BIOLOGY AND COLONIZATION OF THE SAND FLY *Lutzomyia diabolica* (HALL) (DIPTERA: PSYCHODIDAE) WITH NOTES ON ITS POTENTIAL RELATIONSHIP TO HUMAN CUTANEOUS LEISHMANIASIS IN TEXAS, USA

By

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TO MY "FOREVER" FAMILY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

BIOLOGY AND COLONIZATION OF THE SAND FLY *Lutzomyia diabolica* (HALL) (DIPTERA: PSYCHODIDAE) WITH NOTES ON ITS POTENTIAL RELATIONSHIP TO HUMAN CUTANEOUS LEISHMANIASIS IN TEXAS, USA

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A survey for potential sand fly vectors of human cutaneous leishmaniasis was conducted within an endemic focus of the disease in south central Texas, USA. Five species of *Lutzomyia*, including one new species, were collected and eight new county records established. *Lutzomyia diabolica* (Hall), the only anthropophilic sand fly encountered, was the most commonly collected, accounting for 99% of the total catch. This species was taken in light trap collections throughout the frost-free season at a case site in D'Hanis, Texas. No natural leishmanial infections were observed in more than 600 dissections of wild-caught female *Lu. diabolica*. Several natural infections of nonleishmanial parasites are reported for the first time.

The first productive laboratory colony of *Lutzomyia diabolica* was established and detailed studies of the fly's biology were conducted

through 16 generations. A new larval diet, developed from a modified horn fly [Haematobia irritans (Linn.)] diet, reduced the average immature development time by 50%, to about 33 days. Quiescence and diapause occurred in both the egg and larval stages, and lasted as long as 270 days in the egg stage of outside-reared sand flies.

Infection rates of 88% and 95% in Lu. diabolica and Lu. shannoni, respectively, were obtained by feeding the flies on leishmanial histiocytomas of laboratory-infected hamsters. The development of Leishmania mexicana (strain WR-411) in Lu. diabolica is described in detail.

Ultrastructure studies of L. mexicana amastigotes and promastigotes revealed that the subpellicular microtubule number varies widely and is not a good criterion for distinguishing the Leishmania species.

For the first time, transmission experiments demonstrated that Lu. diabolica and Lu. shannoni are able to transmit L. mexicana to hamsters by individual bites. Transmission occurred after a period of parasite multiplication and development, during which time massive infections were established initially in the midgut, then in the cardia and at the stomodeal valve. These gave rise to short-slender, highly active promasitgotes that spread throughout the alimentary tract and invaded the pharynx and mouthparts.

Based on the findings of this study, Lutzomyia diabolica is the suspected vector of human cutaneous leishmaniasis in Texas.

CHAPTER 1
SAND FLIES AND LEISHMANIASIS

Introduction

The common name "sand fly" has been used confusingly in the literature for members of two families of Diptera, the Ceratopogonidae and the Psychodidae. In this work "sand fly" refers only to the members of Psychodidae belonging to the subfamily Phlebotominae.

Sand flies are important as vectors of several human pathogens including phleboviruses (e.g., sand fly fever), bartonellosis (Carrion's disease) and, most notably, leishmaniasis, a complex of diseases caused by various species and subspecies of unicellular hemoflagellates in the genus Leishmania Ross (Adler and Theodor, 1957). Leishmaniasis is widely distributed in most tropical and subtropical countries, extending through Central and South America, Central and Southeast Asia, India, China, the Mediterranean Basin, and Africa (Lainson, 1982). Until recently the disease was believed to be absent from North America north of Mexico, but the confirmation of several autochthonous human cases in Texas since 1968 has dispelled that belief (Simpson et al., 1968; Shaw et al., 1976; Gustafson et al., 1984). In 1981 the World Health Organization (WHO) estimated that 400 thousand new cases of leishmaniasis occur annually throughout the world, but this may be an underestimation. Leishmaniasis is probably second in importance only to malaria among the protozoan diseases in

terms of human suffering and economics (Lainson, 1982). Selection of leishmaniasis by WHO as one of six diseases of man warranting a special program of study has led to increasing interest in sand flies, the only known natural vectors of the disease (Killick-Kendrick, 1978; WHO, 1981). It should be noted that workers in Oklahoma fed nymphal ticks [Rhipicephalus sanguineus (Latrielle)] on dogs infected with L. infantum and found that the ticks remained culture positive for the parasite for at least one month postmolting (Fox, pers. comm., 1984). Culture-positive adult ticks that subsequently fed on uninfected puppies, transfered Leishmania to the puppies. Although this demonstrates that ticks can experimentally transmit leishmaniasis, it may not occur under natural circumstances.

Previous work on sand flies has been mostly taxonomic. More recently, however, interest has turned to the biology of sand flies, the most important practical aspect being their relationship with leishmanial parasites. The role as vectors of most of the 53 species or subspecies of sand flies thought to transmit leishmaniasis to man requires confirmation (Killick-Kendrick, 1978). Of the 21 species of New World sand flies reported or suspected as being natural hosts of Leishmania spp. infecting man, only six have been definitely incriminated as vectors (Table 1-1). Prior to this study no anthropophilic species in the USA had been incriminated in the transmission of leishmaniasis.

Historical Review of the Incrimination of Major Vectors of Leishmaniasis

Cutaneous and visceral leishmaniases are apparently ancient afflictions of man. As early as the first century AD, in Central

Table 1-1. Proven or suspected sand fly vectors of leishmaniasis in the New World, by country (Killick-Kendrick, 1978; WHO, 1984).

