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MORPHOLOGICAL ABERRATIONS INDUCED BY METHOPRENE, A JUVENILE HORMONE ANALOGUE, IN *ANOPHELES DIRUS* S.S. AND *AN. SAWADWONGPORNI* (DIPTERA : CULICIDAE)

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ABSTRACT

Effect of Methoprene (isopropyl 11-methoxy-3,7, 11- trimethyldodeca-2, 4-dienoate), a juvenile hormone analogue, on Anopheles dirus *s.s and* An. sawadwongporni *were investigated under laboratory conditions (28°±1.7° C, 86±3% RH, and 12-h light-dark photophase). Methoprene induced a wide range of morphogenetic aberrations in the 24-h egg treatment in both* An. dirus *s.s. and* An. sawadwongporni. *The majority of mosquito populations died as pupae exhibited various degrees of morphogenetic aberrations. Several other morphogenetic aberrations were noted and were grouped into eight categories according to the stage of death. No significant difference of hatchability of both species was detected when different concentrations of Methoprene between 0.0001 mg/l and 1 mg/l were studied (P<0.5).*

INTRODUCTION

Anopheles (cellia) dirus s.s. (= species A) is widely distributed in Thailand and Malaysian Peninsula and is one of the principal vectors of human malaria[1,2,3,4]. *An. dirus* s.s is abundant in the rainy season and during the late dry season in foothill and forested areas throughout Central and Northeastern Thailand[5].

An. (cellia) maculatus group is widely distributed throughout the oriental zoogeographic region, and is recognized as an important vector of human malaria in Malaysia and in some parts of Indonesia[6-8]. In Thailand, *An. maculatus* complex consists of seven closely related species and two forms[9]. Bionomics of *An. sawadwongporni* sp. nov. (= species A) in Pakchong District, Nakorn Ratchasima Province, Central Thailand were reported[5]. The adults were found in forested and foothill areas, while their immature stages were collected in lightly shaded and slow flowing streams.

Morphogenetic abnormalities in mosquitoes induced by insect growth regulators (IGRs) were reported by several workers. Spielman and Skaff[10] reported the abnormal development in *Aedes aegypti* after treatment with farnesoic acid derivatives. Spielman and Williams[11] found developmental intermediates in *Ae. aegypti* treated with crude synthetic juvenile hormone. Aria and Mulla[12] similarly reported a wide range of morphogenetic changes in *Culex tarsalis* after exposure to Altosid or Methoprene.

In addition, Phonchevin *et al.*[13] reported the biological effects of Cyromazin (OMS-2014 or Neporex^R) and liquid Methoprene (Altosid^R SR - 10) on *An. dirus*, *Ae. aegypti* and *Cx. quinquefasciatus*.

During the course of study on the biological effect of insect juvenile hormone analogues and mimics on anopheline mosquitoes, we have noticed a range of morphogenetic aberrations in the treated populations. Therefore, it was the purpose of this paper to identify and describe the external morphological aberrations and changes induced in larvae, pupae and adults of *An. dirus* s.s. and *An. sawadwongporni* following the egg exposure to Methoprene.

MATERIALS AND METHODS

Maintenance of mosquitoes in the laboratory

An. dirus s.s and *An. sawadwongporni* were colonized in the laboratory at the Center for Applied Malacology and Entomology, Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand. The artificial mating technique was used to induce copulation. The eggs collected from females were floated together on the water, and were surrounded by a square-shaped plastic straw to prevent egg from dessication and to support the larvae to lean on. The hatched larvae were fed daily with ground fish food plus 1% ground dry liver. Daily observation on the conditions of water was made to assure the best survival of instars. The pupae were collected daily and placed in emerging plastic bowls of 20 -cm diameter with 100 ml of water. The female mosquitoes were human-blood fed before copulation, and the eggs were subsequently collected for the study.

Chemical

Comparative testings of liquid formulation and charcoal tablet of Methoprene (isopropyl 11-methoxy-3, 7, 11-trimethyldodeca -2, 4- dienoate) were conducted. The results showed that there was no significant difference as to their efficacy under laboratory experiment. Since the charcoal tablet would be more practical to be applied in the field as a control measure against the anopheline mosquitoes, we decided to select it for the present study.

