rence of overexposure, particularly since compounds selected for clothing repellent tests are also screened for hazardous compounds, toxicants and chemical structure prior to testing.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Johnnie Jackson in conducting these studies.

*References Cited*

Linduska, J. P. and F. A. Morton. 1947. Determining the repellency of solid chemicals to mosquitoes. J. Econ. Entomol. 40:562–564.

Schreck, C. E. 1977. Techniques for the evaluation of insect repellents: A critical review. Annu. Rev. Entomol. 22:101–119.

# EVALUATION OF THE COMBINED EFFECTS OF METHOPRENE AND THE PROTOZOAN PARASITE *ASCOGREGARINA CULICIS* (EUGREGARINIDA, DIPLOCYSTIDAE), ON *AEDES* MOSQUITOES[1]

J. P. SPENCER AND J. K. OLSON[2]

ABSTRACT. Mortality rates for test populations of *Aedes aegypti* were significantly increased with increases in concentrations of methoprene in the larval rearing media from 1.0 ppb (28% average mortality) to 10 ppb (84% average mortality). The mortality rates were not significantly changed when the protozoan parasite, *Ascogregarina culicis*, was used in combination with either concentration of methoprene against *Ae. aegypti* larvae. In contrast, mortality rates for *Ae. epactius* were not only significantly increased with increases in methoprene concentrations from 0.001 ppb (13% average mortality) to 0.01 ppb (53% average mortality), but also, the mortality rates at each IGR concentration were significantly higher when *Ae. epactius* larvae were first exposed to sporocysts of *A. culicis* and then to the IGR. Average mortality rates in this latter case ranged between 73 to 82%. The combined effects of *A. culicis* and methoprene on the mortality rates for *Ae. epactius* appear to be additive. Methoprene appears to have no significant effect either on the infectivity of the sporocyst stage of *A. culicis* or on the level of parasitism that can be established by this parasite in *Ae. aegypti* and *Ae. epactius* populations.

A common approach to integrated control involves the utilization of insecticides and biological control agents in a supplementary and complementary manner. Naturally occurring populations of biological control agents may be used or they may be artificially introduced into a habitat in conjunction with insecticide application, the critical factors being the compatibility of the biological control agent and the chemical insecticide, and the time of application of each. Therefore, preparatory to the integration of any biological and chemical control agents, a thorough study of the combined effects and compatibility of the control agents must be undertaken.

The purpose of this study was to conduct a laboratory investigation of the potential effects that the introduction of

---

[1] This research was conducted in cooperation with the Agricultural Research Service, USDA, and approved as TA 17626 for publication by the Director, Texas Agricultural Experiment Station (TAES).

[2] Present addresses: Screwworm Research Laboratory, ARS, USDA, P.O. Box 986, Mission, TX 78572; and Department of Entomology, Texas A&M University, College Station, TX 77843, respectively.

an insect growth regulator (IGR) into natural systems would have on host-parasite interactions of mosquitoes and their parasites. The IGR selected was methoprene because its effectiveness in controlling mosquito populations has been demonstrated (Schaefer and Wilder 1972, Schaefer and Wilder 1973). The parasite used as the model was the protozoan, *Ascogregarina culicis* (Ross) (formerly *Lankesteria*, see Ward et al. 1982), which appears to be nonpathogenic to its natural host, *Aedes aegypti* (Linn.), but pathogenic to unnatural mosquito hosts such as *Ae. epactius* (Dyar and Knab) (McCray et al. 1970, Walsh and Olson 1976).[3] Experimentation was based around the sequential exposure of larval populations of *Ae. aegypti* and *Ae. epactius* to the infective stage (sporocysts) of *A. culicis* and, then, to the IGR, methoprene. Both methoprene and the sporocysts of the parasite were used at rates equal to or below the $LC_{50}$ rate for each mosquito species so that, when the 2 mortality factors were used in combination, their additive, synergistic or antagonistic effect would be allowed to express itself. In instances where 2 agents are applied in combination to a population of test animals and result in mortality rates greater than the expected sums of the mortality rates when the agents are applied individually to separate test populations, they are said to be synergistic. Conversely, in instances where 2 agents are applied in combination to a population of test animals resulting in mortality rates less than the expected sums of the mortality rates when the agents are applied individually to separate test populations, they are said to be antagonistic. The same distinctions can be used to describe the combined actions of microorganisms and insecticides and did serve as the basis for our assessments of the effects of combining *A. culicis* with methoprene against larvae from laboratory colonies of *Ae. aegypti* and *Ae. epactius*.

