Journal of Chemical Ecology, Vol. 18, No. 7, 1992

EVIDENCE FOR THE ACCESSORY GLANDS AS THE SITE OF PRODUCTION OF THE OVIPOSITION ATTRACTANT AND/OR STIMULANT OF *Lutzomyia longipalpis* (DIPTERA: PSYCHODIDAE)

MARTIN J. DOUGHERTY,* RICHARD D. WARD, and GORDON HAMILTON

Department of Medical Entomology
Liverpool School of Tropical Medicine
Pembroke Place, Liverpool
Merseyside, L3 5QA United Kingdom

(Received July 16, 1991; accepted March 4, 1992)

Abstract—The phlebotomine sandfly *Lutzomyia longipalpis* Lutz and Neiva, the vector of visceral leishmaniasis in South America, has recently been shown to produce an oviposition semiochemical. In the present study it was found that a nonpolar extract of eggs was attractive and/or stimulatory to ovipositing females. A chromatographic investigation indicated the presence of similar compounds in accessory glands and egg extracts. Extract of accessory gland was also found to elicit a positive oviposition response. It is concluded that the pheromone is produced in the accessory glands and is secreted onto the eggs during oviposition.

Key Words—*Lutzomyia longipalpis*, Diptera, Psychodidae, oviposition, pheromone, accessory gland, bioassay.

INTRODUCTION

Pheromones from the eggs of a number of heamatophagus Diptera have been shown to attract ovipositing females; these include *Culex quinquefasciatus* Say Wiedmann (Bruno and Laurence, 1979; Sakaibara et al., 1984; Laurence et al., 1985; Laurence and Pickett, 1985; Hwang et al., 1987). A review of the various *Culex* species oviposition site selection cues, including orientation toward egg pheromone sources, is given by Bentley and Day (1989). Ovipositing *Aedes atropalpus* Coquillett are also attracted to an oviposition attractant produced by immature stages (Kalpage and Brust, 1973). Furthermore, Soman and Reuben

*To whom correspondence should be addressed.

0098-0331/92/0700-1165$06.50/0

(1970), showed that gravid *Aedes aegypti* (L.) females were attracted to water containing immature stages of the same species.

Recently, Elnaiem and Ward (1990, 1991a) showed that eggs of the sandfly *Lutzomyia longipalpis* Lutz and Neiva have an attractant and/or stimulant effect, related to the presence of a semiochemical, perceived by conspecific, ovipositing females. When eggs were washed in a series of polar and nonpolar solvents, the oviposition response was no longer elicited. This demonstrated that the oviposition attractant and/or stimulant effect was chemically mediated, and not due solely to a visual or tactile effect of the eggs.

The field behavior of *L. longipalpis* during oviposition is little studied. The females are known to be gonotrophically concordant and pass through more than one gonotrophic cycle (Guilivard et al., 1980, Dye et al., 1987). A major problem in the study of the transmission of leishmaniasis is the very high mortality rate found at oviposition. This is attributed to the difficulty in matching laboratory and field conditions. Females are known to lay up to 100 eggs in a single oviposition but the normal number is 40. In the wild, the eggs are thought to be laid in microhabitats, such as cracks and crevices (Young et al., 1926; Lewis and Kirk, 1954; Ward, 1974), rich in organic nutrients and are difficult to find in normal circumstances. The eggs are laid in humid conditions and it is known that control of temperature and humidity is an essential requirement of colony maintenance (Killick-Kendrick, 1977).

The photoperiod does not affect the success of oviposition of *L. longipalpis* but does markedly alter the oviposition periodicity (Ready, 1976). Based on laboratory observations it appears that some species are capable of long periods of dormancy (Johnson and Hertig, 1961). While capable of withstanding desiccation, the eggs are resistant to prolonged immersion (Chaniotis, 1967). This is a useful adaptation in a terrestrial insect that may often suffer temporary inundation due to rainfall.

