PROC. ENTOMOL. SOC. WASH.
86(3), 1984, pp. 714–719

AN *IN VIVO* FLUORESCENT MARKER FOR SPERMATOZOA OF THE SCREWWORM (DIPTERA: CALLIPHORIDAE): A FIRST REPORT

RICHARD J. BRENNER

USDA-ARS, Screwworm Research Project, American Embassy–Mexico (Tuxtla), P.O. Box 3087, Laredo, Texas 78041.[1]

Abstract.—Acridine orange (AO) was used as a fluorescent marker for spermatozoa of the screwworm, *Cochliomyia hominivorax* (Coquerel). The chemical was ingested by adult sterile flies in carbohydrate solutions at a concentration of 1.0 mg AO/ml. When reproductive organs of both sexes were examined for fluorescence under longwave ultraviolet light on a Zeiss compound microscope, all reproductive tissues (testes, ovaries, seminal fluid glands, accessory glands, and spermathecae) were marked. Access to AO in 35% honey for 24–48 h was sufficient to mark sperm in the testes for up to 15 days in males (maximum value tested); heads and tails of sperm fluoresced a brilliant green. Neither male adult longevity nor propensity to mate were adversely affected by AO. Spermatozoa within the crushed spermathecae of unmarked females inseminated by marked males fluoresced brilliantly indicating that this technique can be used for mating preference tests. However, false positives were noted; fluorescence of marked spermathecae was transferred to unmarked sperm in the spermathecae soon after mating. Acridine orange at 1.0 mg/ml in the liquid diet of larval screwworms delayed growth in proportion to the duration of exposure; reproductive organs of resultant adults were strongly fluorescent. Fertile flies that fed on AO solutions as adults, mated and oviposited normally; neither sterility nor mutagenic effects were noted. The applications of this technique to the screwworm eradication program, and to biological research are discussed.

The success of the USA-Mexico joint program to eradicate the screwworm, *Cochliomyia hominivorax* (Coquerel), from Mexico north and west of the Isthmus of Teuantepec is dependent on the ability of mass reared sterile male flies to find and mate with native females. Since the conception of the program, the efficacy of any given release strain has been indirectly examined following its release in the field by measuring the proportion of sterile to fertile egg masses collected from sentinel animals.

Decisions on whether to change strains in the mass rearing facilities at Tuxtla Gutierrez, Chiapas, Mexico, have been made by comparing the field effectiveness of a recently produced candidate strain with that of the strain in production. The best available method for comparison involves the simultaneous release of both

[1] Send reprint request % P.O. Box 267, Weslaco, TX 78596. Color transparencies provided with reprint.

strains in "ecologically similar" but separate areas; hence, the comparison is not one of direct competition. Also, factors such as differences in terrain, weather, animal husbandry practices, age structure of the target populations, and irregularities in fly-release flight lanes cannot be controlled. Consequently, the interpretation of data has been difficult and the significance of the results has been questionable.

If the sperm of sterile strains could be differentiated from each other and from that of native flies, these uncontrollable factors would be irrelevant. If their sperm were marked, two or more strains of sterile flies could be released simultaneously in the same region so that sterile males of the test strains would compete for virgin wild females under the same biotic and abiotic conditions. Wild females, trapped at oviposition sites, could be dissected and the sperm in the spermathecae examined to determine the source of male gametes. Thus far, the lack of an accurate method for differentiating irradiated sperm of screwworms from unirradiated (McInnis, 1984), and the lack of any method for differentiating the sperm of two sterile strains, have precluded execution of simultaneous and sympatric releases of sterile strains.

For our purposes, any technique for marking sperm of screwworms must be reliable, easily discernable with readily-available equipment, and usable in the field. Sperm of other insects has been marked using rare elements (Moss and Van Steenwyk, 1982) or radioactive isotopes (Lowe et al., 1974). However, such techniques require special care and equipment, and are thus unsuitable for field studies distant from well supplied laboratories. This paper reports the results of a project undertaken to test the hypothesis that known fluorescent compounds, easily detected in the field with a portable microscope, could be used as *in vivo* markers for spermatozoa of screwworms.

