EFFECTS OF SELECTED INSECTICIDES ON *ROMANOMERMIS CULICIVORAX*, A MERMITHID NEMATODE PARASITE OF MOSQUITO LARVAE

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ABSTRACT. Dosage-mortality tests conducted with preparastic (infective) stage *Romanomermis culicivorax* Ross and Smith showed that insecticide concentrations of 0.0021 ppm chlorpyrifos, 0.018 ppm naled, 0.36 ppm propoxur, 1.06 temephos, 1.10 ppm fenthion, 2.95 ppm methoprene, 4.36 ppm diflubenzuron, and 6.78 ppm malathion caused a 50% loss of swimming activity and, consequently, impaired the ability of the nematode to locate and infect mosquito larvae. Chlorypyrifos was significantly ($P<0.05$) more toxic to the infective stage than the other insecticides tested. Malathion caused significantly ($P<0.05$) less mortality to the infective stage of the nematode than the other insecticides tested.

The infective stage was significantly ($P<0.05$) less susceptible to all the insecticides tested, except naled, at the LC_{90} level than were 2nd instar *Culex quinquefasciatus* Say larvae.

Naled and propoxur significantly reduced ($P<0.05$) the infectivity of the preparasitic stage at the LC_{50} response level established for 2nd instar *Cx. quinquefasciatus* larvae. Chlorpyrifos, malathion, fenthion, temephos, diflubenzuron and methoprene caused no significant reduction ($P>0.05$) of the infectivity of *R. culicivorax* at their respective LC_{50} response levels for 2nd instar *Cx. quinquefasciatus* larvae.

Treatment of parasitized larvae, 24 hr after infection with insecticides, had no adverse effect on the viability of the postparasites that emerged. Juvenile nematodes, less than 7 days after emerging, molted to adults which mated and laid viable eggs after treatment with insecticides.

The rate of egg hatching after exposure to malathion, fenthion, and methoprene was significantly lower ($P<0.05$) than that of eggs exposed to propoxur and naled, however, no significant differences in percent egg hatch ($P<0.05$) existed between eggs treated with insecticides and the untreated eggs.

The prospect of utilizing the mermithid nematode, *Romanomermis culicivorax* Ross and Smith (=*Reesimermis nielseni* Tsai and Grundmann auctt. partim.) as a biological control agent of mosquito larvae has been shown to be quite promising since recent advances in mass rearing techniques (Petersen and Willis 1972a) and field applications (Petersen and Willis 1972 b, 1974, 1975, 1976, and Petersen et al. 1973).

Mitchell et al. (1974) reported that insecticide susceptibility tests with *R. culicivorax* to temephos, dieldrin, and Gamma-HCH at concentrations lethal to mosquito larvae indicated that such treatments had no adverse effect on the infective stages of the nematode. Finney et al. (1977) showed that methoprene at 5 to 50 ppb had no effect on the infectivity of the infective stage and that normal parasitic development occurred in mosquitoes that had been exposed to methoprene at these concentrations. Levy and Miller (1977) showed that 0.001 ppm temephos and chlorpyrifos, 0.1 ppm malathion, 0.003 ppm fenthion, and 0.005 ppm diflubenzuron and methoprene had no adverse effects on infectivity and viable postparasites were produced.

The dependency on chemical control of mosquitoes has resulted often in the development of insecticide resistance. *R. culicivorax* offers a possible alternative control measure that, when included in an integrated control program, could reduce insecticide usage.

This study shows the dosage-mortality responses of infective stage *R. culicivorax* to insecticides currently employed in mosquito control and ascertains the effects of these insecticides on parasitic

stages, postparasitic stages, and hatching of the nematode eggs.

MATERIALS AND METHODS

Emulsifiable concentrate formulations of the organophosphates chlorpyrifos, fenthion, malathion, naled, and temephos; the carbamate propoxur; and the insect growth regulators (IGR's) methoprene and diflubenzuron were used in this study.