Accessory glands of many female insects produce secretions which are present in or on oviposited eggs (Adiyodi and Adiyodi, 1989). In the present study, therefore, we investigated if organic or aqueous extracts of *L. longipalpis* eggs retained biological activity similar to that induced previously by the use of whole eggs. In addition, a chromatographic investigation was carried out to determine if the female accessory glands had a role in the production of the semiochemical.

METHODS AND MATERIALS

Colonization

The sandflies used in the experiments were *L. longipalpis* established from flies collected at Jacobina, Bahia, Brazil, and maintained in the laboratory for

6 years using methods described by Modi and Tesh (1983), at 95% RH, a room temperature of 28°C, and a 12h L:D photoperiod.

Female *L. longipalpis* were fed on Syrian hamsters anesthetized with 12 mg/ml of sodium pentabarbitone and were kept for 4 days to allow for complete defecation and oogenesis before use in bioassays.

Sample Preparation

All eggs used for extraction purposes were taken from colony rearing pots 1 to 2 days after oviposition. To prepare the nonpolar egg extract, 1000 eggs were placed in a 2-ml microvial (Alltech) and left for 24-hr at −70°C in 100 μl of pesticide-grade hexane (BDH Chemicals). Accessory gland extracts were prepared from 50 gravid females that had been blood-fed 4 days previously. The glands were excised under a stereoscopic microscope and the tissue was transferred to a 2-ml microvial using a micropipette and extracted as described above. The remaining female bodies were also similarly extracted, to provide material for a negative control. To prepare the polar extract 1000 eggs were placed in a 2-ml microvial and extracted in 100 μl of double-distilled, deionized H_2O at 3°C for 24 hr; 100 accessory glands and the remainder of the female bodies were also similarly extracted. The preparation of material for HPTLC and GC analysis used the same protocol as described above. Five hundred *L. longipalpis* eggs and 50 excised female accessory glands were extracted in 20 μl of hexane. Further, for GC analysis, five whole male, whole female, and female flies without accessory glands were extracted in 40 μl of hexane. Samples were stored at −70°C.

Bioassay

Only eggs laid in the test and control sites were counted. The bioassay was an adaptation of that used by Elnaiem and Ward (1991). The bioassay chambers were modified polymethylpentene (BDH apparatus) rearing pots. Modifications included the use of two oviposition areas, 2 cm^2, termed test and control, with lightly demarcated perimeter grooves on the plaster of paris base.

Six bioassays were conducted, each with 10 replicates, using 20 females per replicate. In each oviposition chamber a 10 μl aliquot of sample extract was pipetted onto the plaster of paris base at the test site. The control was 10 μl of pure extraction solvent. This test volume consistently gave an equivalent of 100 eggs, accessory glands from five females, or five female bodies without accessory glands. Bioassays performed were (a) nonpolar egg extract versus hexane control, (b) nonpolar accessory gland extract versus hexane, (c) polar egg extract versus water control, (d) polar accessory gland extract versus water, (e) nonpolar extract of females without accessory glands versus hexane, and (f) polar extract of females without accessory glands versus water.

After loading the oviposition areas in the bioassay chambers with the test

and control samples, 20 gravid females were introduced and left for 3 days under normal colonization conditions. The females were provided with a small wad of cotton wool, soaked with a saturated solution of sucrose, placed on top of the oviposition chamber. Provision of a sugar meal is known to increase the number of eggs laid per sandfly (Ready, 1979).

Statistical Analysis

The bioassay design gives a matched pair of results, which was found to be nonparametric. As such, Wilcoxon's rank paired test was employed to a 95% confidence level.