Materials and Methods

Sterile adult screwworms (A-82 or 0-83 strains, originating from material collected in Arriaga, Chiapas and the state of Oaxaca, respectively), were mass reared on liquid medium at the sterile fly production plant in Tuxtla Gutierrez, Chiapas, Mexico, and irradiated as pupae by the standard cesium source. Flies were given continuous access to water and to a 10% sucrose solution (by weight in water) containing 0, 0.01, 0.1, or 1.0 mg of fluorescent compound per ml of solution from emergence through the first five days of adult life. Thereafter, separate containers of water and 10% sucrose were available. All flies were maintained at 23°–28°C in uncontrolled relative humidity under artificial light (10:14 L:D).

Flies were dissected from day four through day 15. Reproductive structures (ovaries or testes, accessory glands or seminal fluid glands, and spermathecae) and parts of the alimentary/excretory systems (midgut, hindgut, and rectal pads) from two males and two females per treatment were excised and placed individually in wells of a porcelain spot plate in 1 ml of physiological saline (Ephrussi and Beadle, 1936) and 10% glycerine. Each organ was then transferred to a clean drop of saline-glycerine solution on a microscope slide, covered with a glass cover slip, and examined for fluorescence at magnifications of 25, 160, and 400 diameters under longwave ultraviolet light (HBO 75W xenon light source) affixed to a Zeiss compound microscope.

Eight fluorescent compounds were screened as described above: ethidium bro-

mide, propidium iodide, fluorescein isothiocyanate, rhodamine B, rhodamine B isothiocyanate, victoria blue, lissamine green, and acridine orange. Of these, only acridine orange (AO) imparted a strong fluorescent color to the reproductive tissues.

Carbohydrate solutions containing the various concentrations of AO were prepared by first dissolving the powder in 1 ml of 95% ethanol and then diluting this to the desired concentrations with the sucrose solution; flies were provided with cotton soaked in the carbohydrate-AO solutions.

In separate tests, larval screwworms were reared on a water-base medium (dried blood, dried milk, powdered egg in a 7:5:3 ratio, respectively, with 1.7 ml formol per liter) containing 0 or 1.0 mg AO/ml of diet. Larvae fed continuously on treated medium from eclosion, 24 h of age, or 48 h of age until the crawl-off stage (96–108 h). Each treatment was replicated 3 times with 75 larvae per replicate. Resultant adults were held at 23°–28°C and 55% relative humidity under artificial light (10:14 L:D) through day 3 of adult life with access to separate containers of water and pure honey. Dissections were made on day 4 of adult life.

Fertile screwworms (strain A-82, non-irradiated) were also fed 1.0 mg AO/ml of 35% honey for 48 h to determine if AO functioned as a chemosterilant. Crosses were made (treated male × untreated female, and untreated male × treated female) and resultant eggs were scored for fertility. Parous females were dissected to verify insemination.

Results

All of the tissues examined were dyed with AO. Fluorescence was proportional to the concentration of the solution. At 0.01 mg AO/ml, fluorescence was barely perceptible at the highest magnification; whereas both the head and tail of spermatozoa from males that had fed on the 1.0 mg AO/ml of carbohydrate solution fluoresced a brilliant green under ultraviolet light at all magnifications used. Regardless of magnification, sperm from untreated males lacked fluorescent color under the same light source. All tests indicated that ingestion of AO at the highest concentration used did not adversely affect longevity (Table 1) nor inhibit mating (Table 2). Dissections through day 15 post-emergence (10 days post-treatment) indicated that the sperm in the testes continued to fluoresce without apparent loss of brilliance. This fluorescence did not appear to be light sensitive, as testes prepared 7–10 days previously still fluoresced. In further testing, similar results were obtained by feeding adults a solution of 1.0, 2.0 or 5.0 mg AO/ml of 35% honey, the standard carbohydrate used for feeding adults prior to aerial release in the eradication program. Results demonstrated that access to AO solutions for 24–48 h was sufficient to mark sperm in the testes for up to 15 days (maximum value tested) without affecting longevity nor propensity to mate.