## METHODS AND MATERIALS

One experiment involving *Ae. aegypti* consisted of the following test groups: (1) mosquitoes exposed throughout their larval development period to only *A. culicis* sporocysts (15 sporocysts/larva); (2) mosquitoes exposed only to either 1.0 ppb or 10 ppb methoprene during the 4th larval instar; (3) mosquitoes exposed to *A. culicis* sporocysts (15 sporocysts/larva) throughout the larval development period *and* to either 1.0 ppb or 10 ppb methoprene during the 4th larval instar and (4) mosquitoes exposed to neither sporocysts nor methoprene which served as controls. Each test group consisted of 4 replicates (10 mosquitoes/replicate) and the total experiment was conducted twice. The source of *A. culicus* sporocysts for this experiment and the others described herein was a laboratory culture established from wild, infected *Ae. aegypti* specimens collected in Houston (Harris Co.) TX in 1970.

All the larvae used in the *Ae. aegypti* experiment were placed as 1st instar larvae into 10 ml beakers (1 larva per beaker) 2/3 filled with deionized water. Larvae in the test groups involving exposure to parasites were placed in beakers each containing 15 *A. culicis* sporocysts in addition to the deionized water. The beakers of larvae were subsequently held in an incubator set at 28°C and a photoperiod of 14:10 hrs (light:dark) and a small amount Tetra-Min®[4] (tropical fish food) slurry was added to each beaker every other day as a food source. Once the larvae in each test group reached the 4th larval instar, they were removed from the beakers and transferred to jars (10 larvae per jar) containing 250 ml of deionized water, and when called for, the appropri-

[3] See Walsh, R. D. 1974. Biological studies of *Lankesteria culicis* (Ross) (Eugregarinida:Diplocystidae) in mosquitoes (Diptera:Culicidae). Ph.D. Dissertation. Texas A&M Univ., College Station, TX. 189 pp.

[4] Mention of a commercial or proprietary product does not constitute an endorsement be USDA or TAES.

ate quantity (1.0 ppb or 10 ppb) of methoprene dissolved in acetone. The jars of larvae were then returned to the incubator and the larvae were fed daily. Mortality and adult emergence rates were recorded throughout the experimental period.

All *Ae. aegypti* specimens in test groups exposed to *A. culicis* sporocysts were dissected and the number of parasites counted. The number of gamonts in dead larvae were counted directly using a stereoscopic dissecting microscope. The number of sporocysts were determined in dead pupae, incompletely emerged adults, and fully emerged adults by dissecting out the Malpighian tubules onto a microscope slide. The Malpighian tubules were then covered with a cover slip and squashed causing the release of the sporocysts from the gametocysts in the Malpighian tubules. The sporocysts were then washed from the slide and cover slip into a small beaker with deionized water. Because sporocyst populations reach such high numbers in *Ae. aegypti,* a blood cell counting chamber (hemacytometer) was used to determine the number of sporocysts present in each infected mosquito.

The experiment involving *Ae. epactius* consisted of the same series of test combinations as that previously described for *Ae. aegypti.* However, in the case of *Ae. epactius,* larvae in the groups to be exposed to *A. culicis* were exposed to 10 sporocysts per larvae and larvae to be exposed to methoprene were exposed to 0.001 ppb and 0.01 ppb of the agent. The rate of 10 sporocysts per larvae was used because, according to Walsh[3], resulting infection levels at this rate of exposure would result in approximately 50% mortality in *Ae. epactius* populations. The lower concentrations of methoprene used in this experiment were based on previous studies which showed *Ae. epactius* to be much more susceptible to methoprene than *Ae. aegypti* (Spencer and Olson 1979). Save for the changes in exposure rates, the *Ae. epactius* larvae used in the experiment were reared, infected with *A. culicis* and exposed to methoprene employing the same methods described for the *Ae. aegypti* experiment.

Each *Ae. epactius* test group consisted of 4 replicates of 10 mosquitoes each, and the total *Ae. epactius* experiment was repeated 3 times. During each experiment, the mosquito mortality occurring in each jar, where *Ae. epactius* larvae were placed after they reached the 4th larval instar, was recorded. In the case of larvae in test groups exposed to *A. culicis* sporocysts, the numbers of gamonts and gametocysts within each mosquito specimen were determined by dissecting out the gut into a microscope slide and counting the parasites using a compound microscope. The parasites present in *Ae. epactius* specimens could be counted directly since they rarely produce sporocysts so the total number of parasites in specimens of this mosquito species was much lower than in infected *Ae. aegypti* specimens.