One hundred milligrams of Methoprene (10% a.i.) was dissolved in 1,000 ml of distilled water to yield the stock solution of 10 mg/l. The concentrations used were 10 -fold dilution of the stock solution.

Biological assay

Biological effects of Methoprene on the mosquitoes were assessed by exposing 20 eggs of *An. dirus* s.s or *An. sawadwongporni* in a Petri dish containing 50 ml of each concentration of Methoprene. Five replicates of each concentration, plus an untreated control, were used. In total, 100 eggs of each mosquito species were used for each concentration. The eggs were floated within a triangle straw until hatching and the straw was then removed. After the 48-h exposure of eggs, the first instar larvae in

each replicate were counted under a stereomicroscope to record viable eggs. Batches of viable eggs were saved to record their hatchability. The hatched larvae were then transferred to a lebelled rearing jar (200 ml) filled with 150 ml water. These rearing jars were set in a room maintained at 28°±1.7°C, 86±3% RH, and a 12- h light-dark photophase. All larvae were daily fed with ground fish food, and dead larvae, pupae, adults, and exuviae were removed and placed in labelled plastic vials containing 70% ethanol.

Representative specimens showing developmental aberrations were photographed under a dissecting microscope. For quantitative studies, dead specimens were categorized according to morphogenetic abberrations in various concentrations of the treated solutions.

Dead specimens were grouped and modified according to those described by Aria and Mulla[12], Phonchevin *et al.*[13], and Yodbutra *et al.*,[14] which were categorized into eight groups, as follows:

Group 1. Normal larva (NL). This group represented the larvae dying after reaching the pre-pupal stage of development. They were classified into four sub-groups as L_1, L_2, L_3, and L_4, representing death in the first, second,third, and fourth instar larvae,respectively.

Group 2. Deformed larva (DL). This group represented the abnormal dead larvae at any of the larval development designated as DL_1, DL_2, DL_3, and DL_4.

Group 3. Pre-pupa and pupa not completely out of the larval exoskeleton (PP). This group represented the pharate pupa and any partially exuviated pupa, dying between the pre-pupal and the pupal stage. At this stage, the larval skin was ruptured and the pupal body was partly protruded from the thoracic split showing the pupal trumpet. The thorax and abdomen adopted the characteristics of pupa with retracted abdomen from the larval skin at the posterior. Some specimens died when the larval head capsule exoskeleton still enclosed the pupal head, and/or died as partially pupa out of the larval exoskeleton leaving larval exuvia attached to the caudal end.

Group 4. White pupa (WP). This group represented the pupa escaping completely from the larval exoskeleton but remaining unmelanized, except for the eye pigments. The pupa died before hardening and darkening of cuticle, and was known as "albino".

Group 5. Deformed pupa (DP). This group denoted the dead pupa freeing completely from the larval exoskeleton, but not normal in appearance. In some cases, the pupa exhibited appearance of an elephant, and was designated as "elephantoid".

Group 6. Dead normal brown pupa (BP). This group denoted the dead pupae that was brown in color and normal in appearance, with either extended or curved body.

Group 7. Adult attached to the pupal case (PA). This group denoted the adult that died after having partly emerged. Its main trunk was exuviated but the remainder was still retained within the pupal exuvia, for example, its tarsi, legs, wings, and abdomen.

Group 8. Normal adult (NA). This group denoted the adult that emerged completely with normal appearance.

RESULTS AND DISCUSSION

Eggs of *An. dirus* s.s. and *An. sawadwongporni* were exposed to various concentrations of Methoprene for 24 hours. The Methoprene could not prevent the hatchability of *An. dirus* s.s. and *An. sawadwongporni* eggs at concentrations from 0.0001 to 1.0 mg/l. The mean numbers of hatchability of the first instar larvae of treated group and untreated control did not differ significantly ($P < 0.5$). The average hatching rates of untreated *An. dirus* s.s. and *An. sawadwongporni* were 60 ± 15.2 and 56.5 ± 12.3 respectively.

The frequency occurrence of different morphogenetic forms and mortality rates of *An. dirus* s.s. and *An. sawadwongporni* are shown in Table 1 and Table 2 respectively. Concerning the external morphology of dead specimens, it was observed that Methoprene induced morphogenetic changes in larvae, pupae, and adults of both species of mosquitoes when they were treated at the egg stage. Forms of the morphological aberrations varied with concentrations of the Methoprene used.