Country	*Leishmania* parasites [1]	*Lutzomyia* sand fly vectors
Belize	*L. m. m.*	*olmeca*[2]
Bolivia	*L. d. c.*	*longipalpis*[3]
Brazil	*L. d. c.*	*longipalpis*[2]
Brazil	*L. b. b.*	*amazonensis*[3]
Brazil	*L. b. b.*	*intermedia*[2]
Brazil	*L. b. b.*	*migonei*[3]
Brazil	*L. b. b.*	*parensis*[3]
Brazil	*L. b. b.*	*pessoai*[3]
Brazil	*L. b. b.*	*wellcomei*[3]
Brazil	*L. b. b.*	*whitmani*[3]
Brazil	*L. b. g.*	*anduzei*[3]
Brazil	*L. b. g.*	*umbratilis*[2]
Brazil	*L. m.*	*flaviscutellata*[3]
Colombia	*L. d. c.*	*longipalpis*[3]
Colombia	*L. b.*	*trapidoi*[3]
Costa Rica	*L. b.*	*shannoni*[3]
Costa Rica	*L. b.*	*ylephiletor*[3]
Dominican Republic	*L. ?*	*christophei*[3]
Ecuador	*L. b.*	*hartmanni*[3]
Ecuador	*L. b.*	*trapidoi*[3]
El Salvador	*L. d. c.*	*longipalpis*[3]
French Guiana	*L. b. g.*	*umbratilis*[3]
Guatemala	*L. d. c.*	*longipalpis*[3]
Guatemala	*L. m.*	*olmeca*[3]

Table 1-1. Continued.

Country	Leishmania parasites[1]	Lutzomyia sand fly vectors
Honduras	L. d. c.	longipalpis[3]
Mexico	L. d. c.	longipalpis[3]
Mexico	L. m.	olmeca[2]
Nicaragua	L. d. c.	longipalpis[3]
Panama	L. b. p.	gomezi[3]
Panama	L. b. p.	panamensis[3]
Panama	L. b. p.	trapidoi[2]
Panama	L. b. p.	ylephiletor[3]
Paraguay	L. d. c.	longipalpis[3]
Peru	L. p.	peruensis[3]
Peru	L. p.	verrucarum[3]
Surinam	L. b. g.	umbratilis[3]
USA	L. m.	diabolica[3]
Venezuela	L. d. c.	longipalpis[3]
Venezuela	L. m. a.	flaviscutellata[3]
Venezuela	L. m. g.	townsendi[3]

[1]Leishmania species: L. m. m. = L. mexicana mexicana, L. d. c. = L. donovani chagasi, L. b. b. = L. braziliensis braziliensis, L. b. g. = L. braziliensis guyanensis, L. m. = L. mexicana, L. b. p. = L. braziliensis panamensis, L. p. = L. peruviana, L. m. a. = L. mexicana amazonensis, L. m. g. = L. mexicana garnhami.

[2]Proven vector.

[3]Suspected vector.

Asia, the cutaneous disease was referred to as "Balkh Sore" (after a town in north Afghanistan, near the Russian border), and by early travelers as "Aleppo Boil" in Syria and "Baghdad Boil" in Iraq (Lainson, 1982). In the Americas, Peruvian and Ecuadorean pottery from the era 400 to 900 AD depicts human faces with mutilations very similar to those caused by cutaneous and mucocutaneous leishmaniasis. Spanish historians at the time of the conquest described severely mutilating sores on the faces of Peruvian Indians (Lainson, 1982).

Because the major signs and symptoms of visceral leishmaniasis resemble those of several other tropical diseases, it is difficult to trace clear-cut references to this disease in ancient writings in either the Old or the New World. The disease first attracted public attention in 1882 when Clark, of the Sanitary Commission of India, gave an account of 100 cases of a severe form of malarial cachexia, depopulating areas of the Garo Hills, Assam. Natives of the area called the disease "kala-azar" (black fever) and it appears that it was known to them as early as 1869 (Strong, 1945). Epidemics of what must have been the same disease, under the name of "Burdwan fever," occurred in lower Bengal from 1854 to 1875, causing a quarter of a million deaths (Strong, 1945). During this same period in the Mediterranean region it was referred to as "infectious splenic anemia" or "infantile splenic anemia" (Lainson, 1982).

Visceral leishmaniasis apparently was unrecognized as a distinct disease in Latin America until the time of the first parasitologically proven case, in Paraguay in 1913 (Migone, 1913). This fact, coupled with clinical, epidemiological, and biochemical similarities between Mediterranean and American visceral leishmaniasis, suggests that it was

introduced since the European discovery of the New World (Lainson, 1982).

Possibly the first written account implicating sand flies in the transmission of human pathogens, including leishmaniasis, appeared in 1764 in a sort of almanac published in Lima, Peru, under the direction of Cosme Bueno, distinguished physician, mathematician and geographer (Herrer and Christensen, 1975). In El Conocimiento de los Tiempos he discussed the folklore regarding the natural transmission of verruga (bartonellosis) and corrosive facial ulcers (uta, a form of cutaneous leishmaniasis), reporting that both diseases originate from the bite of a small insect called "Uta" (sand fly). More than a century later, Mitford reported on cutaneous leishmaniasis in the Middle East and considered the possible participation of some insect in the transmission of Aleppo boil, although its exact role was not clearly indicated (Lewis, 1978). In 1904 Rogers discovered that the causative agent of Indian kala-azar (visceral leishmaniasis) developed into a leptomonad flagellate in culture. He also noted that similar organisms (e.g., Leptomonas) had been found in insects (mosquitoes), tangentially suggesting an insect vector of kala-azar (Rogers, 1904). The ensuing search for vectors of kala-azar (Leishmania donovani) and oriental sore (Leishmania tropica) included a wide range of suspects including bed bugs, fleas, mosquitoes, house flies, sand flies, hippoboscids, and even leeches. In 1905, the Sergents and Pressat, attracted by the coincidental distribution of sand flies and leishmaniasis, independently suggested that these insects were probable vectors of oriental sore (Sergent et al., 1914; Kirk and Lewis, 1955). Wenyon (1912) reviewed the advances made in the knowledge of leishmaniasis in