Sperm in the spermathecae of both marked and unmarked females inseminated by marked males also fluoresced brilliantly. However, since all reproductive organs of treated females were strongly marked, further tests were conducted to determine the existence of "false positives"—i.e. whether sperm from untreated males could become marked by fluids in treated females. All possible crosses were made with marked and unmarked males and females. In blind tests, the source of sperm could easily be determined in crosses involving unmarked females, as sperm from treated males fluoresced brilliantly in females up to 12 days of age (4–6 days post

Table 1. Mortality of sterile adult *Cochliomyia hominivorax* fed 1.0 mg acridine orange per ml of 10% sucrose or 35% honey.

Age of cohort (days)	Mortality ($\bar{x}$% ± SD)[a]			
	Treated		Control	
	Male	Female	Male	Female
5	4.5 ± 4.5	1.8 ± 1.6	3.5 ± 2.4	3.0 ± 2.7
10	17.5 ± 12.0	25.0 ± 2.7	19.0 ± 3.0	15.3 ± 4.3

[a] n = 3 replicates per treatment with 30–60 males and 30–60 females per replicate.

copulation). Thereafter fluorescence was detected in less than 50% of the dissections. In untreated females 11 days old (3 to 5 days post-copulation), fluorescent sperm from marked males was light sensitive; the intensity diminished when tissues were exposed to ultraviolet light for 5–10 minutes. Regardless of pairings, sperm in marked females always fluoresced, thereby confirming the existence of false positives.

Studies on the effect of AO in the larval diet resulted in great variation in survival among replicates, even within controls. Therefore, results are discussed only in general terms. Acridine orange in the larval diet resulted in retardation of development in rates proportional to the time of exposure. Larvae with AO in their diet for the duration of larval development were smaller and required an additional 48–72 h to complete development and pupate. At the time of pupation, the integument of these larvae was characteristically orange. Exposure to AO beginning 24 or 48 h after eclosion resulted in nearly normal survival, slightly smaller pupae, and a total delay of development of 24 h, compared to controls. Resultant adults were dissected and all organs fluoresced, including muscles and pleural integument.

Fertile flies that fed on AO for 48 h post-emergence showed no aberrant behavior. Random samples of both crosses indicated that 96.4 ± 0.42% ($\bar{x}$ ± SD) mated, and 75.6 ± 5.16% of mated females oviposited; 90.2% of the egg masses were fertile.

Discussion

While the entire potential and limitations for this technique are not yet fully evaluated, these studies have demonstrated that acridine orange is a useful vital

Table 2. Mating competence of sterile adult *Cochliomyia hominivorax* fed 1.0 mg acridine orange per ml of 10% sucrose or 35% honey.

Age of cohort (days)	No. inseminated/no. examined	
	Treated[a]	Control[b]
6	0/6	0/6
7	0/6	4/6
8	5/6	5/6
10	5/6	5/6
12	6/6	5/6
14	6/6	5/6

[a] Both sexes were treated.
[b] Neither sex was treated.

stain for research on the mating behavior of screwworms. This technique will be of immediate application to laboratory studies already in progress in our laboratory in Tuxtla Guiterrez and, at Fargo North Dakota, for assessing the mating behavior of both sterile and fertile screwworms. These include studies on mating aggressiveness versus time in colony, multiple matings and sperm displacement, and mating competitiveness of large versus small males.