The results showed that majority of the specimens were identified as brown pupae (BP). At higher concentrations of the Methoprene treatment, the number of dead mosquitoes reaching the pupal stage was greater than those at lower concentrations. The LD-50 of the Methoprene treatment of *An. dirus* s.s. and *An. sawadwongporni* were 0.0033 and 0.00095 mg/l respectively.

Fig. 1 shows morphogenetic changes found in *An. dirus* s.s. at the fourth instar larvae (Figs. 1B and 1C) comparing to the normal fourth instar larvae (Fig.1A). Deformed cuticular formation at abdominal segment (Fig. 1B) and uncompletely melanized at the posterior abdomen of the larvae (Fig. 1C) were observed in most specimens exposed to 0.001 mg/l Methoprene. In addition, abnormal pre-pupal stage with distortion of digestive tract and posterior abdominal segments was found in four specimens after the eggs were treated with 1.0 mg/l (Fig. 1D).

Fig. 2 shows a normal pupal characteristic (Fig 2A); and morphogenetic changes in *An. dirus* s.s. which died as abnormal pre-pupa with characteristics of the fourth instar and pupa, showing a small portion of pupal thorax with thoracic air trumpets protruding when the larval exoskeleton split open in the thorax area (Fig. 2B). Figs. 2C and 2D illustrate a deformed pre-pupa where adult mouthparts and wing pads were not appressed to the body with larval appearance at its posterior end, and a grossly deformed pupa still attached to larval exuvia at the anterior end. Most specimens exposed to 0.0001 mg/l Methoprene exhibited characteristics of pre-pupae and pupae not completely out of the larval exoskeleton.

Table 1. Mortality rates following the 24- hour treatment of eggs of *An. dirus* s.s with graduated doses of Methoprene (N=100).

Doses	Stages at death (%)							Total	Correct
(mg/l)	NL	DL	PP	WP	DP	BP	PA	mortality (%)	mortality (%)
1	13	16	7	14	4	34	2	90	88.9
0.1	10	16	5	13	2	31	4	81	78.8
0.01	10	9	9	7	6	15	4	60	55.5
0.001	5	3	5	8	8	12	5	46	39.9
0.0001	1	0	2	2	9	10	8	32	24.3
Control	8	0	0	0	0	2	0	10	0

LD-50 (mg/l) = 0.0033

Probit regression line (Y) = 5.21 + 0.49 (X-7.96)

Table 2. Mortality rates following the 24- hour treatment of eggs of *An. sawadwongporni* with graduated doses of Methoprene (N=100).

Doses	Stages at death (%)							Total	Correct
(mg/l)	NL	DL	PP	WP	DP	BP	PA	mortality (%)	mortality (%)
1	14	18	5	17	6	30	8	98	97.7
0.1	14	18	4	15	4	30	5	90	88.4
0.01	11	9	9	13	5	28	4	79	75.6
0.001	6	0	4	9	7	20	8	54	46.5
0.0001	3	2	8	7	10	6	4	40	30.2
Control	12	0	0	0	0	2	0	14	0

LD-50 (mg/l) = 0.00095

Probit regression line (Y) = 5.41 + 0.62 (X-7.60)