the Old and New Worlds and supported the suggestion that some sort of insect was involved in its transmission. He and others unsuccessfully attempted to demonstrate leishmaniasis transmission using fleas (Ctenocephalides canis and Pulex irritans), mosquitoes (Stegomyia fasciata = Aedes aegypti), bed-bugs (Cimex sp.), and sand flies (Phlebotomus sp.). On another occasion, Wenyon (1911) dissected a number of wild-caught sand flies from Aleppo and observed "Herpetomonas" flagellates in about 6% of the specimens; he acknowledged the possibility that what appeared to be harmless parasites of sand flies might, in fact, be developmental forms of L. tropica. Wenyon's discovery marked the beginning of intensified efforts by numerous researchers to study all aspects of the parasitic relationship between Leishmania and the sand fly host. For the next 30 years investigations progressed mainly on two fronts, in North Africa and Palestine with oriental sore, and in India with kala-azar.

Old World Leishmaniasis

Leishmania tropica (cutaneous leishmaniasis, "oriental sore"). In a note on the etiology of oriental sore in Mesopotamia, Patton (1919) believed that P. papatasi Scopoli and probably P. minutus Rondani were carriers of the parasite. Acton (1919) showed that the distribution on the body of oriental sores corresponded to the distribution of bites by Phlebotomus. In 1921, Sergent et al., working in Algeria, first described the transmission of oriental sore to a human. They divided 559 sand flies into 23 batches, crushed them in saline and inoculated the resulting suspensions into the arms of 23 volunteers. The flies had been collected in Biskra, an endemic center

of the disease 600 km south of Algiers where the disease does not occur. Only one of the inoculations produced positive results (Adler and Theodor, 1925).

Adler and Theodor (1925), studying Phlebotomus in Jericho (Jordan) found heavy "Herpetomonas" infections in four female sand flies out of nearly 400 dissected. Material from one of these infected flies was inoculated into the forearm of a volunteer and a small papule containing Leishman-Donovan bodies (amastigotes) was subsequently produced. Following this successful transmission of oriental sore, they conducted feeding experiments to determine if "Herpetomonas tropica" was capable of developing in P. papatasi after a feed on an oriental sore, and whether or not the parasite remained infective to man after passing through the sand fly. Dissections were made two to seven days after the infective feed; 16 sand flies were found to be infected. Material from these artificially infected flies was inoculated into volunteers, none of whom subsequently showed signs of infection. Although, Adler and Theodor felt that their experiments had provided sufficient proof of the role of sand flies as vectors of oriental sore, this view was criticized by Wenyon (Lainson, 1982), who emphasized the need to transmit oriental sore by natural bite of the sand fly. Adler and Theodor replied by ingeniously demonstrating the capability of the sand fly to transmit Leishmania by bite, although it was not from man to man (Adler, 1928). Phlebotomus papatasi were fed through a rabbit-skin membrane on a culture of L. tropica, and eight days later were allowed to feed through another membrane on sterile, inactivated rabbit serum. Some of this serum was sown into blood-agar medium used for culturing Leishmania. Flagellates were

present six days later. The same authors may have actually transmitted Leishmania to man by bite of the sand fly one year later. They fed experimentally infected P. sergenti Parrot on a number of volunteers and obtained a positive lesion on the arm of one man. It should be noted that the incubation period was so long that the individual had visited endemic areas of oriental sore before appearance of the lesion and therefore could possibly have acquired the infection there (Adler and Theodor, 1929). Finally, 20 years after Sergent and colleaques artificially transmitted oriental sore by scarification, Adler and Ber (1941) proved without question that artificially infected P. papatasi can transmit oriental sore to a human by bite. The key to this success appears to have been in feeding the sand flies on flagellates suspended in three parts 2.7 percent saline and one part defibrinated blood.

Leishmania donovani (visceral leishmaniasis, "kala-azar"). In 1915 Mackie, convinced that a relationship existed between kala-azar and some biting insect, attempted to make a hut to hut insect census in kala-azar-infected villages. His team collected body lice, head lice, bed bugs, mosquitoes, sand flies and even leeches in the bedding or on the persons of patients who were proven to have active kala-azar; these specimens were carefully examined for Leishmania parasites. All specimens were negative except the sand flies which contained "Herpetomonas parasites" (probably Leishmania), "bodo-like parasites," and "sporozoan-like parasites." Not realizing how close he must have been to linking sand flies with kala-azar, Mackie stated: "This long series of negative results rather tends to check enthusiasm for the insect-borne hypothesis of kala-azar. . . . The only insect

which has given any return for work spent on it is the sand fly and I am of the opinion that the relation of this insect to disease would repay further investigation" (1915, p. 949).

Perhaps stimulated by Mackie's suggestion and following the example of Sergent et al. (1921) in their work on oriental sore in Algeria, Sinton (1922) studied the distribution of Indian sand flies and found that the distribution of kala-azar showed striking congruence with that of P. argentipes Annandale and Brunetti; he considered this sand fly to be the most likely vector. In 1924, a "Kala-azar Commission" was set up under the directorship of Christophers, and its members were soon successful in rearing this most likely suspect in the laboratory. Once a laboratory colony was established, Knowles et al. (1924) fed specimens of these sand flies on patients with kala-azar and found that a heavy flagellate infection developed in P. argentipes; they noted that the same degree of infection did not occur in other Phlebotomus species. The following year Shortt et al. (1926), working with L. donovani, demonstrated that in P. argentipes the infection passes forward in the fly to the buccal cavity and proboscis. For the next five years, Shortt and co-workers of the Kala-azar Commission conducted three series of transmission experiments using human volunteers and Chinese hamsters as hosts. They fed an astounding 79,939 artificially infected P. argentipes on either human volunteers or hamsters but reported negative results as follows:

> The failure of any of the subjects of experiment to show infection with kala-azar . . . is very difficult to explain if the theory of Phlebotomus transmission is to be maintained. We can only suppose that some essential factor in the process of infection has been omitted in our experiments which is present under natural conditions, or that the vast amount of labour expended by us . . . during a period of 5 years has been expended

> on an insect which is not an essential link in the chain of infection. (Shortt et al., 1930, p. 929)

It appears that this final report was written before all the results were in, for on February 19, 1931, the following telegram from New Delhi was received by Nature magazine: "Lieut-Col. Shortt reports successful transmission of Leishmania donovani to Chinese hamsters by bites of artificially infected Phlebotomus argentipes. Hamster bitten repeatedly during twelve months; generalized infection found seventeen months after experiment began" (Shortt, 1931, p. 308). Their persistence had paid off.

The kala-azar commission continued its efforts to transmit kala-azar to man by bite of P. argentipes but met with uniformly negative results (Swaminath et al., 1942). It was only after Smith et al. (1940) devised the technique of keeping the flies alive after oviposition by feeding on the juice of boiled raisins that Swaminath et al. (1942), using this technique, were able to forge the final link of evidence incriminating P. argentipes as the insect vector of kala-azar in India. They suggested that feeding the flies on fruit juices acted either by increasing the virulence of the parasites or increasing the parasitemia, thus enabling them to reach the anterior part of the midgut (cardia) more rapidly.

Leishmania donovani infantum (infantile visceral leishmaniasis). The coincidental distributions of visceral leishmaniasis and the sand fly P. chinensis Newstead in China north of the Yangtze River, pointed to this insect as the vector of the disease. Young and Hertig (1926), in North China, dissected hundreds of field-caught P. chinensis, P. sergenti, and P. perturbans de Meijere and examined them for the presence of flagellates; all specimens were negative. They also attempted to

incriminate these three species by experimentally infecting laboratory-bred sand flies, feeding them on kala-azar patients or infected hamsters, and allowing them to refeed on uninfected hamsters. The rates of infection were 85.3%, <2%, and 0% for P. chinensis, P. sergenti, and P. perturbans, respectively. A small percentage of these infected flies took a second blood meal, but transmission was unsuccessful. Similar studies were conducted by Sun et al. (1936) and Sun and Wu (1937) in which 7 of 21 P. chinensis, collected in houses with cases of visceral leishmaniasis, were infected with promasitgotes. Successful transmission to hamsters by the bite of P. chinensis was finally reported by Feng and Chung (1941).

In the Cévennes, in southern France, Rioux and colleagues accumulated overwhelming epidemiological evidence regarding the suspected role of P. ariasi Tonnoir as the vector of infantile visceral leishmaniasis. Not until 1979, however, did they confirm their suspicions by transmitting the disease to a dog by the bite of an experimentally-infected sand fly (Rioux, et al., 1979).

Leishmania major (cutaneous leishmaniasis, wet sore). It is unclear when L. major was first transmitted experimentally by bite of a sand fly because early workers recognized only one leishmanial species that was divided into two subspecies, "minor" and "major" causing "dry" and "wet" oriental sore, respectively. Perfil'ev (1968) claimed that the first successful transmission of cutaneous leishmaniasis by bite of a sand fly was accomplished in 1941 by Kryukova in experiments with gerbils. Laboratory-bred P. papatasi were infected with cutaneous leishmaniasis by feeding on a histocytoma on a gerbil's ear and were subsequently allowed to take a second blood

meal from an uninfected gerbil. Lesions developed on the second gerbil 15 days post infective feed. In 1927 Koshevnikova and coworkers showed that the principal reservoir host of L. tropica in Central Asia is man and that the host of L. major is the gerbil (Rhombomys opimus) (Perfil'ev, 1968). Therefore, it seems safe to assume that Kryukova's results represent the first experimental transmission of L. major by the bite of a sand fly, because the original pathogen was obtained from gerbils caught in Turkmenia.

New World Leishmaniasis

In the New World, the task of vector incrimination has been complicated by three factors: the extremely wide variety of sand fly species (about 327 Lutzomyia compared to about 101 Phlebotomus in the Old World), the failure to appreciate the multiplicity of leishmanial parasites on the part of some workers, and the difficulty of working in a dense tropical rain forest (Lainson, 1982).

Leishmania donovani chagasi (American visceral leishmaniasis). The peridomestic nature of American visceral leishmaniasis due to L. donovani chagasi facilitated the incrimination of Lu.[1] longipalpis (Lutz and Neiva) as the main vector of this disease. Its geographic distribution coincides with visceral leishmaniasis throughout Latin America.

In 1936, Evandro Chagas found Lu. longipalpis in the house of the first case of visceral leishmaniasis to be studied in South America, in Sergipe, Brazil (Lainson, 1982). This prompted Chagas and others

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[1]The abbreviation Lu. for Lutzomyia is used to avoid confusion with L. for Leishmania.

to feed Lu. longipalpis on infected dogs. Promastigotes developed in the guts of these sand flies and were infective upon subsequent inoculation into uninfected hamsters (Lainson, 1982). With the accidental death of Evandro Chagas in 1940, interest in visceral leishmaniasis also seemed to die, not to be resurrected until 1954 when Deane and Deane, investigating serious outbreaks of the disease in the state of Ceara, Brazil, found wild-caught Lu. longipalpis heavily infected with promastigotes believed to be L. donovani. They also noted highly active flagellates in the biting mouthparts of other Lu. longipalpis they had fed on a naturally-infected fox eight days previously (Lainson, 1982). Although the promastigotes seen by the Deanes were not proven to be L. d. chagasi, the epidemiological evidence was so strong that there remained little doubt as to the importance of this sand fly (Lainson, 1982).