In recent years, some of our research in southern Mexico has focused on developing methodologies to enable us to improve studies on the bionomics and ecology of both sterile and fertile screwworms in the tropical Americas. Field studies conducted in 1982 (Brenner, 1984) demonstrated that sterile screwworms could be ground released in relatively small areas to test mating competence and to collect precise data on the dispersion of sterile flies within the test area. In 1983, this technique was used to compare the effectiveness of two strains of sterile flies in the same test site (Brenner and MacVean, in prep.). While this experimental design reduced or eliminated many of the variables discussed previously, a direct comparison of competitiveness was still not possible since release of the second strain was delayed by a period of four to six weeks in order for the native population to "recover" from the challenge of the first strain. Hence, both weather conditions and the age structure of the target population may have been different when the second strain was released.

The problems inherent to a simultaneous release of 2 or more test strains may be resolved if AO can be shown to be stable and persistant in sterile released flies. A field study has already been designed to examine the limitations of using AO to directly compare the competitiveness of two sterile strains released simultaneously and sympatrically. The results of feeding AO to larvae are encouraging and suggest that large numbers of screwworms can be easily and uniformly marked in this manner. Unfortunately, the existence of false positives will preclude a testing of the hypothesis that sterile females "trap" a significant amount of sperm from native males. Such information would be desirable in assessing the worth of a "males-only" rearing and release program. Ongoing laboratory studies will continue efforts to define the minimum dosage for maximum persistence, while monitoring any deleterious effects on the behavior of marked flies.

Because the acridines bind generally to mitochondrial DNA, or DNA polymerase (Waring, 1968); Simpson et al., 1974; Morales et al., 1972), AO should have a broad applicability for behavioral research on other insects. The ease in marking large populations, in detecting fluorescence with relatively unspecialized portable equipment, the relatively low toxicity, apparent lack of mutagenicity and the ready availability of this inexpensive compound should encourage extensive use of this technology.

Acknowledgments

I thank the technicians, Ernesto Lée Bosque, Eric Rubén Suriano Guzmán, and Sergio Zepeda Hernández, for their competent assistance. I am most grateful to numerous ARS scientists at Tuxtla Gutierrez; Fargo, North Dakota; Kerrville, Texas: and Gainesville, Florida for their suggestions of candidate fluorescent compounds. I also thank O. H. Graham, J. D. Hoffman, R. L. Mangan, and L. G. Zárate for reviewing an earlier version of this manuscript.

Literature Cited

Brenner, R. J. In press. Dispersal, mating and oviposition of the screwworm (Diptera: Calliphoridae) in southern Mexico. Ann. Entomol. Soc. Am.

Brenner, R. J. and C. M. MacVean. In prep. A field comparison of 2 strains of sterile screwworms (Diptera: Calliphoridae) using ground release technology.

Ephrussi, B. and G. W. Beadle. 1936. A technique of transplantation for *Drosophila.* Amer. Nat. 728: 218–225.

Lowe, R. E., H. R. Ford, A. L. Cameron, B. J. Smittle, D. A. Dame, R. S. Patterson, and D. E. Weidhaas. 1974. Competitiveness of sterile male *Culex pipiens quinquefaciatus* Say released into a natural population. Mosq. News 34: 447–453.

McInnis, D. O. 1984. A limited technique for distinguishing normal from irradiated sperm in screwworm flies, *Cochliomyia hominivorax* (Diptera: Calliphoridae). J. Med. Entomol. 21: 116–117.

Morales, N. M., F. W. Schaefer, III, S. J. Keller, and R. R. Meyer. 1972. Effects of ethidium bromide and several acridine dyes on the kinetoplast DNA of *Leishmania tropica.* J. Protozool. 19: 667–672.

Moss, J. I. and R. A. Van Steenwyk. 1982. Marking pink bollworm (Lepidoptera: Gelechiidae) with cesium. Environ. Entomol. 11: 1264–1268.

Simpson, L., A. M. Simpson, and R. D. Wesley. 1974. Replication of the kinetoplast DNA of *Leishmania tarentolae* and *Crithidia fasciculata.* Biochem. Biophys. Acta 349: 161–172.

Waring, M. J. 1968. Drugs which affect the structure and function of DNA. Nature 219: 1320–1325.