Chemical Analysis

High-Performance Thin-Layer Chromatography (HPTLC). High-performance TLC plates were 10 × 20 cm, coated with a 200-μm-thick layer of silica (Whatman Lab Supplies). The developing solvent was a 1 : 1 chloroform/hexane solution (BDH Chemicals), described by Kates (1986), for nonpolar lipids. HPTLC plates were prepared by baking at 200°C for 24 hr and then cleaned by running twice in the solvent system. Twenty-microliter aliquots of egg and accessory gland extract (25 female equivalents) were applied to the plates with 2-μl capillary tubes. Twenty micrograms of each of five standards (cholesterol, cholesterol linolenate, cholesterol stearate, squalene and caryophyllene oxide; Sigma Chemicals) was applied to the plate. Plates were visualised by ultraviolet light at 302 nm, followed by charring with concentrated H_2SO_4/EtOH solution (1 : 1, v/v) and baking at 110°C for 10 min. Comparisons of samples and standards were carried out using R_f values.

Gas Chromatography. Gas chromatography was performed on a Shimadzu GC-15a (Dyson Instruments), fitted with a Grob splitless injection system with a 0.6-min sampling time. The column used was a 30-m, fused silica DB1 capillary column, with a 0.25-μm film thickness (J. and W. Scientific). The temperature program was as follows: 45°C initial, increased at 15°C min^{-1} to 250°C, held for 4 min, increased at 10°C min^{-1}, and then held at 350°C for 4 min. The carrier gas was helium at a pressure of 4.5 kg cm^3 and the injection block and detector temperatures were 250 and 350°C, respectively. Integration was carried out with the Shimadzu CR5a Chromatopac (Dyson Instruments).

Only nonpolar extracts were analyzed by GC. Samples included extracts of eggs, female accessory glands, whole males, whole females, and females without accessory glands prepared as described above. Before being analyzed by GC, extracts, cooled on ice, were reduced under N_2 to 1 μl.

RESULTS

Bioassays

The bioassay results are summarized in Table 1. The response of *L. longipalpis* during oviposition to hexane extracts of egg and accessory gland material showed a highly significant difference between the test and the control sites. Neither the hexane nor the aqueous extracts of female bodies without accessory glands produced a statistically different number of eggs at the test and control sites. Aqueous extracts of both egg and accessory glands failed to induce an oviposition response. Thus nonpolar extracts of both egg and accessory gland material were the only samples to elicit the biological response which has previously been associated with whole eggs.

Thin-Layer Chromatography

The HPTLC analysis demonstrates chemicals present in both egg and accessory gland samples that are of a very similar polarity (Table 2). A compound which has a R_f value similar to that of cholesterol, lane 1 ($R_f = 0.12$), is present in both, as is a compound that is more polar than cholesterol. Two other pairs of matching spots are observed on the HPTLC plate. The spots at $R_f = 0.51$ (accessory, lane 6) and $R_f = 0.50$ (egg, lane 7) are only slightly less polar than caryophyllene oxide, lane 4 ($R_f = 0.48$). At $R_f = 0.62$ (accessory) and $R_f = 0.66$ (egg) two spots were exhibited of a polarity similar to that of cholesterol stearate, lane 5, and cholesterol linolenate, lane 1.

TABLE 1. OVIPOSITION BIOASSAY WITH POLAR AND NONPOLAR EXTRACTS OF EGGS, ACCESSORY GLANDS, AND WHOLE FEMALE *L. longipalpis* WITHOUT ACCESSORY GLANDS[a]

Extract	Solvent	Oviposition response (mean ± SD)		*P*
		Test	Control	
Egg	Hexane	95.40 ± 13.79	20.80 ± 8.68	0.006
Egg	Water	22.00 ± 8.16	26.20 ± 4.76	0.186[b]
Accessory gland	Hexane	98.80 ± 12.31	26.40 ± 11.54	0.006
Accessory gland	Water	23.60 ± 6.26	24.3 ± 4.90	0.610[b]
Whole ♀ without gland	Hexane	25.90 ± 6.49	26.50 ± 6.98	0.799[b]
Whole ♀ without gland	Water	25.70 ± 5.60	23.50 ± 4.20	0.262[b]

[a]Each bioassay was composed of 10 replicates employing 20 females in each. *P*, probability. Consistently 100 egg equivalents, 5 female equivalents of accessory gland material, and 5 whole-female equivalents without glands were used in each respective bioassay.
[b]Nonsignificant by Wilcoxon ranked pair test.