Further evidence to incriminate this peridomestic vector came in 1977 when Lainson and colleagues achieved five separate transmissions of L. d. chagasi in hamsters, by bite of laboratory-bred insects which had previously ingested amastigotes in artificially infected rabbit blood (Lainson et al., 1977).

Leishmania mexicana (American cutaneous leishmaniasis, bay sore, chiclero's ulcer). Experimental infection of sylvatic sand flies with parasites causing American cutaneous leishmaniasis has been accomplished in a wide range of species, but experimental transmission has been difficult to achieve because of the wide choice of potential vectors. Strangways-Dixon and Lainson (1962) infected wild-caught females of nine species of sand flies in Belize by feeding them on hamsters exhibiting skin lesions due to L. m. mexicana. The flies then

were fed on human volunteers, one of whom developed a small lesion 17 days after one insect made a 30 second bloodless probe. The vector was initially identified as "P. paraensis" Costa Lima, but was later reexamined by Williams (1983) and determined to be most similar to Lu. panamensis (Shannon) rather than paraensis. To demonstrate a natural infection of leishmaniasis in sand flies and to strengthen their case for incriminating the vector (Lu. panamensis), Strangways-Dixon and Lainson (1962), collected approximately 270 wild female sand flies, triturated them in sterile Locke's solution and inoculated half of the suspension into the back of an uninfected hamster. Two months later a small dermal swelling was noted at the site of the inoculation. Stained smears from the lesion revealed leishmaniae (Strangways-Dixon and Lainson, 1962). This experiment showed that some wild-caught sand flies were naturally infected, but since all specimens collected were pooled, it was not determined which species was (were) infected. Subsequent dissections of 334 newly caught, man-biting Lutzomyia revealed epimastigote-like infections in two specimens, one each of Lu. ovallesi (Ortiz) and Lu. cruciata (Coq.). In view of the scanty infections, the authors felt that these more likely represented Leptomonas or Herpetomonas rather than Leishmania promastigotes.

In Brazil, wild-caught Lu. longipalpis and Lu. renei (Martins, Falcao, and da Silva), allowed to feed on a strain of Leishmania isolated from a human patient in Belize, also successfully transmitted the parasite (Coelho and Falcão, 1962).

Williams (1966a) collected wild sand flies in Belize and fed them on a leishmanial lesion on a hamster. Two to three days later, the

flies fed on the forearm of a human volunteer. Two lesions were produced about four weeks later, and in both cases the vector fly was Lu. cruciata.

In the same country, Disney (1966) designed a trap to catch sand flies attracted to rodents and found that Lu. olmeca olmeca (Vargas and Najera) was highly attracted to them. Later, using this technique, he found Lu. o. olmeca naturally infected with L. m. mexicana (Disney, 1968). In neighboring Yucatan Peninsula, Biagi et al. (1965) confirmed the importance of Lu. o. olmeca as a vector of leishmaniasis, and transmitted the parasite to a volunteer by bite of a naturally infected fly (Lainson, 1982).

Lainson and Shaw (1968) demonstrated that the vector of L. mexicana amazonensis in the Amazon forests of Brazil is Lu. flaviscutellata (Mang.), a species highly attracted to the rodent Proechimys guyanensis, the principal reservoir host. Of 7,322 flies dissected between 1968 and 1973, 45 or 0.6% were infected with promastigotes. Parasites from 18 of these infected flies were inoculated into hamsters and 15 of the inoculations produced typical L. mexicana amazonensis infections (Ward et al., 1973). These workers also transmitted the disease from hamster to hamster on four occasions using laboratory-bred Lu. flaviscutellata (Ward et al., 1977). From these findings it was concluded that Lu. flaviscutellata is the principal, and probably only, vector of L. mexicana amazonensis in the Amazon region (Lainson and Shaw, 1979).

Leishmania braziliensis panamensis (Panamanian cutaneous leishmaniasis). Natural flagellate infections have been recorded in numerous neotropical sand fly species, but it was not until extensive

studies were carried out at the Gorgas Memorial Laboratory in Panama that they were proven to be Leishmania (Hertig and McConnell, 1963; Johnson et al., 1963). After many years of patient work, these researchers found natural promastigote infections in more than 400 man-biting Panamanian sand flies and incriminated three species, Lu. trapidoi (Fairchild and Hertig), Lu. ylephiletor (Fairchild and Hertig) and Lu. gomezi (Nitz.) as vectors of Panamanian cutaneous leishmaniasis, L. braziliensis panamensis (Johnson et al., 1963). Christensen et al. (1969) also found Lu. panamensis infected with promastigotes, but there is some question as to the identity of those promastigotes. Lainson (1982) believed that some of these infections were due either to Endotrypanum (a blood parasite of sloths which also develops as promastigotes in sand flies) or to nonhuman Leishmania, such as Lu. hertigi of porcupines.

McConnell (1963) cultured flagellates from wild-caught sand flies and determined, without question, that Lu. trapidoi harbored promastigotes of L. b. panamensis. Furthermore, the observation that Lu. trapidoi is largely arboreal led to the incrimination of sloths as the major reservoir of L. braziliensis panamensis (Lainson, 1982).

Leishmania braziliensis guyanensis ("pian-bios"). In Surinam, Wijers and Linger (1966) caught large numbers of anthropophilic sand flies off human bait in areas where "pian-bois," due to L. b. guyanensis, is endemic. The most common species recorded was Lu. squamiventris (Lutz and Neiva), but all dissections of this species proved negative for leishmaniae. Numerous promastigote infections were found in dissections of another species, "Lu. anduzei," found resting on tree trunks. Their "anduzei" probably represented

Lu. umbratilis (Ward and Fraiha) since it was found resting on tree trunks (Ward and Fraiha, 1977). However, the role of this sand fly as a vector remained speculative, since attempts to infect a hamster with the parasite failed.

