

Changes in susceptibility to pyriproxyfen, a JH mimic,  
during late larval and early pupal stages of  
*Culex pipiens molestus*

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**Abstract:** Effects of pyriproxyfen were investigated in 5 strains of *Culex pipiens molestus* containing an organophosphorus insecticide resistant strain and a low-level resistant strain against methoprene and pyrethroids. Pyriproxyfen showed potent JH mimic activity in all strains regardless of insecticide resistance. When the susceptibility to pyriproxyfen was examined during the fourth instar to early pupa by 1 hr immersion test method, the susceptibility changed markedly depending on the developmental stages. The maximum susceptibility appeared from the fourth day of the fourth instar to 30 min after pupation, and the susceptibility was very low before and after that period. The stage of maximum susceptibility to pyriproxyfen did not coincide with that to methoprene; from the third day of the fourth instar to pupation, suggesting the involvement of different factors in the mode of action. Results of injection test in early pupa indicated that decline in the JH mimic susceptibility mainly depends on the decrease of cellular sensitivity to the JH mimics in the pupa, not on the change in their permeability through the cuticle.

#### INTRODUCTION

Insecticide resistance has been a serious impediment in controlling the vectors of human diseases. In mosquitoes, many instances of the resistance to conventional insecticides have been reported. Insect growth regulators such as juvenile hormone (JH) mimics have an advantage in control of resistant mosquitoes because of their unique mode of action (Spielman and Williams, 1966) and relatively low toxicity to non-target organisms (Steelman and Schilling, 1972; Steelman *et al.*, 1975). However, the stage of mosquitoes at which the insecticide is applied is important in practical use of JH mimics since the susceptibility changes greatly during de-

velopmental stages (Noguchi and Ohtaki, 1974).

Pyriproxyfen, a newly developed JH mimic, which has potent activity against mosquitoes (Hatakoshi *et al.*, 1987; Ali *et al.*, 1995), has been used as a mosquito larvicide (Kawada *et al.*, 1988; Suzuki *et al.*, 1989; Chavasse *et al.*, 1995). In the present paper, the susceptibility to pyriproxyfen in several strains of *Culex pipiens molestus* including insecticide-resistant ones, and the change in susceptibility during late larva to early pupa were investigated.

#### MATERIALS AND METHODS

*Insects.* Five strains of *C. p. molestus* maintained in our laboratory were used for the experiments. The Tokyo U, Totsuka and

Sumitomo strains are susceptible to insecticides, the Shinjuku strain is resistant to organophosphorus insecticides (Kono and Tomita, 1993) and the Shizuoka strain is resistant to pyrethroids and methoprene at low level (Yasutomi, unpublished data). Insect diet (Oriental Yeast Co.) and sucrose solution (3%) were fed to the larvae and the adults, respectively.

**Insecticides.** Pyriproxyfen (98.4% in purity) donated by Sumitomo Chemical Industries, Ltd., was used in the experiments. As reference chemicals, methoprene (92.8%, Otsuka Chemical Co., Ltd.), JH-III (88.0%, Sigma Chemical Co.) and fenitrothion (99.0%, Sumitomo Chemical Co., Ltd.) were used.

**Test methods.** Three methods described below were adopted for the experiments.

(1) Appropriate concentrations (series of five to seven different concentrations) of chemicals were dissolved in 100 ml of distilled water (containing ethanol less than 0.2% as a solvent) in a plastic cup where 20 mosquito larvae at 7 days after the hatch (mostly third day of the last instar) were released. Susceptibility of larvae of the five strains to pyriproxyfen and methoprene was compared using this method.

(2) Twenty to forty larvae or pupae were released into a plastic cup containing 100 ml aqueous solution of the test chemicals (containing ethanol or acetone less than 0.1%) for 1 hr, then the insects were washed with tap water on gauze sheet and released again into the distilled water. Using this method, susceptibility of newly emerged pupae to pyriproxyfen, methoprene and JH-III was tested. Changes in susceptibility during late larval and early pupal stages were also tested by the method in Tokyo U and Totsuka strains.

(3) Using a microsyringe with a fine glass tip, 0.05  $\mu$ l of pyriproxyfen aqueous solution was injected into the body cavity of the pupa of Tokyo U strain which were kept on ice. The treated pupae were released into 100 ml of distilled water in a cup.

A small amount of diet (Insect diet, Oriental Yeast Co.) was fed to the larvae and the effect of the chemicals was assessed after all the adults emerged in the control plot. As for JH and JH mimics, individuals with various degrees of defect at adult eclosion and those that died during larval and pupal stages, were counted as effective. For fenitrothion, dead individuals were counted 1 day after the treatment. Based on the numbers of affected individuals being corrected by Abbott's formula [ $\{(\% \text{ effect}) - (\% \text{ dead in control})\} / \{100 - (\% \text{ dead in control})\}$ ], EC-50's within 95% confidence limits were calculated according to a computer program of probit analysis.