Gas Chromatography

The retention times of major peaks found during the GC investigation are shown in Table 3. Chromatograms of the following extracts are shown in Figures 1A–C; females without accessory glands, eggs, and accessory glands. There is a similar pattern to all the traces, showing a series of compounds common to

TABLE 2. R_f VALUES OF STANDARDS AND EXTRACTS OF ACCESSORY GLAND AND EGG SAMPLES BY HPTLC[a]

Sample	1	2	3	4	5	6	7	8
Cholesterol linolenate						0.69		
Cholesterol	0.12							
Squalene							0.72	
Caryophyllene				0.48				
Cholesterol sterate						0.67		
Accessory gland	0.12				0.51	0.62		
	0.03							
Egg	0.12				0.5	0.66		
	0.06							

[a] Plates were loaded at 20 μl standards, 500 equiv. eggs, 25 female equiv. accessory glands. Solvent phase, 1 : 1 chloroform/hexane, with a silica gel plate.

TABLE 3. CHROMATOGRAM RETENTION TIMES FOR EXTRACTS OF EGG, ACCESSORY GLAND, WHOLE-FEMALE, AND WHOLE-MALE SAMPLES BY GAS CHROMATOGRAPHY[a]

	Retention time (min)					
	11	12	13	14	15	16
Egg	11.86		13.52	14.82		16.21
				14.99		
Accessory gland	11.89		13.50	14.84		16.20
				14.99		
Whole ♀	11.88		13.34	14.86	15.00	16.2
			13.50			
Whole ♂	11.05			14.85		16.19
	11.51			14.99		
Corresponding number on chromatogram[a]	1		2	3		4

[a] Numbers correspond to peaks on chromatograms in Fig. 1.

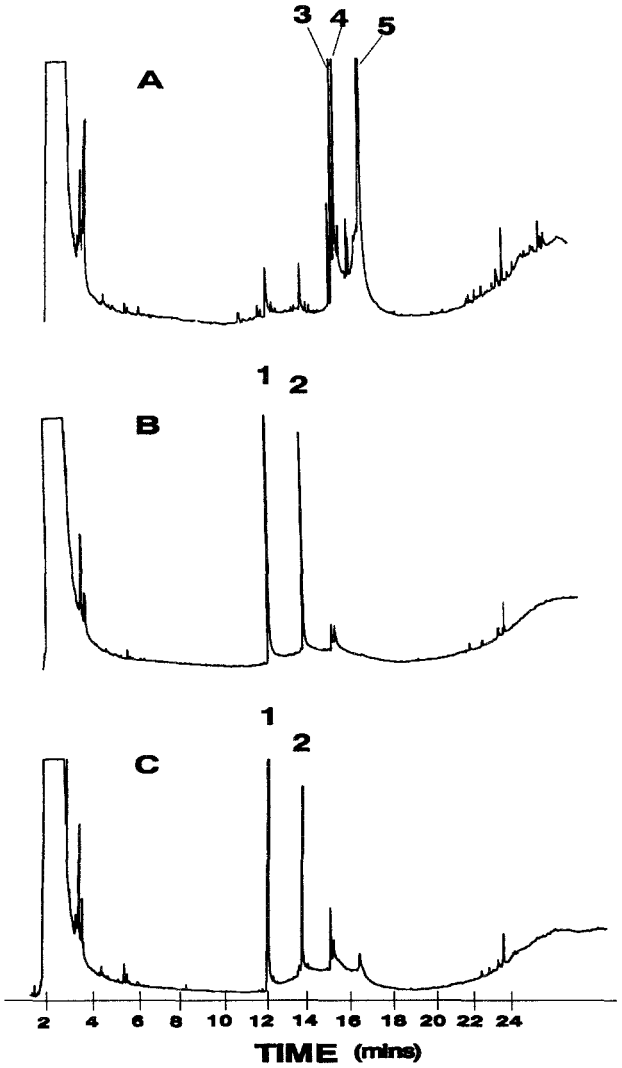

FIG. 1. Gas chromatograms of hexane extracts of (A) females without accessory glands, (B) eggs, and (C) accessory glands. Peak numbers correspond to those given in Table 3. The experimental conditions are given in the text.