The infective stage of the parasite was obtained in moist sand that contained eggs and adults from the U.S.D.A. Gulf Coast Mosquito Research Laboratory, Lake Charles, Louisiana. Aliquots of moist sand were flooded with sodium thiosulfate treated water (0.15 ml of 5.0% solution per 3.78 liters of tap water). Suspended eggs were allowed to settle to the bottom (approximately 30 min) after which the water was carefully aspirated with a disposable pipet to remove previously hatched infective stage nematodes. The sample was reflooded and 4 hr were allowed for hatching of the eggs. The number of infective stage nematodes per ml of suspension was estimated using the procedure described by Petersen and Willis (1970).

A test replicate consisted of a control plus 5 insecticide concentrations prepared by serial dilution using dechlorinated water. The serial dilutions were prepared in 20 ml glass vials that contained 1.0 ml of insecticide solution to which was added 20–30 infective stage nematodes in 1.0 ml of water. Thus, each vial contained a final volume of 2.0 ml. The vials were tightly capped and held for 24 hr at 27±2°C.

Loss of normal motility was used as a criterion to determine the effect of the insecticide on the infective stage nematodes. The contents of the vials were examined by placing 0.2 ml of the test concentrations in each of the 10 depressions of a Boerner slide. The numbers of actively swimming or moribund infective stage nematodes were counted on a black microscope stage using an illuminator at 7.5 v intensity, spaced approximately 30 cm from the slide at an angle at 45°. The criteria for determining moribundity were one or more of the following: (a) the inability of the nematode to move out of a 1.0 mm radius circle within 30 sec after being touched by a probe, (b) the loss of normal undulating motion, (c) swimming in small circles or constant coiling (the loss of directed movement), and (d) the presence of any indentations in the cuticle which caused a "bent-like" appearance.

The dosage-mortality response of 2nd instar *Culex quinquefasciatus* Say larvae from a susceptible laboratory colony was determined using the procedure described by the World Health Organization (1963) for determination of susceptibility or resistance of mosquito larvae to insecticides.

Second instar mosquito larvae were used because this stage is the most susceptible to the infective stage nematode (Petersen and Willis 1970); and the data obtained later were used for tests of infectivity following exposure to the various insecticides.

Dosage-mortality regression lines were determined using the probit analysis program described by Daum (1970). Dosage-mortality data that varied to the extent that nonsignificant regression lines resulted were "eyefitted" on log probit paper.

To determine the effects of the insecticides on the parasitic stage of the nematode, 1,000 *Cx. quinquefasciatus* larvae and 20,000 6 hr-old infective stage *R. culicivorax* were added to a 28 × 17 × 4.5 cm enameled pan that contained 1.0 liter of water. After 24 hr exposure, the nematodes were removed by washing the mosquito larvae on a No. 60 Tyler sieve. Groups of 100 mosquito larvae were placed in beakers that contained 24 ml of dechlorinated water and then were poured into 600 ml beakers that contained 226 ml of dechlorinated water. A 1.0 ml solution of the appropriate insecticide was then added to make a final volume-concentration of 250 ml. The in-

secticide concentrations used were the LC_{50}'s that had been previously established by the authors for 2nd instar *Cx. quinquefasciatus* larvae. The beakers that contained mosquito larvae were placed in a water bath at 27±2°C for 24 hr. Dead mosquito larvae were counted and removed. The surviving larvae were poured into pans that contained 1.0 liter of water and then were reared to the 4th instar on a diet of 0.1 g ground rabbit pellets. The water in each pan was aerated and diet added daily. Six days after exposure to both the nematodes and insecticide, the dead and surviving larvae were counted and placed in holding containers to allow the postparasitic stages to emerge. The postparasitic stage nematodes then were sexed and counted.

The effect of the selected insecticides on the postparasitic stage nematodes was determined by exposing the nematodes within 1 week after they had emerged to the insecticides in groups of 20 females and 20 males in 600 ml glass beakers that contained 250 ml of insecticide solution (or 250 ml of untreated water for the checks). The concentrations corresponded to the maximum allowable insecticide label rate of application for 0.4 ha of water 0.15 and 0.30 m in depth. Tests were 24 hr in duration at 27±2°C and each test was replicated twice. The postparasitic stage nematodes were washed in 250 ml of water and transferred to 30 ml plastic cups that contained 1.0 cm of sterile coarse sand in 10 ml of water. After 2 weeks, the dead nematodes and the water were removed leaving the sand moist but not flooded. The containers were placed inside a covered desiccator that had a water saturated atmosphere so that the sand remained moist. After 4 weeks, the sand cultures were flooded with 10 ml of water and the nematodes eggs were incubated for 8 hr. Estimates of the number of infective stage nematodes for each culture were made according to the procedure described by Petersen and Willis (1970). These tests were conducted in a completely randomized design and the data were analyzed using a nested analysis of variance with Tukey's "w" procedure used to test for differences between treatment means.