Lainson et al. (1976), observed a 7% infection rate in "Lu. anduzei" (now Lu. umbratilis Ward and Fraiha) taken mostly from large tree trunks during their studies of the epidemiology of "pian-bois" in the Monte Dourado region, Pará State, Brazil. Intradermal inoculations of the flagellates into hamsters produced infections in all cases. The parasite was shown to be identical biologically and biochemically with that causing the disease in man. Lu. umbratilis was subsequently incriminated as the vector of L. b. guyanensis in the neighboring state of Amazonas, Brazil, by Arias and Freitas in 1977. Lainson et al. (1976) reported heavy promastigote infections in seven specimens of Lu. whitmani (Antunes and Coutinho), another tree trunk-inhabiting sand fly. Unlike Lu. umbratilis, this sand fly was not particularly anthropophilic, however, and they suggested that its importance is probably limited to secondary transmission among wild animal reservoirs.

Leishmania braziliensis braziliensis (mucocutaneous leishmaniasis, "espundia"). Incrimination of sand fly vectors of L. b. braziliensis has been more difficult than with the other American forms because of the poor growth of the parasite in laboratory animals and in culture media, and because of difficulties in establishing productive colonies of suspected vectors for transmission experiments. Forattini et al. (1972), however, successfully infected hamsters with promastigotes (believed to be L. b. braziliensis) found in two

naturally infected sand flies, Lu. intermedia (Lutz and Neiva) and Lu. pessoai (Coutinho and Barretto) in Brazil. The former species is found in low, secondary forests and is known also to invade houses, while the latter is essentially sylvatic, but has been collected in houses up to 300 m from the forest edge. With this in mind, it was suggested that cutaneous leishmaniasis in southern Brazil may have a peridomestic transmission, the original source of the infection being in nearby wooded areas (Lainson, 1982).

In Serra dos Carajás, Pará State, North Brazil, where both cutaneous and mucocutaneous leishmaniasis are serious public health problems, Lainson et al. (1973) concluded that Lu. welcomei (Fraiha, Shaw, and Lainson) was a major vector to man. Attempts to infect hamsters by inoculation with parasites isolated from naturally infected Lu. wellcomei were largely unsuccessful, but it was shown that the parasite was the same as that infecting man in the same area. Lu. wellcomei was considered to be of particular importance because of the avidity with which it attacks man both during the night and the day.

Leishmaniasis in the United States of America

Few studies have been conducted on the epidemiology of leishmaniasis in the USA. Prior to 1976 leishmaniasis was not generally thought to occur autochthonously in the USA, and potential sand fly vectors were reported so rarely as to be considered of little medical consequence.

McEwen (1914) reported the first case of leishmaniasis in the USA, referring to it as "oriental sore." There is little doubt that

he was dealing with American cutaneous leishmaniasis since the patient acquired the lesion while traveling in South America (Stewart and Pilcher, 1945). At that early date, differentiation between oriental sore and American cutaneous leishmaniasis had not been made, nor had any vectors been incriminated.

In 1919, Parman drew attention to a "Phlebotomus" species in Uvalde, Texas, and noted that it attacked man. He did not attempt to implicate it in disease transmission. This fly, described and named Phlebotomus (Brumptomyia) diabolicus by Hall in 1936, represented the first confirmed anthropophilic species of the genus Phlebotomus (now Lutzomyia) from the USA. Lindquist (1936) stated that this species was not a major pest in Uvalde, but that it frequently caused some annoyance to people in southwestern Texas. He described a peridomestic fly that often entered dwellings and other buildings to feed on man and domestic animals. At that time, species of Phlebotomus had been incriminated in the transmission of Phlebotomus fever but were only suspected to play a role in the transmission of leishmaniasis. Consequently, the fly was considered to be of little importance other than being a nuisance.

Prior to 1943, approximately 30 human cases of cutaneous leishmaniasis (oriental sore) had been reported from the USA and Canada (Dwork, 1942), none of which (with one possible exception), were autochthonous. The possible exception was a case reported by Gelber (1942) in a 53-year-old woman from southern California who had a typical leishmanial lesion on her left cheek. This case was thought to be autochthonous because the woman had not left the country for 13 years. But since she had made an earlier tour of Europe and the

Mediterranean, the possibility cannot be ruled out that she might have contracted the disease outside the USA.

Only three cases of mucocutaneous leishmaniasis had been reported in the USA prior to 1943, one of which was presumed to be autochthonous (Stewart and Pilcher, 1943). Benedek (1940) reported a case of mucocutaneous leishmaniasis in a man from Chicago, which he considered the first autochthonous case of leishmaniasis in the USA. However, there is some question as to the extent of the patient's travel, and thus there is room for doubt.

Stewart and Pilcher (1943) reported on a case of cutaneous leishmaniasis in a 6-year-old Mexican-American boy who lived on a ranch near Alice, Texas and had never traveled more than 60 miles from his home. The authors, obviously aware of the recent incrimination of sand flies as the vectors of both visceral and cutaneous leishmaniasis in the Old World, mentioned that three species of "Phlebotomus" (Lutzomyia) had been identified in the USA, all of which were found within or near Texas. They astutely concluded that the apparent rarity of the disease in the United States was probably not real and that the return of military and civilian personnel from endemic centers was further reason for keeping American leishmaniasis in mind. This was probably the first truly autochthonous case of leishmaniasis reported in this country, although Wenyon disputed the identity of the parasite stating: "The microphotograph illustrating the paper is a good one, but though suggestive of Leishmania, it is not absolutely convincing" (1945, p. 712).