all the samples, with peaks 1 and 2 being specific to eggs and accessory glands. Male *L. longipalpis* has two major peaks, at 11.052 and 11.511. These are known to originate from the tergal glands (Hamilton, personal communication). Whole-female extract has major peaks at 11.887 and 13.509, which are not

present in the whole male samples. These two peaks (peaks 1 and 2 on the chromatogram) do occur in female accessory gland extract (11.896 and 13.502) and in egg material (11.862 and 13.521) and are greatly reduced in samples of females with excised accessory glands.

DISCUSSION

This is the first demonstration that the oviposition semiochemical present on the eggs of *L. longipalpis* may be extracted in hexane, indicating that the molecule is a relatively nonpolar organic compound. Furthermore, the extract does not lose its oviposition attractant and/or stimulant properties when bioassayed. The hexane extract of the accessory gland material also elicited similar biological activity. In contrast, hexane extracts of female bodies from which accessory glands had been excised did not elicit an oviposition response, nor did any of the aqueous extracts.

The gas chromatographic analysis revealed that two compounds which occur in extracts of whole female flies and accessory glands are also present in extracts of eggs. These compounds are absent in females without accessory glands and from whole males. The HPTLC results indicate that two compounds of comparable polarity are present in egg and accessory gland material. These compounds are of a polarity similar to that of cholesterol linolenate or cholesterol stearate and caryophyllene oxide, all of which are oxygenated compounds.

Chromatographic and bioassay evidence suggests that the semiochemical(s) present on *L. longipalpis* eggs is produced by the accessory glands of the female. It appears that as eggs are laid, the semiochemical is coated onto them from this gland. It is known that secretions from the accessory gland include a sticky substance, which is used to adhere the egg to oviposition substrates (Davis, 1967; Wu and Tesh, 1989). Adiyodi and Adiyodi (1989) reviewed the role of the accessory glands of female insects, noting them to have a secretory role in both egg maturation for ootheca production and during oviposition.

In contrast, the presence of oviposition-deterring pheromones in sticky secretions used to adhere eggs to oviposition substrates has been shown in the sorghum shoot fly, *Atherigona soccata* (Raina, 1981). Here, a water-soluble glue is used to stick eggs to the leaves of sorghum.

Little is known about the attractant and/or stimulant role of the *L. longipalpis* oviposition pheromone. It is possible that the pheromone acts over a limited range, with the female being directed to oviposition sites by long-range volatile chemicals from organic substrates and physical characteristics of the oviposition substrate. In this situation the pheromone would be the final phase in the oviposition site selection mechanism. Elnaiem and Ward (1991b) produced some circumstantial evidence for the above hypothesis, showing that

oviposition *L. longipalpis* females were attracted to an aqueous extract of rabbit feces, a component of the laboratory larval rearing medium. Schlein et al. (1990) also found that *Phlebotomous papatasi* chose oviposition sites by physical and chemical stimuli in the oviposition substrate. Furthermore, it is known that *Culex* females select oviposition sites by environmental, physical, chemical, and pheromonal cues.

