CONTROL OF LARVAE AND PUPAE OF *ANOPHELES QUADRIMACULATUS* AND *ANOPHELES CRUCIANS* IN NATURAL PALUDAL PONDS WITH THE MONOMOLECULAR SURFACE FILM ISOSTEARYL ALCOHOL CONTAINING TWO OXYETHYLENE GROUPS

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ABSTRACT. The efficacy of the monomolecular surface film isostearyl alcohol containing 2 oxyethylene groups (ISA-20E) for controlling mixed populations of *Anopheles quadrimaculatus* and *An. crucians* was evaluated in standing paludal ponds. Results of hand spray application indicated that 90–100% mortality of larvae and pupae could be achieved in 48–72 hr post-treatment at a surface dosage of 0.3–0.4 ml/m² (0.32–0.42 gal/acre). The effects of dosage, habitat surface characteristics, dissolved oxygen levels, meteorology, and instar sensitivity on the impact of ISA-20E on immature stages of the *Anopheles* spp. are discussed.

Observations on immature stages of *Culex erraticus* indicated that larvae of this species were significantly more tolerant to the surface film than larvae of the *Anopheles* spp. An explanation for this effect is presented.

Results of preliminary tests against *Anopheles* and *Culex* spp. with automatic ISA-20E-dispensing systems and with slow-release solid formulations of ISA-20E are also presented.

The use of the organic surface film isostearyl alcohol containing 2 oxyethylene groups (ISA-20E)[3] for controlling natural populations of immature mosquitoes in a variety of habitats has been reported by Levy et al. (1980a, 1980b, 1981, 1982). Results of their ground and aerial spray tests with ISA-20E at application rates of 0.2–0.45 ml/m² (0.21–0.48 gal/acre) against larvae and pupae of *Aedes, Culex, Psorophora,* and *Uranotaenia* spp. have indicated that effective control of larvae and pupae (>90% mortality) could usually be achieved within 24 to 72 hr post-treatment. Laboratory and simulated field trials to control 4th instar larvae and pupae of *Anopheles quadrimaculatus* Say (Garrett 1976, White and Garrett 1977) and *An. stephensi* Liston (Garrett unpublished) with ISA-20E at a surface dosage of 0.04 ml/m² (0.043 gal/acre) resulted in 99–100% mortality of immatures within 24 hr post-treatment.

Observations in habitats sprayed with ISA-20E to control immature mosquitoes have indicated that this monomolecular surface film will cause no phytotoxicity, and little or no adverse affects on non-target invertebrate and vertebrate organisms (Levy et al. 1981, White and Garrett 1977).

Since preliminary studies indicated that 4th instar larvae and pupae of *Anopheles* spp. were highly sensitive to ISA-20E, field trials were conducted to determine if this film could be used to control natural populations of *Anopheles* spp. in selected habitats under a variety of environmental conditions. Data from these trials could then be used to determine the feasibility of using ISA-20E for control of vectors of malaria.

METHODS AND MATERIALS

Paludal ponds (38.9–138.6 m²) constructed in the natural substrata at the Lee County Mosquito Control District were used as test sites to evaluate the effi-

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cacy of ISA-20E in controlling mixed natural populations of *Anopheles quadrimaculatus* Say and *An. crucians* Wiedemann. Since some ponds contained mixed populations of larvae and pupae of *Culex erraticus* (Dyar and Knab) along with the *Anopheles* spp., data were also obtained on the effects of ISA-20E on this species. A control pond was used to monitor natural fluctuations in the populations of the *Anopheles* and *Culex* spp.

The level of ground water in the experimental ponds fluctuated in relation to rainfall (0.25–0.75 m deep). Water temperature, dissolved oxygen, conductivity, and pH of the ponds ranged from 28–32°C, 10–12.1 ppm, 110–210 μmhos/cm, and 7.3–8.9, respectively. Natural populations of nontarget vertebrate and invertebrate organisms, and submerged, surface, and emergent vegetation were present in the ponds during the experiments.

ISA-20E was applied at a surface dosage of 0.3–0.4 ml/m² (0.32 and 0.42 gal/acre) with a non-pressurized plastic hand-sprayer along the upwind edge and around the vegetative perimeter of a pond and allowed to spread over the entire surface of the water. Preliminary investigations were also conducted on possible techniques for slow-continuous release of ISA-20E for prolonged mosquito control. In these experiments, 2 automatic ISA-20E-dispensing systems and 2 solid/semi-solid formulations of ISA-20E were evaluated against the *Anopheles* and *Culex* spp. in the laboratory and in the experimental ponds to determine the efficacy of the techniques.