Addis (1945a) described a new species of sand fly from Texas, "Phlebotomus anthophorus" (Lu. anthophora), from specimens collected

at Uvalde, Texas, in the same locality where Lu. diabolica was found. The female flies were collected in the mornings while feeding on domestic rabbits. Attempts to feed this fly on man were unsuccessful.

Packchanian (1946) reviewed distributions and habits of the six species of sand flies then known to occur in the USA. He drew attention to the fact that sand flies were known vectors of leishmaniasis in the Old World and were known to have been infected naturally or experimentally with leishmaniasis in numerous localities in Latin America. He stated that in all probability the species found in the USA represented potential vectors of leishmaniasis and that this important problem remained to be investigated under rigid experimental conditions.

In 1968, Simpson et al. reported the first well documented, and undisputed, autochthonous case of leishmaniasis in the USA. The patient was a 64-year-old Mexican-American woman from San Benito, Texas, who had a 57 year history of chronically active, disseminated anergic cutaneous leishmaniasis.

Shaw et al. (1976) described two more autochthonous human cases of cutaneous leishmaniasis in Texas. The first occurred in 1972 in a 74-year-old woman from Dilworth, Gonzales County, and the second in a 56-year-old man from Kenedy, Karnes County. The first patient owned a ranch a few miles from her home and visited it daily to feed the cattle and some stray dogs. She related that she frequently saw swarms of gnats and small black flies, but did not remember any specific insect bites, especially in relation to her skin lesions. Aside from short visits to northern Mexico she had had no foreign travel (Shaw et al., 1976). The second patient lived in a primitive

shack in rather unsanitary conditions. He reported no travel outside the USA except for two half-day visits to Nuevo Laredo, Mexico. The authors conducted epidemiologic studies in the neighborhoods and communities surrounding the two case sites but collected no sand flies. They concluded that conditions may be proper in south central Texas for arthropod-borne transmission of cutaneous leishmaniasis, and that the suspected endemicity of the disease should be confirmed by further studies.

Interest in the epidemiology of leishmaniasis in Texas mounted slowly until, in 1980, cutaneous leishmaniasis (L. mexicana mexicana) was diagnosed in a 11-year-old boy from Uvalde, Texas, the same locality in which the first confirmed anthropophilic species of sand fly in the USA (Lu. diabolica) was found (Gustafson et al., 1984). The boy was presumed to have contracted the disease near his home or while on camping trips in south central Texas, since his travel had been limited.

In that same year, Anderson et al. (1980) reported endemic canine leishmaniasis in dogs near Oklahoma City, Oklahoma. The parasite most closely resembles L. donovani infantum (Kocan et al., 1984). These instances further emphasize the need to study the epidemiology of leishmaniasis in the USA.

In 1981, Perkins (1982) demonstrated for the first time that an anthropophilic USA sand fly, Lu. shannoni, could be experimentally infected with an indigenous strain of L. mexicana (strain WR-411, Uvalde, Texas) by feeding them on histocytomas on infected hamsters. Although transmission was not accomplished at that time, the ground work was laid for further studies. Later that same year Endris et al.

(Endris, pers. comm., 1984) demonstrated the ability of the non-anthropophilic, rodent-feeding sand fly, Lu. anthophora, to transmit L. mexicana (strain WR-411, Uvalde, Texas) from infected to uninfected hamsters by bite. This was the first report of a native USA species of sand fly transmitting leishmaniasis by bite. The authors suggested that L. mexicana could be maintained in a wild rodent population by Lu. anthophora from which it could then be transmitted to man by other sympatric anthropophilic sand flies such as Lu. diabolica.

Finally, in 1983, Gustafson reported three confirmed and one suspected case of cutaneous leishmaniasis from south central Texas (Gustafson et al., 1984).

The vectors of this disease in Texas are unknown and until the present study, no attempts had been made to incriminate any anthropophilic sand fly from areas of Leishmania endemicity in the USA.

Statement of Objectives

This study was undertaken to investigate the life history and biology of the sand fly Lutzomyia diabolica (Hall) and its possible role in the transmission of human cutaneous leishmaniasis in Texas. For comparison, the vector capacity of Lu. shannoni (Dyar), another anthropophilic sand fly, was investigated in conjunction with that of Lu. diabolica. The specific objectives of the study were to

1. conduct a field survey of potential vector sand flies in vicinities of recent human case sites of leishmaniasis in Texas;

2. study the field biology of Lu. diabolica and collect wild stock for a laboratory colony;

3. establish a productive colony of Lu. diabolica in the laboratory;

4. study the biology and life history of Lu. diabolica under laboratory conditions;

5. investigate, under controlled laboratory conditions, the vector capacity of Lu. diabolica, as compared with Lu. shannoni, for leishmaniasis; and

6. examine by means of the electron microscope the morphology of L. mexicana promastigotes found in the sand fly vector.

CHAPTER 2
SAND FLIES ASSOCIATED WITH HUMAN CUTANEOUS LEISHMANIASIS IN TEXAS: OBSERVATIONS ON THEIR BIOLOGY WITH SPECIAL REFERENCE TO *Lutzomyia diabolica* (HALL)

Introduction

General

Recent reports of human cutaneous leishmaniasis acquired in south central Texas strongly suggest that the disease is endemic there (Shaw *et al.*, 1976; Gustafson *et al.*, 1984). Because sand flies are the only known natural vectors of leishmaniasis, a knowledge of their field biology and host associations is of paramount importance in epidemiologic studies of this disease. Six species of sand flies are known to occur in Texas; these are *Lutzomyia anthophora* (Addis), *Lu. californica* (Fairchild and Hertig), *Lu. diabolica* (Hall), *Lu. oppidana* (Dampf), *Lu. texana* (Dampf), and *Lu. vexator* (Coq.) (Young and Perkins, 1984). Only one, *Lu. diabolica*, is known to be anthropophilic. This field study is the first to investigate sand flies in Texas associated with specific human case sites of cutaneous leishmaniasis.