Use of an oviposition pheromone by *L. longipalpis* may lead to larval aggregation, which may be advantageous in overcoming factors such as predation and parasitism (Manning, 1979). However, attempts to locate sandfly larval breeding sites in soil samples have only shown sparsely scattered immature stages (Hanson, 1961; Thatcher, 1968; Rutledge and Mosser, 1972). However, Bettini et al. (1986), using emergence traps, were successful in collecting 27,405 emergent flies from a 25-m^2 breeding site in 1 year. Further investigation is therefore needed to clarify the apparent disparity between the results from these two sampling techniques and to investigate the role of the oviposition pheromone in the field. It is recognized that some oviposition pheromone-like activity has been associated with the presence of volatiles from bacterial activity, as with *Lucilia cuprina* Wiedmann (Emmens and Murray, 1983). As accessory gland extract of female *L. longipalpis* elicited a response and samples of female bodies without these glands did not, it is thought that this phenomenon is not occurring here. Further work is currently being carried out to clarify this point and to determine the structure of the components of egg and accessory gland material that are responsible for the biological activity.

Acknowledgments—This work was carried out with financial support from the Wellcome Trust, London.

REFERENCES

BENTLEY, M.D., and DAY, J.F. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Annu. Rev. Entomol.* **34**:401–421.

BETTINI, S. 1988. Sandfly breeding sites, pp. 179–188, *in* D.T. Hart (ed.). Leishmaniasis, the Current Status and New Strategies for Control. Plenum Press, New York and London.

BRUNO, D.W., and LAURENCE, B.R. 1979. The influence of the apical droplet of the *Culex pipens fatigans* (Diptera: Culicidae). *J. Med. Entomol.* **16**:300–305.

CHANIOTIS, B.N. 1967. The biology of California *Phelebotomus* (Diptera: Psychcodidae) under laboratory conditions. *J. Med. Entomol.* **4**:221–233.

DAVIS, N.T. 1967. Leishmaniasis in the Sudan republic. Anatomical studies on *Phlebotomus orientalis parroti* and *P. papatasii* Scopoli. *J. Med. Entomol.* **4**:50–65.

DYE, C., GUY, M.W., ELKINS, D.B., WILKES, T.J., and KILLICK-KENDRICK, B. 1987. The life expectancy of phlebotomine sandflies: first field estimate from Southern France. *Med. Vet. Entomol.* **1**:417–425.

ELNAIEM, D.A., and WARD, R.D. 1990. An oviposition pheromone on the eggs of sandflies (Diptera: Psychodidae). *Trans. R. Soc. Trop. Med. Hyg.* **84**:456–475.

ELNAIEM, D.A., and WARD, R.D. 1991a. Response of the sandfly *Lutzomyia longipalpis* to an oviposition pheromone associated with conspecific eggs. *Med. Vet. Entomol.* **5**:87–91.

ELNAIEM, D.A., and WARD, R.D. 1991b. Oviposition attractants and stimulants for the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.* **29**:5–12.

EMMENS, R.L., and MURRAY, M.D. 1983. Bacterial odours as oviposition stimulants for *Lucilia cuprina* Wiedmann (Diptera: Calliphoridae), the Australian sheep blowfly. *Bull. Entomol. Res.* **73**:411–415.

GILLOT, C. 1989. Arthropoda—Insecta, pp. 371–384, *in* K.G. and R.G. Adiyodi (eds.). Reproductive Biology of Invertebrates, Vol. III. Accessory Glands. John Wiley and Sons, Chichester, New York, Brisbane, Toronto, and Singapore.

GUILIVARD, E., WILKES, T.J., KILLICK-KENDRICK, R., and RIOUX, J.A. 1980. Ecologie des leishmanioses dans le sud de la France. Derovlement des cycles gonotrohphique chez *Phlebotomous ariasi* Tonnoir, 1921 et *Phlebotomus mascitti* Grassi, 1908, en Cenennes Corollaire epidemioloque. *Ann. Parasitol.* **55**:659–664.

HANSON, W.J. 1961. The breeding places of Phlebotomus in Panama (Diptera: Psychodidae). *Ann. Entomol. Soc. Am.* **54**:317–322.

HAWANG, Y.S., MULLA, M.S., CHANEY, J.D., LIN, G.G., and XU, H.J. 1987. Attractancy and species specificity of 6-acetoxy-5-hexadecanolide, a mosquito oviposition attractant pheromone. *J. Chem. Ecol.* **13**:245–252.