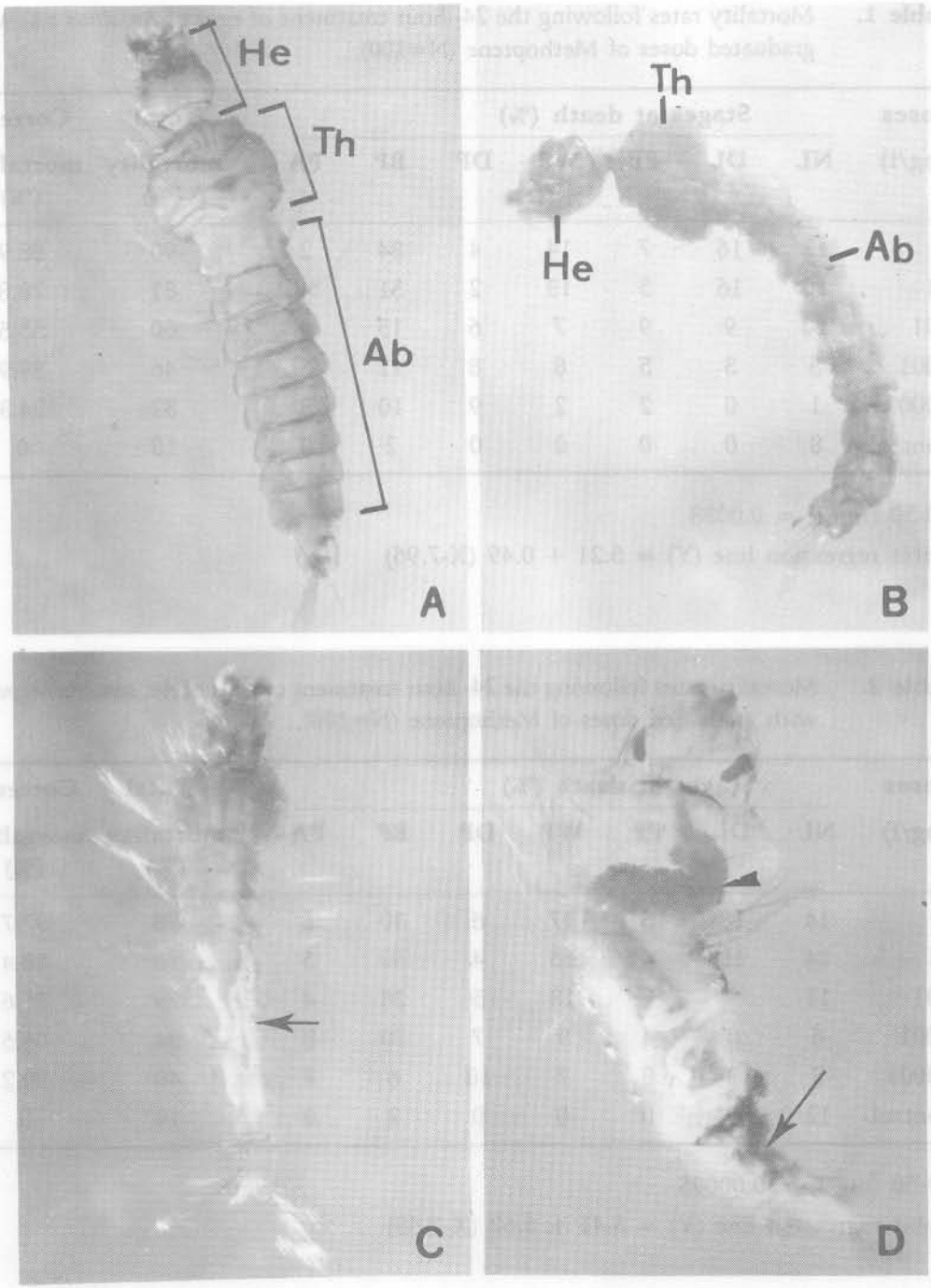

Fig. 1. Morphogenetic changes found in *An. dirus* s.s after the 24 -hour treatment of eggs at various concentrations of Methoprene. **A**, normal fourth instar larva (He = head, Th = thorax, Ab = abdomen) ; **B**, deformed fourth instar larva; **C**, fourth instar larva, not completely melanized (arrow); **D**, abnormal pre-pupa with distortion of digestive tract (arrowhead) and posterior segments of abdomen (arrow) (A-D x16).