Larvae and pupae were collected pre- and post-treatment from vegetative areas within and around the perimeter of the ponds with a pint dipper at 24 hr intervals (20 dips/test site). Pre-treatment samples in spray trials ranged from 1.3–1.8 immatures/dip for *Anopheles* spp. and 0.9–1.1 immatures/dip for the *Culex* sp. Percentage reduction of larvae according to instar and pupae at each 24 hr post-treatment sampling period was used to evaluate the efficacy of ISA-20E and the control techniques. Data were analyzed by analysis of variance. Persistence of ISA-20E was determined at each sampling interval with an oleyl alcohol indicator oil[4] in a manner previously described (Levy et al. 1980b).

RESULTS AND DISCUSSION

Results of hand-sprayer field trials are presented in Table 1. When ponds 1 and 2 were sprayed with ISA-20E at a surface dosage of 0.3 ml/m², mean mortality of immatures at 24 and 48 hr post-treatment was 20 and 65%, respectively. In tests at the 0.4 ml/m² dosage, combined larval and pupal mortality of 57.6 and 81.4% were recorded in ponds 3 and 4 at 24 and 48 hr post-treatment, respectively. One hundred percent control was achieved in pond 4 at 48 hr post-application and in ponds 1, 2 and 3 at 72 hr. Development of *Anopheles* and *Culex* spp. larvae to higher instars in control ponds seemed to be more accelerated when compared to the larvae in treated ponds. Control immatures ranged from 1.4–2.1 per dip throughout the course of the experiment.

No effective control of mixed larval instars of the *Anopheles* spp. (>90% mortality) occurred in the field trials within 24 hr post-treatment. Except for pond 4, effectiveness was not observed until 72 hr post-treatment. However, delayed effectiveness for larvae (i.e., >90% of larvae occurring 1–3 days post-treatment) was shown to frequently occur in tests to control *Aedes, Culex* and *Psorophora* spp. Factors contributing to this delayed effect are reported by Levy et al. (1980b, 1981).

Garrett (1976) and White and Garrett (1977) reported that 90% or greater control of 4th instar larvae and pupae of *An. quadrimaculatus* could be achieved within 24 hr after ISA-20E treatment at a surface dosage of 0.043 gal/acre; however, sensitivity of *Anopheles* larvae to such sur-

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[4] Manufactured as a cosmetic and pharmaceutical ingredient under the trade name ®Adol 85 N.F. by Sherex Chemical Co., Inc., P.O. Box 646, Dublin, OH 43017.

Table 1. Percent reduction of *Anopheles* spp. and *Culex erraticus* larvae and pupae after spray application of ISA-20E in standing paludal ponds.[1]

Pond number	Surface dosage (ml/m²)	Hours post-treatment	Percent reduction of larvae by instar and pupae (P); Total (T)					
			1	*2*	*3*	*4*	*P*	*T*
					Anopheles spp.			
1	0.30	24	33.3	0	0*	0*	—	0*
		48	100	0	12.5	100	—	32
		72	100	100	100	100	—	100
2	0.30	24	100	0	33.3	100	—	40
		48	90	88.2	83.3	100	—	88.6
		72	100	100	100	100	—	100
3	0.40	24	100	23.1	0*	100	100	53.6
		48	100	46.2	0*	100	100	60.7
		72	100	100	100	100	100	100
4	0.40	24	100	57.9	40	50	—	61.3
		48	100	100	100	100	—	100
					Culex erraticus			
1	0.30	24	0*	0*	0	0*	—	0*
		48	—	0*	0	40	—	0*
		72	0*	0*	33	100	—	22.2
		96	—	14.3	40	100	—	50
3	0.40	24	100	0*	50	100	100	16.1
		48	100	43.5	0	100	100	51.6
		72	100	91.3	0*	100	100	77.4
		96	100	91.3	0*	100	100	80.6

[1] Wind speed < 2–10 mph; multidirectional
* Increase over pre-treatment samples.

face concentrations was not observed in the paludal pond tests conducted against mixed instars of *Anopheles* spp. at significantly higher dosages. This was attributed to differences in instar sensitivity and to natural habitat characteristics. The tolerance of 2nd-3rd instar larvae of *An. quadrimaculatus* and *An. crucians* to prolonged exposure to ISA-20E was observed in laboratory tests (Garrett 1976). Bioassays at surface dosages of 0.04–0.25 ml/m² (0.043–0.26 gal/acre) against mixed *Anopheles* spp. instars (3rd-4th) collected from the experimental ponds showed that 3rd instar larvae could survive 1–2 days longer than 4th instar larvae. A direct relationship between ISA-20E dosage and acute larvicidal action was not shown in these tests or in tests against larvae of *Aedes, Culex,* and *Psorophora* spp. (Levy et al. 1981, 1982). However, dosage was found to be important in the persistence of the non-toxic physicochemical effect of ISA-20E on larvae, and was therefore important in obtaining effective delayed mortality of mixed *Anopheles* instars.