The mortality data from tests involving the exposure of *Ae. aegypti* and *Ae. epactius* to various combinations of parasites and methoprene were analyzed using a completely randomized design with a factorial treatment structure. The parasite data were analyzed using two-way analysis of variance and Duncan's Multiple Range Test.

To determine the effect of methoprene on *A. culicis* infectivity, sporocysts (the infectious stage) from the stock culture of the parasite were exposed to methoprene for 24, 48 and 72-hour periods. The sporocysts for each test group (including the controls) were counted using a compound microscope and then transferred with micropipettes to 10 ml beakers 2/3 full of deionized water. Fifteen sporocysts and one 1st instar larva were added to each beaker. Forty larvae were exposed to sporocysts from each given time interval of methoprene exposure. Forty larvae were also exposed to sporocysts removed from the vial before the methoprene was added and served as the controls. The beakers containing the larvae were placed in covered plastic rectangular boxes where they were held in an incubator until the mosquitoes reached the adult

stage. The larvae were fed a slurry of Tetra-Min on alternate days. Upon reaching the adult stage, the mosquitoes were removed from the plastic boxes with a hand vacuum aspirator and placed inside a deep freezer until they were immobilized. Then each mosquito was dissected and the sporocysts inside the Malpighian tubules counted with a hemacytometer.

After the sporocysts were counted, a 2nd generation of *Ae. aegypti* larvae was infected with the sporocysts dissected from the 1st generation to see if there was any carry-over effect of the methoprene upon their infectivity. The larvae were infected and reared using the same techniques employed for the 1st generation. The adult mosquitoes emerging from the 2nd generation were, again, dissected and the sporocysts counted. The data ultimately obtained from these experiments were statistically analyzed using a two-way analysis of variance program.

## RESULTS AND DISCUSSION

Analysis of the data derived from experiments involving the sequential exposure of *Ae. aegypti* to *A. culicis* sporocysts and methoprene revealed that, while mortality rates for test populations of this species were significantly increased ($p = 0.01$) with increases in methoprene concentrations from 1.0 ppb to 10 ppb, there was not a significant change in the mortality rates when *A. culicis* was used in combination with either concentration of this compound (Table 1). The results indicate that there is no apparent interaction between the parasite and methoprene as far as mortality rates are concerned in *Ae. aegypti* populations. These results correspond to those of McCray et al. (1970) who found no significant differences in mortality rates between *Ae. aegypti* larvae infected and uninfected with *A. culicis* when they were exposed to such insecticides as DDT, dieldrin, malathion, carbaryl and temephos.

Mortality rates for experimental populations of *Ae. epactius* exposed to various combinations of methoprene and *A. culicis* sporocysts are summarized in Table 2. Statistical analysis of these data revealed that mortality rates within populations of this mosquito species were not only increased significantly ($p = 0.01$) by increases in the concentration of methoprene, but also, the mortality rates at a given concentration of this compound were significantly greater ($p = 0.01$) for populations exposed as larvae to both the sporocysts of *A. culicis* and to the IGR. Further analysis of these data indicated that, on the basis of resulting mortality rates within target populations of *Ae. epactius*, there was no antagonistic or synergistic interaction between methoprene and the parasite, but rather, the effects of these 2 agents when used in combination against this particular mosquito

Table 1. Mortality rates within test populations of *Aedes aegypti* exposed to 1.0 ppb or 10 ppb methoprene only, to parasites only, and to combinations of methoprene and parasites.[a]

| | Average percent mortality for populations exposed to:[b] | | | | |
|---|---|---|---|---|---|
| | Methoprene only | | | Parasites + Methoprene | |
| Test (4 rep./test) | 1.0 ppb (%) | 10 ppb (%) | Parasites only (%) | 1.0 ppb Methoprene (%) | 10 ppb Methoprene (%) |
| I | 37 | 87 | 0 | 45 | 66 |
| II | 59 | 82 | 8 | 59 | 79 |
| Average | 28 | 84 | 3 | 52 | 72 |

[a] Parasites = sporocysts of *Ascogregarina culicis*.
[b] Percent mortalities corrected by Abbott's formula.

Table 2. Mortality rates within test populations of *Aedes epactius* exposed to .001 ppb or .01 ppb methoprene only, to parasites only, and to combinations of methoprene and parasites.[a]

| Test (4 rep./test) | Methoprene only 0.001 ppb (%) | Methoprene only 0.01 ppb (%) | Parasites only (%) | Parasites + Methoprene 0.001 ppb Methoprene (%) | Parasites + Methoprene 0.01 ppb Methoprene (%) |
|---|---|---|---|---|---|
| I | 11 | 37 | 69 | 86 | 89 |
| II | 11 | 69 | 22 | 69 | 83 |
| III | 18 | 53 | 55 | 63 | 74 |
| Average | 13 | 53 | 49 | 73 | 82 |

Average percent mortality for populations exposed to:[b]

[a] Parasites = sporocysts of *Ascogregarina culicis.*
[b] Percent mortalities corrected by Abbott's formula.

species appear to be additive. It was thus concluded that the 2 agents were compatible, each being responsible for a certain level of mortality occurring within the target population of mosquitoes.