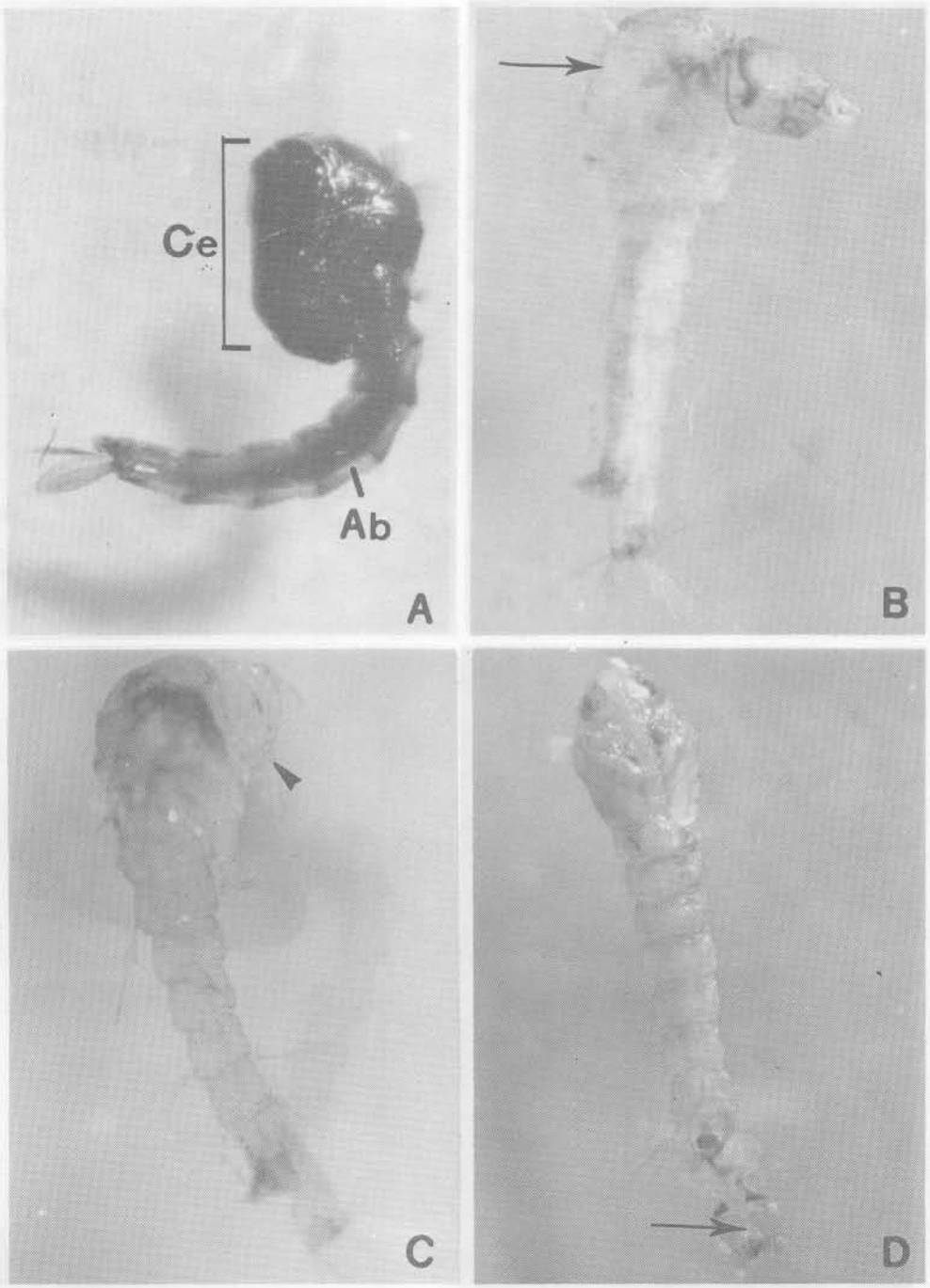

Fig. 2. Gradation of morphogenetic changes of *An. dirus* s.s treated with Methoprene at 0.0001 mg/l: **A**, normal pupa (Ce = cephalothorax,Ab = abdomen); **B**, abnormal pre-pupa with deformed cephalothorax (arrow); **C**, deformed pre-pupa (arrowhead); **D**, grossly deformed pre-pupa with attachment of larval exuvia (arrow) at the posterior end. (A-D x16)