The stress response of larvae exposed to ISA-20E in bioassays appeared to vary with instar. The addition of 0.25 ml/m² of ISA-20E to beakers caused 4th instar larvae of the *An. quadrimaculatus* to immediately drop from their normal horizontal respiratory position at the surface. Although they contorted in repeated attempts to reattach to the interface to obtain air, the reduced surface tension inhibited their buoyancy at the surface. They quickly fatigued, dropped to the bottom of the container in a moribund condition and died. Comparable dosages of ISA-20E also caused some 1st-3rd instar *Anopheles* larvae to drop from the surface in an elongated fashion while others were observed to drop from the surface and form an "O" shape in an attempt to remove the film from their siphon with their mouth parts. However, they were usually able to reattach their siphons to the interface in an "abnormal" culicine resting position. In this position, some larvae were observed to rapidly undulate the posterior portion of their abdomens. Some larvae did not lose siphonal contact with the interface but were observed to shift their horizontal surface position to a modified culicine pose. Presumably, in this manner these larvae were able to penetrate the interface at various intervals to obtain air and prolong survival. In addition, little hyperactivity of early instars exposed to ISA-20E was noted when compared to 4th instar larvae. However, pupae and 4th instar larvae appeared to be acutely sensitive to ISA-20E. Data from laboratory observations indicated that 4th instar larvae and pupae were significantly more sensitive than 1st-3rd instar larvae of the *Anopheles* spp. Although data from field trials indicated that 1st instar larvae were also acutely sensitive to ISA-20E (Table 1), laboratory observations did not confirm this. Molting of larvae to the higher instars and/or egg hatch are presumed to have caused the observed increases over pre-treatment samples and the reduction in 1st instar larvae in several field tests (Table 1). In general, sensitivity of immature *Anopheles* to ISA-20E appeared to follow the general trend of 1st instar $<$2nd instar $<$3rd instar $<$4th instar $\cong$pupae.

Differences in morphological structures on the dorsal surface of *Anopheles* larvae could be related to the observed differences in the sensitivity of 4th instar *Anopheles* to ISA-20E when compared to the earlier instars. The palmate and filamentous setae of the dorsal surface of 4th instar larvae are larger, more numerous and in different locations and arrangements than 1st-3rd instars. Therefore, the wetting action of ISA-20E on the setae of 4th instar larvae and the reduced surface tension effect may account for the acute sensitivity of this instar. Thus, molting of the 1st-3rd instars (morphologically less sensitive to ISA-20E) to the more sensitive 4th instar could be an important factor for obtaining effective delayed mortality of *Anopheles* larvae and further emphasize the importance of ISA-20E persistence to achieve effective control of *Anopheles* spp. under field conditions.

The high dissolved oxygen concentration of the paludal ponds (10–12 ppm) may also contribute to prolonged survival of larvae exposed to ISA-20E. Reiter (1978) indicated that low dissolved oxygen levels were necessary to obtain effective control of *Anopheles* spp. larvae with a monolayer. Presumably, delayed larval mortality was related to cuticular respiration and air storage in the tracheal system. It is interesting to note that data by Reiter (1978) on the comparative sensitivity of 2nd and 4th instar larvae of *An. stephensi* to low dissolved oxygen levels suggest that size differences (and surface to volume ratio) are relatively unimportant in determining the survival time of submerged larvae.