The effect of the selected insecticides on nematode egg hatching was determined by flooding aliquots of culture sand that contained *R. culicivorax* eggs with insecticide concentrations which corresponded to the maximum allowable insecticide label rate of application for 0.4 ha of water 0.15 and 0.30 m in depth. The suspension was stirred and 0.2 ml subsamples were pipetted into the depressions of a Boerner slide. The nematode eggs were counted using a stereoscopic dissecting microscope at 15x magnification after all preflood infective stage nematodes had been removed. The Boerner slides were placed in tightly covered desiccators as previously described. The number of infective stage nematodes and unhatched eggs were counted after 8 hr. The statistical analyses were similar to those previously described.

RESULTS

The responses of infective stage *R. culicivorax* and 2nd instar *Cx. quinquefasciatus* to selected insecticides after 24 hr exposure are shown in Table 1. The slopes of the regression lines indicated that the responses of *R. culicivorax* to organophosphate and carbamate compounds were generally more heterogeneous than the responses of 2nd instar *Cx. quinquefasciatus* larvae. The responses of *R. culicivorax* to diflubenzuron and methoprene were more homogeneous than the responses of 2nd instar *Cx. quinquefasciatus* larvae which had been reported by Hsieh and Steelman (1974). With the exception of the IGR's and malathion, the slopes of the ld-p lines for the mosquito larvae were steeper than those for the infective stage nematodes.

No significant difference ($P>0.05$) was detected between the LC_{50} responses of *R. culicivorax* to fenthion and temephos. There were, however, significant differences ($P<0.05$) between all other chemicals.

Table 1. Susceptibility of infective stage *R. culicivorax* and 2nd instar *Cx. quinquefasciatus* larvae to selected insecticides at the LC_{50} response level.

Chemical	*R. culicivorax*			*Cx. p. quinquefasciatus*			Difference Factor
	LC_{50}[1] (ppm)	95% Confidence Interval	Slope	LC_{50} (ppm)	95% Confidence Interval	Slope	
Chlorpyrifos	0.0021[a]	0.0018–0.0024	2.65	0.00024[h]	0.00022–0.00025	8.48	8.7
Naled	0.018[2]		1.36	0.076[k]	0.052 –0.093	11.4	0.24
Propoxur	0.36[b]	0.1900–0.5700	2.24	0.28[l]	0.25 –0.31	4.32	1.0
Temephos	1.06[c]	0.6700–1.4500	1.03	0.00028[h]	0.00025–0.00032	4.68	3786.0
Fenthion	1.10[c]	0.9800–1.2100	3.39	0.0089[j]	0.00860–0.0093	7.56	124.0
Methoprene	2.95[d]	2.4300–3.4700	2.40	0.0026[i3]	0.0009 –0.005	0.68	1135.0
Diflubenzuron	4.36[e]	3.9200–4.8000	4.93	0.000075[g3]	0.00005–0.0001	1.38	58,100.0
Malathion	6.78[f]	5.4500–7.9200	2.90	.051[k]	0.043 –0.063	2.88	133.0

[1] Those LC_{50} values followed by the same letter are not significantly different at the $P<0.05$ level (Tukey's test).
[2] Eye fitted line.
[3] Hsieh and Steelman (1974).

Naled was 4x more toxic to *R. culicivorax* than to *Cx. quinquefasciatus* at the LC_{50} response level. No significant difference ($P>0.05$) was detected between the LC_{50} levels of *R. culicivorax* and *Cx. quinquefasciatus* to propoxur. Significantly less ($P<0.05$) chlorpyrifos was required to cause 50% mortality to *R. culicivorax* than the other insecticides. However, the chlorpyrifos concentration required to kill 50% of the *Cx. quinquefasciatus* larvae was 9x greater than that required for *R. culicivorax.* The other chemicals were many times more toxic to 2nd instar *Cx. quinquefasciatus* larvae than to infective stage *R. culicivorax,* especially at levels required to cause 50% mortality.