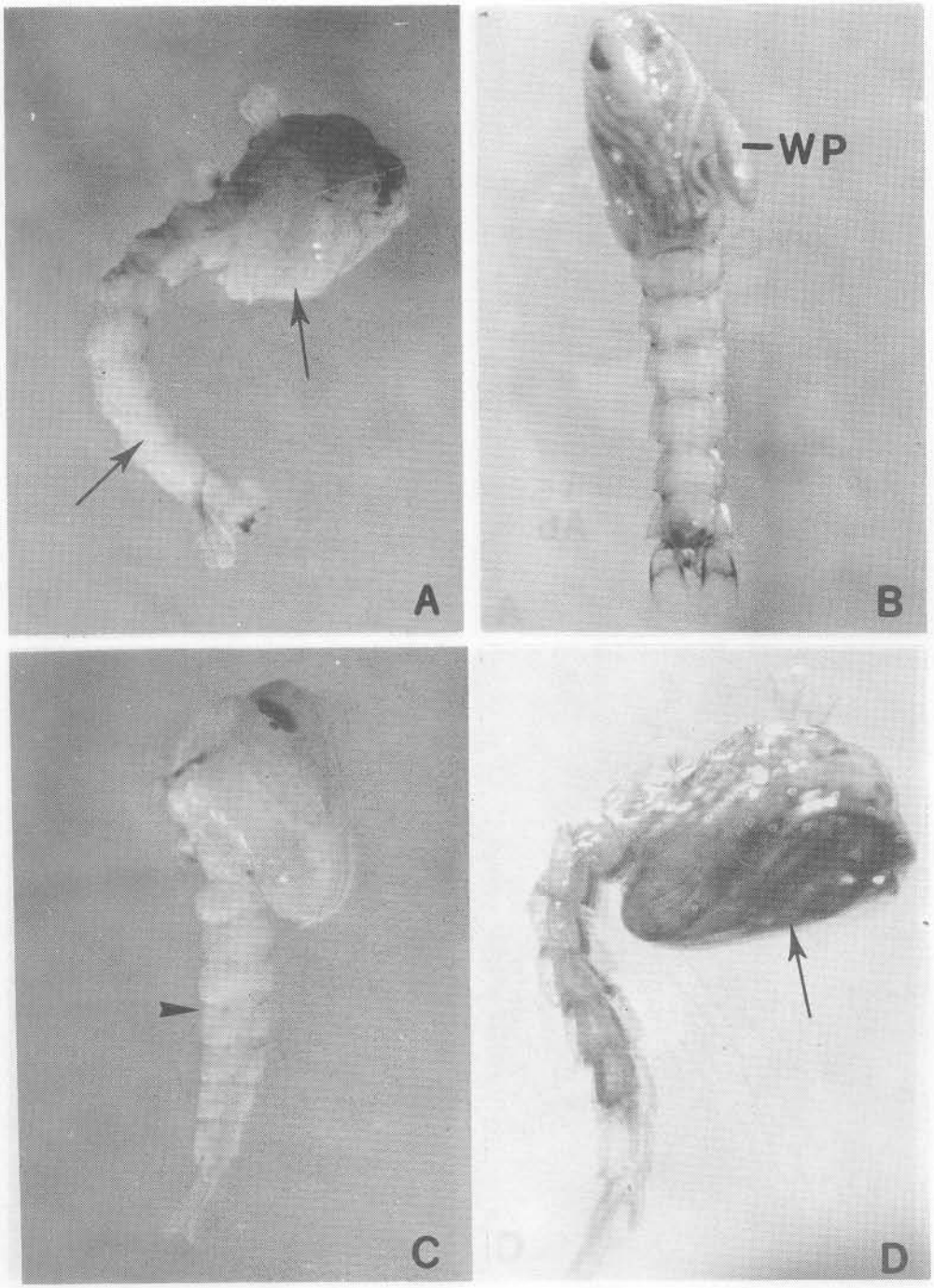

Fig. 3. Various forms of morphogenetic aberrations induced by Methoprene in pupae of *An. dirus* s.s : **A**, pupa, not completely melanized or hardened (arrows); **B**, deformed albino pupa where wing pads (WP) are not appressed to the body; **C**, extended albino pupa (arrowhead); **D**, brown pupa with enlarged cephalothorax (arrow) (A-D x16).