The field data indicated a significant difference in percentage mortality of immatures at the post-treatment intervals based on dosage; however, this effect was partially attributed to certain habitat characteristics of the test ponds. Observations on the amounts of surface and emergent vegetation in ponds 1–4 during the tests indicated that ca. 5–10%, 40–50%, 20–30% and 80–90% of the water was covered with surface and emergent vegetation, respectively. These observations suggested the interrelated importance of dosage and the amount of surface and emergent vegetation in a habitat and the percentage of ISA-20E-induced mortality of the *Anopheles* spp. observed at the sampling periods (Table 1). Persistent and unidirectional wind fetch will cause downwind compaction of ISA-20E and will usually result in poor control of immatures due to incomplete surface film coverage (Levy et al. 1981). These observations indicated that at low to moderate wind speeds, emergent and surface vegetation could act as wind baffles and keep a significant portion of the surface film from being translocated, thereby achieving better surface coverage and mosquito control. Tests with ISA-20E at 0.32 and 0.48 gal/acre in paludal ponds having moderate and little or no emergent or surface vegetation under conditions of constant unidirectional winds gusting to 15–20 mph during the test resulted in 0–35% control of immature stages of mixed populations of *An. quadrimaculatus* and *An. crucians* at 72 hr post-treatment. Under these conditions, persistence of the film was only indicated along portions of the downwind perimeter. Poor control and no film persistence was observed in these habitats at 144 hr post-treatment. Since the wind speed had subsided (<2–10 mph), the experimental habitats were retreated at the original dosages, resulting in 93–100% mortality of the immature *Anopheles* spp. 72 hr after the 2nd treatment. Dosages of 0.043–0.26 gal ISA-20E/acre were insufficient to maintain an effective film in natural paludal ponds having moderate to high vegetation before sufficient control of early instar *Anopheles* spp. could be achieved; however, satisfactory persistence of ISA-20E (3–7 days) and <90% kill of 1st-4th instar larvae and pupae of *Anopheles* spp. was observed at surface dosages of 0.32–0.48 gal/acre.

In the ponds treated with ISA-20E at 0.3 and 0.4 ml/m^2 (Table 1) increased larval mortality with time was the general rule. Persistence of ISA-20E was indicated in all ponds at the termination of the experiments; however, at least 24 hr of extended ISA-20E persistence was observed at the higher dosage. It is presumed that film loss from wind-induced compaction against the embankment and deposition on aquatic vegetation would be less severe at the higher dosage since more "lenses" of excess surface film would be available to resupply the film pressure of the habitat and insure increased persistence.

Larvae and pupae of *Cx. erraticus* were also present in ponds 1 and 3 during the *Anopheles* tests (Table 1). Observations indicated that larvae of this *Culex* sp. were significantly less susceptible to ISA-20E than the *Anopheles* spp. Although control of 4th instar larvae and pupae were observed within 72 hr post-treatment, poor control of 2nd-3rd instar larvae of *Cx. erraticus* was achieved even though presence of adequate amounts of ISA-20E was indicated. Laboratory observations indicated that larvae of this species are able to obtain oxygen from the water for extended periods without contacts with the water surface. Larvae of *Cx. pilosus* (Dyar and Knab) were observed to react in a similar manner; however, pupae of both *Culex* spp. were highly sensitive to ISA-20E.

Experiments to obtain prolonged mosquito control with ISA-20E were also conducted in the ponds with an automatic ISA-20E dispensing technique that was developed to provide continuous film coverage and thereby counteract the effects of film disruptive processes such as wind and rain. ISA-20E was sorbed into an oleophilic polyurethane foam (OPF) (reticulated, 80 pores per linear inch) and anchored with a wax-coated string at the upwind side of a pond. Since the porous sorbent holds a volume of ISA-20E equal to its own, pieces were cut to hold the requisite quantity of surface film. Laboratory studies indicated that the sponge-like sorbent would float at the water surface and release surface film on demand when the surface pressure of the ISA-20E decreased as the film was slowly degraded by natural processes.

Field tests with the OPF were conducted at dosages of 0.043–0.32 gal ISA-20E/acre. Results of trials against the *Anopheles* spp. with this technique were less successful; percentage mortality ranged from 27–84% and was stage dependent with 4th instar larvae and pupae accounting for most of the control. Persistence of the film ranged from <24 to <72 hr and was related to the amount of material sorbed into the sponge. Poor control of mixed instars of *Anopheles* spp. was related to failure of the OPF to release all of the ISA-20E. Squeezing the sponge at various post-treatment periods where no film was present resulted in the release of a sufficient quantity of ISA-20E to resupply the test habitat at high film pressure for as long as 2 days.