The effect of the use of methoprene on the level of parasitism by *A. culicis* occurring within test populations of *Ae. aegypti* exposed of various combinations of these 2 agents is summarized in Table 3. In order to compare the numbers of parasites found in the different mosquito stages examined, all data were converted to average number of gamonts per mosquito specimen. In the case of *Ae. aegypti, A. culicis* gamonts were actually found only in the larval stages of the mosquito. The parasites were found to have advanced to the sporocyst stage in the pupal and adult mosquito specimens of *Ae. aegypti* examined. Conversion of the sporocyst numbers in these latter mosquito specimens back to gamont equivalents was accomplished by dividing the number of sporocysts observed in a given mosquito by 250 and then multiplying the resulting dividend by 2. This method of converting the sporocyst data into gamont equivalents was based on the fact that, according to the life cycle of *A. culicis* described by Wenyon (1926), 2 gamonts fuse to form 1 gametocyst and each gametocyst will subsequently give rise to 250 sporocysts. In accordance with the gamont equivalent data thus derived, no significant difference in parasitemia was

Table 3. Average number of *Ascogregarina culicis* gamonts recovered from *Aedes aegypti* specimens exposed to 15 sporocysts per larva and subsequently treated with 1.0 ppb or 10 ppb methoprene.[a]

| Mosquito stage examined | Parasites only | Parasites + 1.0 ppb Methoprene | Parasites + 10 ppb Methoprene |
|---|---|---|---|
| Dead larvae | 57 (1) | 99 (3) | 100 (4) |
| Dead pupae | 54 (3) | 56 (35) | 58 (55) |
| Incompletely emerged adults | 58 (1) | 58 (5) | 63 (2) |
| Males | 66 (27) | 58 (11) | 60 (9) |
| Females | 65 (48) | 60 (25) | 64 (10) |

Average number of gamonts per mosquito specimen exposed to:[b]

[a] Average of 2 tests.
[b] Numbers in parentheses indicate number of mosquitoes examined.

found to exist between populations of *Ae. aegypti* exposed only to *A. culicis* sporocysts and populations exposed both to the parasite and to either of the 2 concentrations of methoprene used in the experiments.

Levels of parasitism observed for populations of *Ae. epactius* exposed to various combinations of *A. culicis* and methoprene are summarized in Table 4. Again, the data concerning *A. culicis* were converted into gamont equivalents for comparative purposes. In the case of *Ae. epactius*, only the gamont and gametocyst stages of the parasite were found to be present. Gamonts were observed not only in *Ae. epactius* larvae, but also in the pupal and adult stages of this mosquito species as well. In certain instances, the parasite was found to have advanced to the gametocyst stage of its developmental cycle in some of the pupal and adult specimens of *Ae. epactius* examined; however, in no instance were sporocysts observed. Also, it was not uncommon to find melanized gamonts and gametocysts in the pupal and adult specimens of this mosquito species. These observations on the status of parasite development and the occurrence of parasite melanization of *Ae. epactius* are similar to those reported by Walsh[3].

The gametocyst data for *Ae. epactius* was converted to gamont equivalents by multiplying numbers of gametocysts by 2 since, theoretically, 2 gamonts fuse to form 1 gametocyst (Wenyon 1926). A two-way analysis of variance of the *A. culicis* gamont equivalent data for *Ae. epactius*, as summarized in Table 4, revealed no significant difference in parasite numbers at a given stage of mosquito development between mosquitoes exposed to methoprene and those not exposed to methoprene. However, subjecting the data for each test group of mosquitoes to Duncan's New Multiple Range Test revealed that, regardless of treatment, the numbers of parasites found in emerging male and female *Ae. epactius* from a given test population were significantly lower ($p = 0.05$) than those occurring in the dead mosquito larvae and pupae from the same population. These observations again correspond to those reported by Walsh[3].

