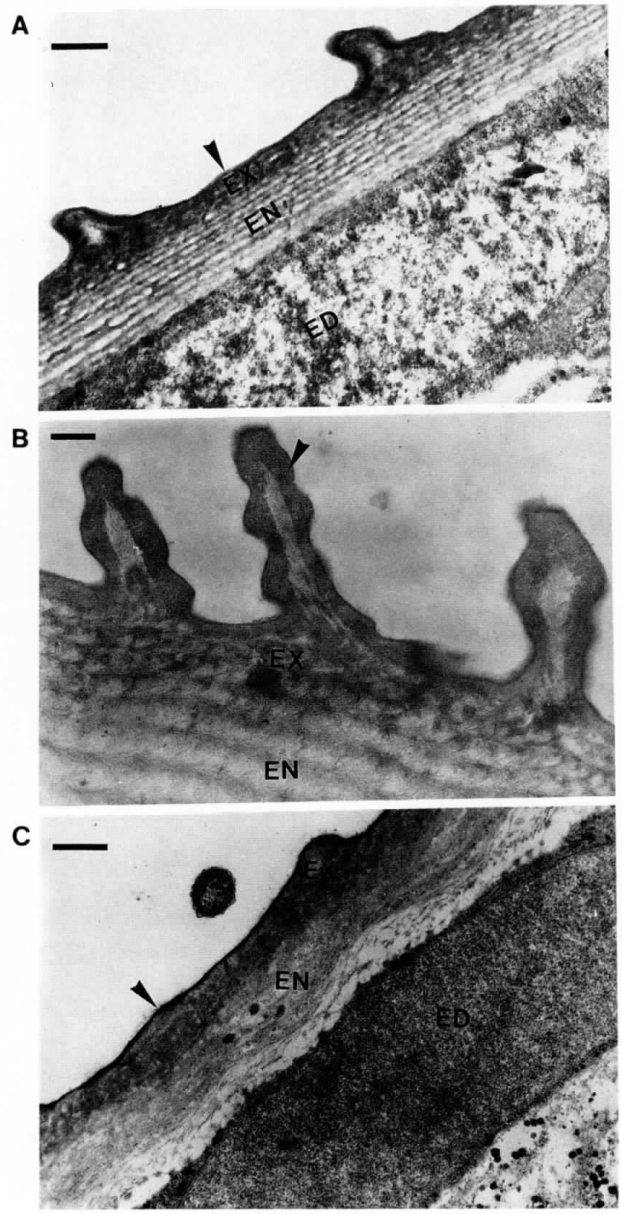

Fig. 4. A-C. Electron micrograph of the integument is untreated and Dimilin (0.125 ppb) treated larvae.

A. Transverse section of the thoracic cuticle of *A. albopictus*. The exocuticle (EX) appeared electron dense and closely adhered to the epicuticle (arrow), light and dark bands formed the laminae of endocuticle (EN) (Bar=60 μm).

B. Higher magnification of the exocuticle (EX) and laminar endocuticle (EN) of untreated larvae (Bar=200 μm).

C. Exocuticle (EX), endocuticle (EN) and epidermis (ED) of 4th-instar larvae from 3rd-instar larve treated with Dimilin for 24 hours. Note that loss of delicate laminar layers in both of the exocuticle and endocuticle (Bar=60 μm).

Discussion

The present study demonstrated that Dimilin had a potent inhibiting effect on the development of *A. albopictus*. A variety of abnormalities occurred in larvae, pupae and adults after the larvae of this species were exposed in Dimilin for 24 hours, although younger instars were more susceptible than the older ones. Histological examinations indicated that the internal organs and tissues in a majority of the larvae were not damaged unless the cuticle of larvae with delayed development failed to apolyze from the epidermis. Scanning and transmission microscopic studies revealed a splitting of the integument as well as obscured laminae in the endocuticle of these abnormally developing larvae. These observed symptoms in *A. albopictus* resulted from Dimilin treatment were not greatly different from those reported in 16 other species of mosquito (Busvine *et al.*, 1976). They suggested that the mode of action of Dimilin is not species and stage specific.

The obscured laminae of newly formed cuticle in the larvae after Dimilin treatments is similar to that reported in the development of the embryonic cuticle of *Leptinotarsa* (Grosscurt, 1977). Cuticular growth is known to occur during the intermolt period by the additional laminae at the epidermal surface (Condoulis and Lock, 1966). Accordingly our results suggested that Dimilin may cause the interferance of chitin synthesis. The deformed new cuticles may thus not resist the increase internal body, leading to the splitting of the integument after molting.

Although the direct effect of Dimilin remained to be determined, three modes of action have been proposed. First, it acts directly on the chitin synthase (Cohen and Casida, 1980; Deul *et al.*, 1978; Hajjar and Casida, 1978; Van Eck, 1979); Second, it inhibits glycosyltransferase instead of chitin synthase (Mayer *et al.*, 1981). Third, it blocks the synthesis of imaginal cuticle by preventing formation of the adult epidermis in the pupal stage (Meola and Mayer, 1980). Our present results did not readily provided a definitive support for these proposed modes of action. Since our specimens were treated at larval stage for only 24 hours, it is unlikely that the effect of Dimilin can maintained till imaginal epidermis is formed. It is more likely that Dimilin may inhibit chitin synthase or glucosyltransferase. These two latter hypothesis will be given priority in our current research.

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幾丁質合成抑制劑二福隆對白線斑紋幼蟲之生長與發育之影響

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本研究係根據實驗室內以二福隆處理白線斑蚊幼蟲後之蟲體發生異常的時期及比例評估此藥劑對蚊之效應。同時以石蠟切片，掃描及穿透式電子顯微技術觀察此藥劑對幼蟲之表皮及其他組織之作用。

試驗證實以二福隆處理各齡的幼蟲一天，即可使幼蟲或其蛻變而成之蛹或成蟲之發育異常。愈幼齡之幼蟲感受性愈大。當第二、三、四各齡的幼蟲經中間致死濃度之二福隆處理後，前二者多於幼蟲蛻皮時死亡，第四齡的幼蟲之死亡時間則延至幼蟲—蛹之中間期，甚至到成蟲羽化之時。將幼蟲自第四齡開始至蛹生成之期間均置於濃度爲 12.5 ppb 之二福隆的水中飼養，取樣作成石蠟切片，顯示此批幼蟲的內部器官及組織均未損傷，僅看到發育遲滯的幼蟲之腹部在蛻皮時有表皮增厚、表皮與眞皮細胞未順利分離的現象。以 0.125 ppb 之二福隆處理第三齡之幼蟲一天，取倖存至第四齡的幼蟲以掃描電子顯微鏡觀察，發現部分幼蟲之胸部皮層破裂，或尾鰓腫脹。經此種處理的幼蟲之超薄切片呈現表皮紋理較爲模糊之情形。

以上結果雖然尚不足以證明二福隆能直接抑制幾丁質的合成，但已顯示舊表皮與眞皮細胞之延遲分離，與新表皮的改變可能是此藥劑阻礙蚊蟲蛻皮或發育之重要原因。有關此藥劑之作用機制，文末亦略加討論。