Prolonged control (90–100%) of mixed populations of immature *Anopheles* spp. and *Cx. erraticus* was achieved for 30 days when ISA-20E was drip-dispensed into 65–70 m^2 moderately vegetative paludal ponds at the upwind side with one gallon capacity multiple valve gravity-feed oilers. Although the drip rate utilized in the initial 30-day experiment was too high (i.e., ca. 0.5 gal/acre/24 hr) in relation to the surface area of the experimental habitats, the data indicated that a drip-dispenser could be used to maintain high film pressure on the water surface for prolonged periods. It should be noted that similar levels of control were obtained when a 50:50 formulation of ISA-20E and 2-propanol was used to reduce the active treatment dosage in the small habitats. Although the technique was effective, these commercial oilers are expensive, cumbersome, difficult to calibrate and set up in the field, and can produce erratic drip dosages when low application rates are required. The potential of inexpensive dripper devices such as modified infusion and plasma bottles is currently being evaluated in the field. It is expected that this type of ISA-20E-dispensing system will be useful only in specialized mosquito control situations (e.g. in sewage treatment and irrigation systems).

To date, the use of solid/semi-solid formulations of ISA-20E has been the most effective and practical experimental technique for prolonged release of ISA-20E. In these formulations, several binder components were formulated with 40 and 50% ISA-20E to achieve biodegradable 270–290 g multi-component solid/semi-solid matrices designed to float on the water surface and release ISA-20E. Preliminary tests against *Anopheles* spp. and *Culex erraticus* in paludal ponds (ca. 70 m^2) with the 2 formulations have indicated that ISA-20E can be released for at least 6 months and control 95–100% of the larvae and pupae. It should be noted that each formulation was placed in a nylon net and anchored at the upwind portion of the experimental habitat. Although effective under the experimental conditions, problems concerning block consistency after prolonged field exposure and minimal release rates must be solved before a solid formulation can be considered suitable for operational use in mosquito control. These formulations can be fabricated into beads, pellets, blocks, etc., to facilitate ground or aerial application and have an extremely stable shelf-life. Twenty-five experimental solid formulations are currently being evaluated in the laboratory and in semi-permanent and permanent mosquito habitats in cooperation with Sherex Chemical Company, Inc.

In conclusion, spray tests to control *An. quadrimaculatus* and *An. crucians* in natural paludal ponds with ISA-20E at a surface dosage of 0.32 and 0.42 gal/acre indicated that 100% mortality of 1st-4th instar larvae and pupae could be achieved within 48–72 hr post-treatment. However, at these dosages ISA-20E was ineffective in controlling mixed populations of *Cx. erraticus*. Tests to control immature stages of the *Anopheles* spp. with an automatic OPF ISA-20E dispensing system were unsuccessful due to failure of the OPF to release sufficient quantity of the sorbed dosage. However, preliminary tests in paludal ponds with an automatic ISA-20E drip-dispensing system and 2 slow-release solid formulations of ISA-20E provided 90–100% control of larvae and pupae of *An. quadrimaculatus*, *An. crucians*, and *Cx. erraticus* for periods of 1 and 6 months, respectively under a variety of natural conditions.

No phytotoxicity and little or no adverse effects on fish and wildlife were noted in habitats treated with spray applications of ISA-20E. Georghiou (1980) reported 44 species of *Anopheles* have developed resistance to certain organochlorine and/or organophosphate pesticides; however, no resistance to this non-petroleum organic surface film is expected to develop since the larvicidal and pupicidal action of ISA-20E is due to the physical factors of habitat surface tension reduction and suffocation. Continuous challenge tests against laboratory-reared *Culex quinquefasciatus* Say with ISA-20E for 15 generations have indicated that larval susceptibility to this surface film has not decreased. It should be noted that resistance of *Cx. quinquefasciatus* to 3 types of petroleum hydrocarbons was not induced after more than 2 years by continuous selection pressure (Micks et al. 1968). Therefore, ISA-20E may be useful in malaria control programs where certain conventional pesticides are no longer effective against the *Anopheles* spp. ISA-20E resists oxidative rancidity and therefore should have an almost indefinite storage or shelf-life at typical ambient temperatures (21–49°C) encountered in many tropical and subtropical areas where high rates of malaria prevail. ISA-20E is classified as non-toxic orally (acute oral LD_{50} rats = 20,000 mg/kg) and non-irritating to the eyes and skin and is expected to pose little or no ecological hazards when used to control mosquitoes at recommended dosages in the aquatic ecosystem. In general, the effectiveness of ISA-20E against the immature stages of certain *Anopheles* spp. at low dosages, the ease of application, and safety to applicators and the environment makes this monomolecular organic surface film an excellent candidate for use in malaria vector control programs.

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