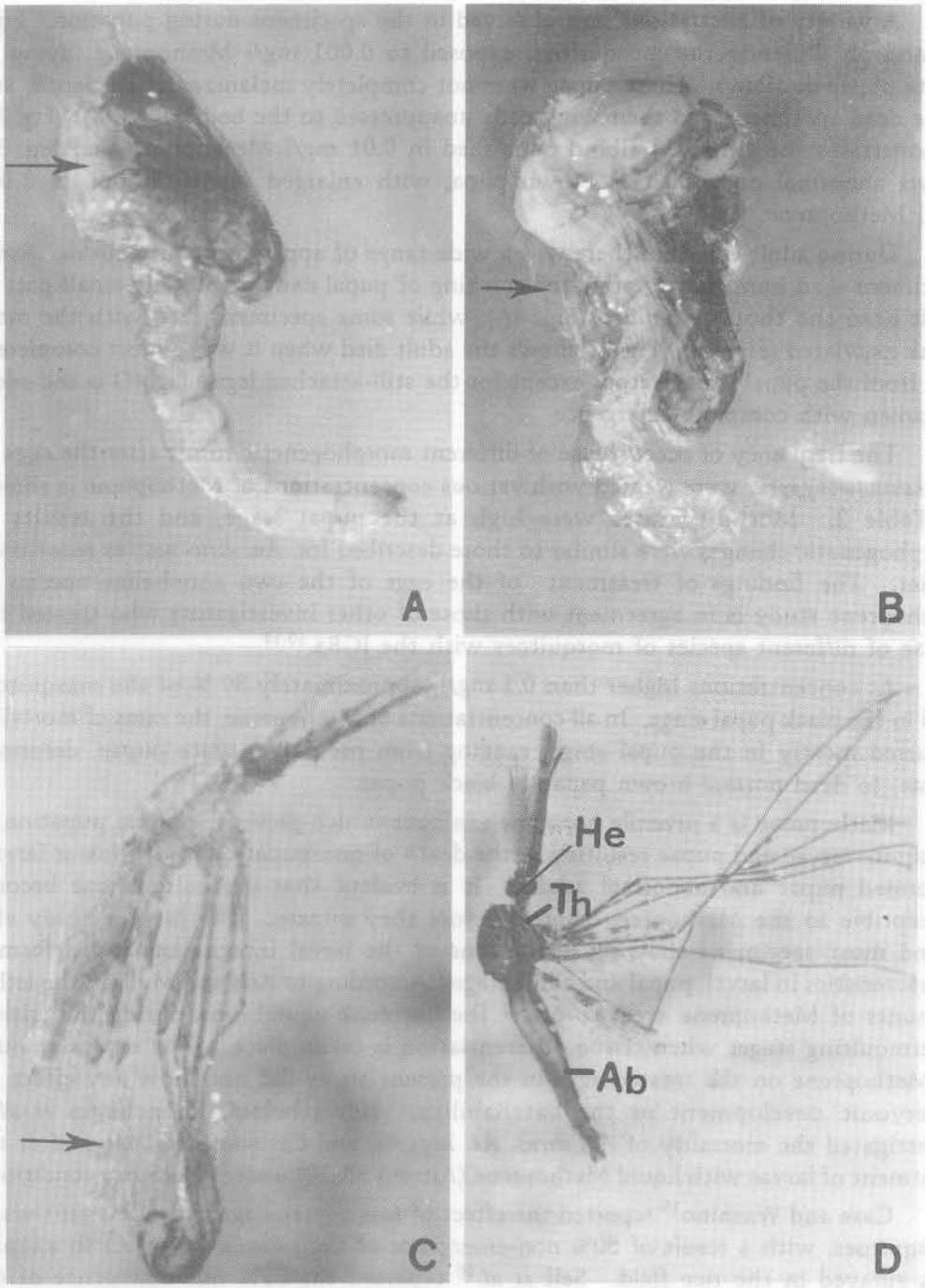

Fig. 4. Morphogenetic aberrations induced by Methoprene in adult mosquitoes when eggs of *An. dirus* s.s were treated: **A**, a specimen died immediately after cracking of pupal exuvia and adult thorax partially extruded (arrow); **B**, adult's main trunk partly exuviated (arrow); **C**, adult completely exuviated but still attached to pupal exuvia by tarsi (arrow); **D**, adult completely exuviated (He = head, Th = thorax, Ab = abdomen). (A-C x 16, D x 10).

A variety of aberrations was observed in the specimens during pupation. Figs. 3A and 3B illustrate the mosquitoes, exposed to 0.001 mg/l Methoprene, dying as white pupae or albino. These pupae were not completely melanized or hardened, and some dead specimens had their wing pads unappressed to the body (Fig. 3B). Fig. 3C demonstrates the extended albino pupa died in 0.01 mg/l Methoprene, and Fig. 3D shows abnormal pupa died as brown pupa, with enlarged cephalothorax, in 0.001 mg/l Methoprene.