## RESULTS

### 1. Effect of pyriproxyfen and methoprene on different strains

Effect of pyriproxyfen and methoprene on the larvae treated 7 days after hatch was estimated in the 5 strains (Table 1). The two JH mimics both showed very potent insecticidal activity to the mosquito larvae. EC-50 values of pyriproxyfen varied between 0.15 (Sumitomo) and 0.67 ppb (Tokyo U) among the strains regardless of insecticide resistance, while the values of methoprene varied more widely between 0.23 (Tokyo U) and 5.32 ppb (Shizuoka). Against methoprene, the Shizuoka strain showed the highest EC-50 value (5.32 ppb) 20 times higher than EC-50 value of Tokyo U, indicating low level of resistance. The value of the Shinjuku strain (4.89 ppb) followed that of Shizuoka. When newly emerged pupae within 30 min following pupation were immersed for 1 hr in the test solutions (method 2, Table 2), difference in the effective concentration between pyriproxyfen and methoprene became greater than in the test of continuous immersion (method 1) in all strains. Effect of pyriproxyfen was not significantly reduce in the 1 hr immersion test comparing with the results in the continuous immersion test, while effect of

Table 1. EC-50 of pyriproxyfen and methoprene in continuous immersion test in 5 strains of *Culex pipiens molestus*.

Strain	EC-50 (95% confidence limits) in ppb	
	Pyriproxyfen	Methoprene
Tokyo U*	0.67 (0.45-0.94)	0.23 (0.09-0.43)
Totsuka*	0.16 (0.08-0.25)	2.99 (2.08-4.30)
Sumitomo*	0.15 (0.12-0.19)	0.57 (0.28-1.51)
Shinjuku**	0.29 (0.18-0.45)	4.89 (3.73-6.42)
Shizuoka***	0.54 (0.44-0.66)	5.32 (4.36-6.50)

Larvae 7 days after hatch (mostly third day of the last instar) were used for the continuous immersion test.

\* susceptible to insecticides.

\*\* resistant to organophosphorus insecticides.

\*\*\* resistant to pyrethroids and methoprene.

Table 2. EC-50 of pyriproxyfen, methoprene and JH-III in 1 hr immersion test in 4 strains of *C. p. molestus*.

Strain	EC-50 (95% confidence limits)		
	Pyriproxyfen (ppb)	Methoprene (ppb)	JH-III (ppm)
Tokyo U	0.56 (0.45-0.70)	22.9 (16.8-31.2)	12.4 ( 3.8-106 )
Totsuka	0.28 (0.22-0.38)	9.1 ( 6.7-12.0)	21.9 ( 9.8- 40.2)
Shinjuku	0.14 (0.10-0.19)	24.9 (16.8-41.2)	36.4 (18.3- 75.1)
Shizuoka	0.33 (0.24-0.43)	57.9 (42.8-82.5)	33.2 (11.8-180 )

Pupae within 30 min following pupation were used for the test.

Table 3. Change of EC-50 of pyriproxyfen and methoprene during 4th instar and early pupa.

Chemical	Strain	EC-50 (95% confidence limits)						
		4th instar				pupa		
		1st day	2nd day	3rd day	4th day	5-0 hr before pupation	0-30 min after pupation	3-4 hr after pupation
Pyriproxyfen (ppb)	Tokyo U	≥32	>32 (14.8%)	6.2 (3.9-17.3)	0.62 (0.53-0.72)	0.63 (0.36-1.19)	0.56 (0.46-0.70)	>32 (27.8%)
	Totsuka	>32 (10.5%)	13.7 (7.4-32.7)	1.3 (0.17-2.1)	0.24 (0.18-0.58)	0.41 (0.25-0.56)	0.28 (0.22-0.38)	>16 ( 6.7%)
Methoprene (ppb)	Tokyo U		>100 (15.4%)	9.6 (5.5-14.0)	7.5 (5.0-9.9)	11.4 (7.9-14.8)	22.9 (16.8-31.2)	≥100
Fenitrothion (ppm)	Tokyo U		0.10 (0.10-0.12)	0.11 (0.10-0.13)	0.11 (0.10-0.12)		1.8 (1.3-2.6)	>16 (13.3%)

One-hour immersion test was adopted.

methoprene in the 1 hr immersion test was reduced obviously; EC-50 values were 3 to 10 times higher than those in the continuous immersion test.