No significant differences ($P>0.05$) were obtained between the following pairs of insecticides at the LC_{90} response level for *R. culicivorax:* malathion and temephos, fenthion and propoxur, or diflubenzuron and methoprene (Table 2). At the LC_{90} response level, chlorpyrifos was significantly more toxic ($P<0.05$) to *R. culicivorax* than the other insecticides.

There was a 2.5X difference in the toxicity of naled and propoxur with respect to *R. culicivorax* and *Cx. quinquefasciatus* larvae at the LC_{90} level. The infective stages of *R. culicivorax* were about 20x more tolerant to chlorpyrifos at the LC_{90} response level than were 2nd instar *Cx. quinquefasciatus* larvae (which were significantly different, $P<0.05$). The toxicities of the remaining chemicals to *Cx. quinquefasciatus* larvae were significantly higher ($P<0.05$) than to *R. culicivorax* at the LC_{90} level. The difference factors for these chemicals were greater than 50x with respect to the infective stage nematodes and *Cx. quinquefasciatus* larvae.

No significant difference ($P>0.05$) was detected between the LC_{90} response of 2nd instar *Cx. quinquefasciatus* and the LC_{50} response of infective stage *R. culicivorax* to propoxur. It is also notable that naled was nearly 5x more toxic to infective stage *R. culicivorax* at the LC_{50} response level than to *Cx. quinquefasciatus* larvae at the LC_{90} level.

The parasitic stage of the nematodes

Table 2. Susceptibility of infective stage *R. culicivorax* and 2nd instar *Cx. quinquefasciatus* larvae to selected insecticides at the LC_{90} response level.

Chemical	*R. culicivorax* LC_{90}[1] (ppm)	*R. culicivorax* 95% Confidence Interval	*Cx. p. quinquefasciatus* LC_{90} (ppm)	*Cx. p. quinquefasciatus* 95% Confidence Interval	Difference Factor
Chlorpyrifos	0.0063[a]	0.00050–0.0090	0.00034[e]	0.00031–0.00038	18.5
Naled	0.25[2]		0.099[g]	0.085 –0.43	2.5
Propoxur	1.36[b]	0.8000–5.0300	0.55[h]	0.47 –0.71	2.5
Fenthion	2.63[b]	2.2000–3.4900	0.013[f]	0.012 –0.015	202.0
Diflubenzuron	7.93[c]	7.0400–9.3300	0.00064[e3]	0.00038–0.0015	124,000.0
Methoprene	10.1[c]	8.1400–13.400	0.19[g]	0.077 –0.97	53.2
Malathion	18.8[d]	15.700–24.700	0.14[g]	0.10 –0.28	134.0
Temephos	18.8[d]	11.100–47.100	0.00053[e]	0.00045–0.00068	35,550.0

[1] Those LC_{50} values followed by the same letter are not significantly different at the $P < 0.05$ level (Tukey's test).
[2] Eye fitted line.
[3] Hsieh and Steelman (1974).

survived each insecticide treatment of the host mosquito larvae (Table 3). The rate of infection for all those surviving the insecticide treatments was 100%. The large variation in the number of postparasitic stages that emerged was a result of the variation in the response of the mosquito larvae to the LC_{50} concentration rates of the insecticides after 24 hr exposure. The effects of diflubenzuron were not apparent until 6 days after exposure. The number of postparasites that emerged from the surviving larvae averaged 3.60 per host larva and ranged from 2.47 for the check to 5.90 for the propoxur treatment. All the postparasites appeared healthy and viable. The control mortality after 6 days was 18% and was the lowest except for the temephos treatment (12%). The differences among the treatments appeared to be small.

The postparasitic stage nematodes were able to molt, mate, and lay viable eggs after exposure to the insecticides (Table 4). The yield of infective stage nematodes varied considerably among the treatments and among some of the replicates. The analysis of variance

Table 3. The effects of selected insecticides on the parasitic stage of *R. culicivorax* at the LC_{50} level for 2nd instar *Cx. quinquefasciatus* larvae.