During adult eclosion, there was a wide range of apparent abnormalities. Some specimens died immediately after the cracking of pupal exuvia and only small part of adult head and thorax extruded (Fig. 4A), while some specimens died with the main trunk exuviated (Fig. 4B). Fig 4C shows the adult died when it was almost completely free from the pupal exoskeleton, except for the still-attached legs. Fig 4D is the adult specimen with complete emergence.

The frequency of occcurrence of different morphogenetic forms after the eggs of *An. sawadwongporni* were treated with various concentrations of Methoprene is shown in Table 2. Mortality rates were high at the pupal stage, and the results of morphogenetic changes were similar to those described for *An. dirus* s.s., as mentioned earlier. The findings of treatment of the eggs of the two anopheline species in Methoprene study is in agreement with those of other investigators who treated the larvae of different species of mosquitoes with the IGRs.[10-14]

At concentrations higher than 0.1 mg/l, approximately 30 % of the mosquitoes died in the black pupal stage. In all concentrations of Methoprene, the rates of mortality occurred mostly in the pupal stage, ranging from pre-pupae,white pupae, deformed pupae, to dead normal brown papae or black pupae.

Methoprene is a juvenile hormone analogue which prevents normal pupation of mosquito larvae and pupae resulting in the death of non-pupating fourth-instar larvae, deformed pupae and abnormal adults. It is evident that mosquito larvae become susceptible to the Methoprene shortly before they pupate. The present study also found most specimens showing disruptions of the larval integument and deformed characteristics in larval, pupal and adult stages. According to Aris and Mulla[12], the lethal amounts of Methoprene seem to offset the hormone equilibrium during the critical intermoulting stages when tissue differentiation is taken place. The excess amount of Methoprene on the treated eggs in the present study did not show any effect on embryonic development or the hatchability. Nevertheless, Phonchevin *et al.*[13] investigated the mortality of *An. dirus*, *Ae. aegypti*, and *Cx. quinquefasciatus* after the treatment of larvae with liquid Methoprene (Altosid SR-10) under laboratory conditions.

Case and Washino[15] reported the effect of Methoprene against permanent water mosquitoes, with a result of 50% non-emergence of *Cx. tarsalis* when 0.1 lb a.i./acre was sprayed in the rice field. Self *et al.*[16] reported the 52% non-emergence of *Cx. quinquefasciatus* at 35 days after treatment with 1 mg/l Methoprene in septic ditches. Comparative mortality rates of *Cx. quinquefasciatus* exposed to the water solution (Altosid SR-10) and to the experimental wettable power (ZPA-1019) in septic ditches were reported by Williams and Palmisana.[17]

In Thailand, larvicides are widely used for mosquito control. Questions have been raised as to whether the larvicides applied would cause environmental pollution and hazards to man and non-target organisms. Methoprene and a similar type of insect juvenile hormone analogue are a novel group of insecticides that are very safe to man and environment. To evaluate the appropriate dosage rates for the anopheline control, especially these two species which are not commonly found in the residential area, baseline studies on the field application, population dynamics of the vectors in the studied areas, and the epidemiology of related public health diseases should be carried out simultaneously. Thus, in the future, Methoprene may be useful as a candidate larvicide for the mosquito control programme.

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บทคัดย่อ

จากการศึกษาผลของสารเมโทพรีนต่อการฟักออกจากไข่และการเจริญเติบโตของยุงก้นปล่องสองสปีชีส์, *Anopheles dirus* และ *An. sawadwongpporni* ในห้องปฏิบัติการ (28±1.7 °ซ, ความชื้นสัมพัทธ์ 86±3% เวลาที่มีแสง 12 ชั่วโมง) พบว่าสารเมโทพรีนที่ความเข้มข้นระหว่าง 0.0001-1 มก/ลิตร ไม่มีผลต่ออัตราการฟักออกจากไข่ของลูกน้ำยุงก้นปล่องทั้งสองสปีชีส์ แต่มีผลต่อการเจริญเติบโตและเมเทมอร์โฟซิส

ลูกน้ำยุงก้นปล่องที่ฟักออกมาจากไข่ซึ่งแช่ในสารเมโทพรีนเป็นเวลา 24 ชั่วโมง ส่วนใหญ่จะตายในระยะดักแด้โดยมีความผิดปกติทางรูปร่างที่แตกต่างกัน