2. Change in susceptibility to JH mimics during late larva and early pupa  
When 1 hr immersion test (method 2)

was carried out during developmental stages from the first day of fourth instar to several hours after pupation, the effect of JH mimics greatly fluctuated (Table 3). The susceptibility to pyriproxyfen and methoprene was very low at early fourth instar (first and second days). The susceptibility to pyriproxyfen became maximum

Table 4. Effect of pyriproxyfen injection to newly pupa on the metamorphosis.

Stage (after pupation)	Dose (ng)	Number treated	Number of			% effective*
			Normal adults	Abnormal adults	Deaths in pupa	
0-30 min	1.0	13	1	1	11	89.7
	0	12	9	1	2	0
2-3 hr	1.0	9	5	2	2	25.9
	10.0	11	4	2	5	51.5

Pupae of Tokyo U strain were used.

\* with Abbot's correction.

at the last day of larva and the maximum continued until just after pupation, while the maximum susceptibility to methoprene appeared at the last two days of the final instar and decreased at around pupation. The susceptibility to both JH mimics decreased to very low level after 3-4 hr following pupation.

On the contrary, the susceptibility to fenitrothion, a typical conventional insecticide, was constantly maintained throughout the last larval instar, but decreased after pupation.

### 3. Effect of injection of pyriproxyfen

Susceptibility to pyriproxyfen was tested by injecting to the early pupae in order to know the cause of its remarkable reduction at pupal stage (Table 4). One ng of pyriproxyfen showed to be highly effective to newly emerged pupae within 30 min following pupation, but 10 ng of pyriproxyfen did not show complete effect to the pupae at 2-3 hr following pupation. The result indicates that the decrease in susceptibility depends on factors other than pupal cuticle hardening.

## DISCUSSION

Pyriproxyfen showed potent JH-like activity against not only susceptible strains but also insecticide-resistant strains of *C. p. molestus*. It is noteworthy that pyriproxyfen was effective to a methoprene-resistant strain, Shizuoka. It is well-known that effect of JH mimics fluctuates depending on the developmental stage of

target insects, and becomes maximum at the late stage of last instar larva or nymph when no JH exists and molting hormone exists in the haemolymph (Shimada, 1982). Susceptibility of *C. p. molestus* to two JH mimics, pyriproxyfen and methoprene, also reached the maximum at the late fourth instar. If the fluctuation of JH mimics activity depends only on the change in hormonal sensitivity to JH and the presence of molting hormone in insects, the maximum susceptibility of the mosquito to two JH mimics coincides with each other and with that of JH. However, the stages of maximum susceptibility to two JH mimics did not coincide precisely; that to pyriproxyfen extended to just after the pupation, while that to methoprene began on the third day of fourth instar (one day earlier than pyriproxyfen) and lasted until pupation. Furthermore, no correlation was found between the activity of two JH mimics among the strains. The discrepancy in susceptibility to two JH mimics suggests that other mechanisms such as degradation and permeability of the JH mimics or more complicated mechanisms are important factors in the expression of the JH mimic activity. The results that the effect of pyriproxyfen in 1 hr immersion is comparable to that in continuous immersion, though the effect of 1 hr immersion with methoprene was much less than the effect of continuous immersion, support at least the existence of a different degrading system to two JH mimics.

As for the sudden decline of JH effect

after pupation, results of injection of pyriproxyfen into the pupa indicate that innate sensitivity to JH decreased after pupation regardless of the cuticular hardening. Insensitivity to JH mimics at pupal stage seems to be characterized by a hormonal circumstances without both JH and molting hormone, though that at early last instar, by the presence of both hormones. Susceptibility to fenitrothion, an organophosphate, also decreased at this stage when the apolysis begins. Susceptibility to insecticides seems to change through the reconstruction of tissues including targets of insecticides.

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## 摘 要

チカイエカの終齢幼虫期から蛹初期における  
ピリプロキシフェン感受性の変化

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ピリプロキシフェンのチカイエカに対する効力を検討した。有機りん剤抵抗性および、ピレスロイド、メトブレン低レベル抵抗性系統を含む5系統で浸漬試験を行ったが、薬剤抵抗性には関係なく、どの系統に対しても高い効力が認められた。薬液に1時間浸漬する方法により終齢幼虫初期から蛹初期のピリプロキシフェン感受性を調べたところ、感受性は発育段階に従って大きく変化した。感受性ピークは終齢4日目から蛹化後30分まで続いたが、その前後では著しく低かった。一方、メトブレンに対する感受性ピークの期間は終齢3日目から蛹化前までで、ピリプロキシフェンとはずれていた。ピリプロキシフェン感受性の蛹化後の急激な低下が蛹皮の硬化と関連があるかを注射によって確かめたが、結果は否定的で、原因は薬剤の表皮透過性が低下するためではなく、この時期に体内のJH感受性が急激に低下するためと考えられた。