JOHNSON, P.T., and HERTIG, M. 1961. The rearing of *phlebotomus* sandflies (Diptera: psychodidae). 11. Development and behaviour of Panamanian sandflies in laboratory culture. *Ann. Entomol. Soc. Am.* **54**:764–776.

KALPAGE, K.S.P., and BRUST, R.A. 1973. Oviposition attractant produced by immature *Aedes atropalpus*. *Environ. Entomol.* **2**:729–730.

KATES, M. 1986. Techniques of Lipidology: Isolation, Analysis and Identification of Lipids, 2nd. rev. ed. Elsevier, Amsterdam.

KILLICK-KENDRICK, R. 1978. Recent advances and outstanding problems in the biology of phlebotomine sandflies. *Acta Tropica* **35**:297–313.

LAURENCE, B.R., and PICKETT, J.A. 1985. An oviposition pheromone in *Culex quinquefasciatus say* (Diptera: Culicidae). *Bull. Entomol. Res.* **75**:283–290.

LAURENCE, B.R., MORI, K., OTSUKA, T., PICKETT, J.A., and WADHAMS, L.J. 1985. Absolute configuration of mosquito oviposition attractant pheromone, 6-acetoxyhexadecanolide. *J. Chem. Ecol.* **11**:643–648.

LEWIS, D.J., and KIRK, R. 1954. Notes on the phlebotominae of the Anglo Egyptian Sudan. *Ann. Trop. Med. Parasitol.* **48**:33–45.

MANNING, A. 1979. An Introduction to Animal Behaviour, 3rd. ed. Edward Arnold, London.

MODI, G.B., and TESH, R.B. 1983. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *J. Med. Entomol.* **20**:568–569.

RAINA, A.K. 1981. Deterrence of repeated oviposition in sorghum shoot fly, *Atherigona soccata*. *J. Chem. Ecol.* **7**:785–790.

READY, P.D. 1976. Studies on the Biology of Phelbotomid Sandflies, Ph.D. thesis. University of London, London.

READY, P.D. 1978. The feeding habits of laboratory bred *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.* **14**:545–522.

RUTELEDGE, L.C., and MOSSER, H.L. 1972. Biology of phlebotomine sandflies on the open forest floor in Panama. *Environ. Entomol.* **1**:300–309.

SAKAKIBARA, M., IKESHOJI, T., MACHIYA, T., and ICHIACOTO, I. 1984. Activity of four sterioisomers of 6-acetoxy-hexadecanolide, the oviposition pheromone on Culicine mosquitos. *Jap. J. Sanit. Zool.* **35**:401–403.

SCHLEIN, Y.S., BOURT, S., and JACOBSON, R.L. 1990. Oviposition diapause and other factors affecting the egg-laying of *Phlebotomous papatasi* in the laboratory. *Med. Vet. Entomol.* **4**:69–78.

SOMAN, R.S., and REUBEN, R. 1970. Studies on the preference shown by ovipositing females of *Aedes aegypti* for water containing immature stages of the same species. *J. Med. Entomol.* **7**:785–789.

THATCHER, V.E. 1986. Arboreal breeding sites of Phlebotomine sandflies in Panama. *Ann. Entomol. Soc. Am.* **61**:1141–1143.

WARD, R.D. 1974. Studies on the Adult and Immature Stages of Some Phlebotomid Sandflies (Diptera: Phlebotominae) in Northern Brazil, Ph.D. thesis. University of London, London.

WU, W.K., and TESH, R.B. 1989. Experimental infection of Old and New World Phlebotomine sandflies (Diptera: Psychodidae) with *Ascogregarine chagasi* (Eugregarinorida: Lecudinidae). *J. Med. Entomol.* **26**:237–247.

YOUNG, T.C.M., RICHMOND, A.E., and BRENDISH, G.R. 1926. Sandflies and sandfly fever in the Peshawar district. *Indian J. Med. Res.* **13**:961.