Statistical analysis of the data derived from the experiment involving exposure of *A. culicis* sporocysts to 5 ppm methoprene for up to 72 hrs revealed no significant difference ($p = 0.01$) in the rates of *Ae. aegypti* infection between treated and untreated sporocysts. The average number of $F_1$ sporocysts occurring in mosquito populations exposed to the methoprene-treated sporocysts ranged between 12,200 (24-hr methoprene exposure) and 13,700 per mosquito (72-hr methoprene exposure). The average number of $F_1$ sporocysts occurring in

Table 4. Average number of *Ascogregarina culicis* gamonts recovered from *Aedes epactius* specimens exposed to 10 sporocysts per larva and subsequently treated with 0.001 ppb or 0.01 ppb methoprene.[a]

| Mosquito stage examined | Average number of gamonts per mosquito specimen exposed to:[b] | | |
|---|---|---|---|
| | Parasites only | Parasites + 0.001 ppb Methoprene | Parasites + 0.01 ppb Methoprene |
| Dead larvae | 10 (16) | 8 (40) | 10 (33) |
| Dead pupae | 14 (38) | 11 (38) | 8 (63) |
| Incompletely emerged adults | 12 (7) | 5 (10) | 2 (4) |
| Males | 3 (24) | 3 (9) | 2 (4) |
| Females | 4 (32) | 3 (22) | 4 (14) |

[a] Average of 3 tests.
[b] Numbers in parentheses indicate number of mosquitoes examined.

mosquitoes exposed to untreated sporocysts was 12,200 per mosquito. When $F_1$ sporocysts dissected from the 1st population of mosquitoes were used to infect another population, it was found that the number of $F_2$ sporocysts which developed in the 2nd mosquito population did not differ significantly from either the controls or from the number of $F_1$ sporocysts developing in the first population of mosquitoes indicating no carry-over effect.

The data reported herein indicate that methoprene and *A. culicis* are compatible under the experimental conditions employed in our study and that an additive mortality effect may be obtained when they are applied in combination against certain mosquito species. Preliminary studies also indicate that methoprene may be applied (at 5 ppm or larger) to mosquito habitats without disrupting the infectivity of *A. culicis*.

In other studies involving IGR's, Wright and Spates (1972) and Wilkinson and Ignoffo (1973) demonstrated the compatibility of these agents with hymenopteran parasitoids. Also, Finney et al. (1977) showed that methoprene was not only compatible with the nematode parasite of mosquitoes, *Romanomermis culicivorax*, but also that mosquito mortality was actually increased when the IGR and the parasite were used concurrently against mosquito populations. These workers also found that methoprene neither affected the infectivity of the preparasitic stage of the nematode nor did the methoprene exposure affect the normal parasitic development in the mosquito host. These studies along with the one described herein lend credence to the idea that an integrated approach to mosquito control might be developed utilizing mosquito parasites in combination with IGR's without deleterious effects to the biological control agent.

*References Cited*

Finney, J. R., R. Gordon, W. J. Condon and T. N. Rusted. 1977. Laboratory studies on the feasibility of integrated mosquito control using an insect growth regulator and a mermithid nematode. Mosq. News 37:6–11.

McCray, E. M., R. W. Fay and H. F. Schoof. 1970. The bionomics of *Lankesteria culicis* and *Aedes aegypti*. J. Invertebr. Pathol. 16:42–53.

Schaefer, C. H. and W. H. Wilder. 1972. Insect developmental inhibitors: a practical evaluation as mosquito control agents. J. Econ. Entomol. 65:1066–71.

Schaefer, C. H. and W. H. Wilder. 1973. Insect development inhibitors. 2. Effects on target mosquito species. J. Econ. Entomol. 66:913–6.

Spencer, J. P. and J. K. Olson. 1979. Baselines of the susceptibility of four species of floodwater mosquitoes to methoprene. Southwestern Entomol. 4:86–88.

Walsh, D., and J. K. Olson. 1976. Observations on the susceptibility of certain culicine mosquito species to infection by *Lankesteria culicis* (Ross). Mosq. News 36:154–60.

Ward, R. A., R. V. Melville, N. D. Levine and G. B. Craig, Jr. 1982. *Ascogregarina* nom. nov. for *Ascocystis* Grasse 1953 (Apicomplexa, Eugregarinida). J. Parasitol. 68: 1331.

Wenyon, C. M. 1926. Protozoology. William Wood and Co., N.Y. (Reprinted by Hafner Publishing Co. N.Y. 1965):1121–5.

Wilkinson, J. D. and C. M. Ignoffo. 1973. Activity of a juvenile hormone analogue on a parasitoid, *Apanteles rubecula*, via its host, *Pieris rapae*. J. Econ. Entomol. 66:643–6.

Wright, J. E. and G. E. Spates. 1972. A new approach in integrated control: insect juvenile hormone plus a hymenopteran parasite against the stable fly. Science. 178:1292–3.