Insecticide	Conc. (ppm)	Number Larvae survived 24 fr	Number Larvae survived 6 days	Number Postparasites Emerged Total	Males	Females	Mean number Postparasites per host
Diflubenzuron	0.00010	90	53	196	180	16	3.70
Chlorpyrifos	0.00024	49	19	54	48	6	2.84
Temephos	0.00028	65	57	241	224	17	4.23
Methoprene	0.0030	89	66	208	197	11	3.15
Fenthion	0.0089	14	11	48	48	0	4.36
Malathion	0.051	70	56	161	146	15	2.88
Naled	0.076	51	40	116	106	10	2.90
Propoxur	0.28	26	10	59	58	1	5.90
Check (H_20)		94	77	190	170	20	2.47
							Mean = 3.60

Table 4. The effects of selected insecticides on the postparasitic stage of *R. culicivorax*.

Insecticide	Mean Concentration[a] (ppm)	Average yield of infective stages
Malathion	0.30	3733[b]
Naled	0.15	4116[b]
Propoxur	0.038	5460[b]
Chlorpyrifos	0.030	14959[c]
Fenthion	0.022	7334[b]
Temephos	0.022	3917[b]
Methoprene	0.015	8228[b]
Diflubenzuron	0.015	16333[c]
Control (H_2O)		3367[b]

[a] Maximum allowable insecticide (AI) label rate of application for 0.4 ha of water 0.15 in depth.

[b, c] Means not followed by the same letter are significantly different from each other at $P<0.05$ level of probability.

showed that there were no significant differences ($P>0.05$) between the concentrations within chemicals. However, a highly significant difference ($P<0.01$) was detected among chemicals. Postparasites exposed to propoxur, malathion, temephos, naled, and the dechlorinated water check yielded significantly fewer ($P<0.05$) infective stage nematodes 6 weeks after flooding than chlorpyrifos and diflubenzuron. The yields obtained for the methoprene treatment did not differ significantly ($P>0.05$) from all the other insecticides.

The percentage hatch of eggs exposed to selected concentrations of insecticides is shown in Table 5. The percent hatching rate was quite low for eggs exposed to the various insecticides and the untreated eggs (below 35%) and apparently was caused by undeveloped eggs. The range was from 6.4 to 34.5% with a mean of 19.1% for those exposed to insecticide. The untreated control egg hatch averaged 15.8%. No significant differences ($P>0.05$) existed among concentrations of any insecticide. The rate of hatching was significantly different ($P<0.01$) among insecticides. The rates of hatching were significantly lower ($P<0.05$) for eggs exposed to malathion, fenthion, and

Table 5. The effects of selected insecticides on the hatching rate of *R. culicivorax* eggs.

Insecticide	Mean Concentration[a] (ppm)	Percent Hatched
Malathion	0.30	12.2[b]
Naled	0.15	29.8[c]
Propoxur	0.038	33.6[c]
Chlorpyrifos	0.030	17.6[b]
Fenthion	0.022	12.2[b]
Temephos	0.022	19.6[b]
Diflubenzuron	0.015	18.4[b]
Methoprene	0.015	10.4[b]
Control (H_2O)		15.8[b]

[a] Maximum allowable insecticide (AI) label rate of application for 0.4 ha acre of water 0.15 in depth.

[b, c] Means not followed by the same letter are significantly different from each other at $P<0.05$ level of probability.

methoprene compared to propoxur and naled treatments. Significantly ($P<0.05$) more eggs hatched that had been exposed to propoxur (33%) than in the untreated control (15.8%).

DISCUSSION

The results of this study indicated that insecticide concentrations of 0.0021 ppm for chlorpyrifos, 0.018 ppm for naled, 0.36 ppm for propoxur, 1.06 ppm for temephos, 1.10 ppm for fenthion, 2.95 ppm for methoprene, 4.36 ppm for diflubenzuron, and 6.78 ppm for malathion caused a 50% loss of motility in the infective stage nematodes. The infectivity of the nematode would be impaired at these rates. The data reported in this study in part agree with the recent findings of Levy and Miller (1977) except for the acute mortality response of the infective stage nematodes to 0.004 ppm chlorpyrifos. They reported that this concentration of chlorpyrifos did not affect the swimming activity of the infective stage nematodes. However, they stated that 0.004 ppm chlorpyrifos caused a significantly lower infection rate in the mosquito larvae compared to their controls. One can predict from the results of the present study that 0.004 ppm chlor-

pyrifos causes between 50 and 90% loss of swimming activity in the infective stage nematode. This does not support the "delayed inhibitory effect on host penetration" as they reported for 0.004 ppm chlorpyrifos.

The dosage mortality data showed that the infective stage nematodes were not capable of surviving treatment that would provide concentrations equivalent to that in an 0.4 ha of water 0.15 m in depth treated at the maximum rate on the insecticide label for naled and chlorpyrifos. On the other hand, fenthion, malathion, temephos, propoxur, diflubenzuron, and methoprene would not have any detrimental effects on the motility of the infective stages if the conditions described above were used, nor would there be any adverse effects on the egg or postparasitic stages. When propoxur and naled were used at rates lethal to 2nd instar mosquito larvae, significant loss of infective stage motility occurred.

Although viable postparasites were recovered from infected mosquito larvae that had been treated with lethal concentrations of insecticides, the abundance of the postparasitic stage nematodes was limited because many of the larvae were killed before the postparasitic stage emerged. The results showed that IGR's such as methoprene and diflubenzuron have the advantage of allowing more of the postparasitic stage nematodes to emerge from the mosquito larvae than conventional insecticides, because the IGR's tend to lengthen the larval life cycle allowing the development of immature parasites before the treated larvae die.

It is not certain from these results that the effects of the insecticides on the infected mosquito larvae altered the sex ratio of the postparasitic stages. The abundance of males and paucity of females may not have been the result of the infection rate that averaged 3.6 parasites per host. Petersen (1972) observed a higher abundance of males only when the infection ratio of parasites to host greatly exceeded 3.0; and he also noted that stressing conditions such as starvation and crowding induced a higher ratio of males to females. The results indicated that stress was placed on the mosquito or nematode larvae. Although the control yielded slightly more females, it was not significant. It appeared likely that the small numbers of females that were obtained from the treatments with fenthion and propoxur were not attributable to random sampling error. Larval mortality 24 hr after treatment with propoxur and fenthion was extremely higher than the LC_{50} for uninfected larvae; and this suggests that infected larvae are more susceptible to fenthion and propoxur than noninfected larvae. Therefore, there is no doubt that the infected larvae were stressed resulting in more male adults of *R. culicivorax* than females.

It was demonstrated in this study that a 2nd generation of infective stage nematodes still could be obtained after treating less than 1 week old juveniles (pre-molt postparasitic stage). The juveniles were always alive 24 hr after exposure. Infective stage nematodes were recovered 6 weeks after treatment, evidence that the juveniles molted to adults which mated and deposited viable eggs. However, there was considerable variation between chemical treatments not attributable to random sampling error. Since the dechlorinated water treatments (controls) yielded the least number of infective stages, it appears unlikely that the observed variation between the insecticide treatments was a result of any adverse effects of the insecticides on the postparasitic nematodes. What caused this variation is not certain. In some of the treatments and controls dead adults were observed in a mass on the surface of the sand. The cause of such mass mortality of postparasites is unknown. One explanation may be that too many postparasitic stages were confined in a small volume of water, and consequently they succumbed to their own waste and metabolic products. This still leaves the question as to why this occurred in only about one-half of the treatments. Therefore, insecticides at the concentrations tested did not interfere with the recycling potential of the nematode.

None of the insecticides at the concentrations tested had detrimental effects on the egg hatching of *R. culicivorax*. The number of eggs that hatched in the propoxur treatment was significantly higher ($P<0.05$) than the control, and this may be an indication that propoxur stimulated or increased the egg hatching rate.

Data obtained in this study indicate that *R. culicivorax* could be used in an integrated mosquito control program provided the application of insecticides was carefully monitored. This could reduce the amount of insecticides used and allow the biological agent to become established in the breeding area.

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