The investigation was initiated in 1982 with a survey trip in late spring and early summer (4-28 June) to south central Texas. The principal study site was Garner State Park near Concan in Uvalde County. Secondary sites included Rio Frio in Real County, Seminole Canyon State Park in Val Verde County, and Fawcett Boy Scout Camp near

Barksdale in Edwards County. The field work was conducted in cooperation with Drs. D. G. Young, G. B. Fairchild, and R. G. Endris, all from the University of Florida, Gainesville, FL. A second survey trip was taken in early fall of 1983 (19-30 September), the principal field study site being in and around the rural community of D'Hanis in Medina County. Secondary sites included Garner State Park, the Romer Ranch south of Devine in Medina County, and two sites within the city limits of San Antonio in Bexar County. During the second trip the able assistance of Mr. T. Long, zoonotic technician, Region 9, Texas State Health Department, was greatly appreciated. The objectives were to

1. conduct a survey of potential vector sand flies in the vicinities of recent human case sites of leishmaniasis in Texas (A case site, as used herein, is defined as the home environs of a confirmed leishmaniasis patient, or localities where that individual camped or otherwise visited within three months prior to the onset of disease symptoms.); and

2. study the field biology of Lu. diabolica and collect wild stock for a laboratory colony.

Human Case Histories

Eight autochthonous human cases of cutaneous leishmaniasis have been reported in south central Texas, four of which have occurred since 1980 (Gustafson et al., 1984) (Fig. 2-1). These latter four were the only cases investigated during this study and are described below. Portions of the following unpublished case histories were graciously provided by Dr. T. Gustafson, the investigating Texas State epidemiologist. The

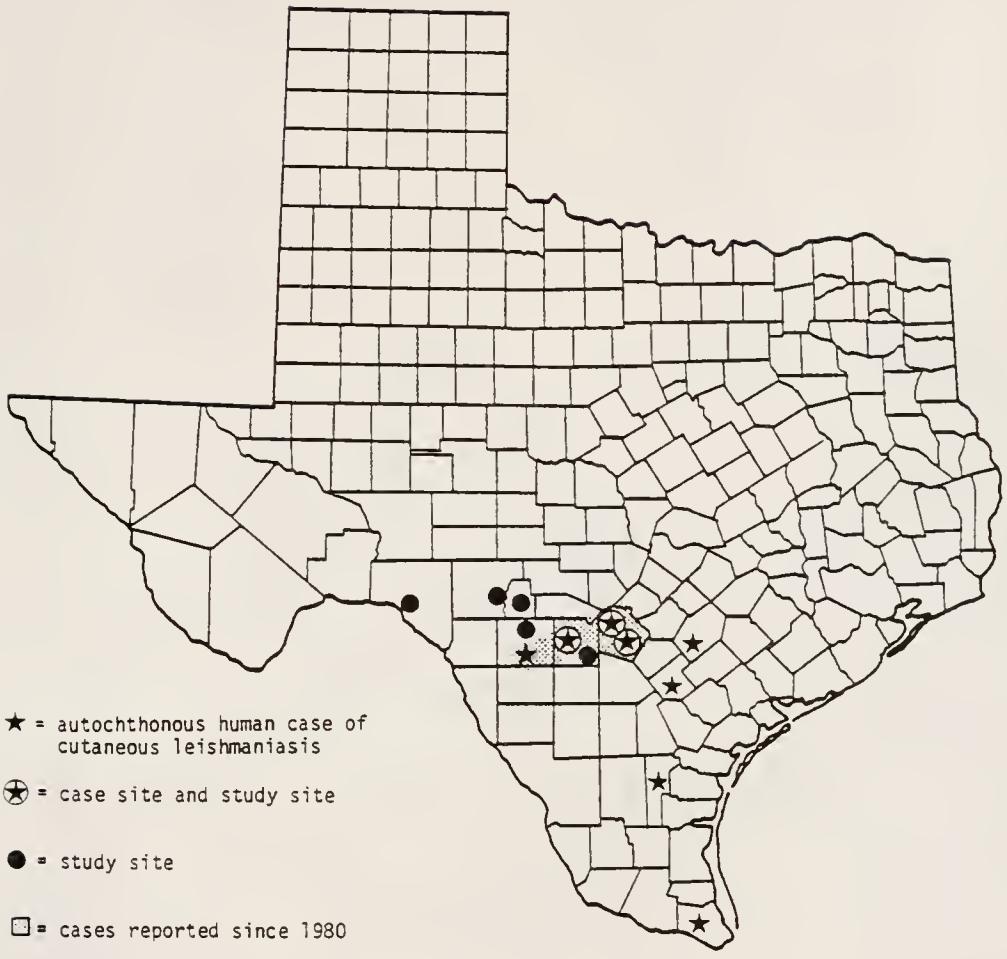


Figure 2-1. Distribution of autochthonous human cases of cutaneous leishmaniasis in Texas and locations of study sites.

remaining information was obtained through personal interviews with patients or their family members. A complete discussion of the case histories and results of serologic tests are provided by Gustafson et al. (1984).

Patient A. The patient was an 11-year-old white male from Uvalde, Texas, who noticed an ulcerating lesion on his left cheek beginning February, 1980. In May, 1980, a biopsy was performed which showed amastigotes in dermal macrophages. Leishmania mexicana was cultured from biopsy material that was sent to Walter Reed Army Institute of Research. Patient A had never traveled outside the USA except for occasional one-day trips to the border city of Ciudad Acuna, Mexico. In the month prior to onset, he had participated in a camping trip at Fawcett Boy Scout Camp in Edwards County. The family had no pets.

Patient B. The patient was a 56-year-old white female from a suburban neighborhood in southeast San Antonio, Texas, who first noticed a small lesion on her left ear in November 1982 (Fig. 2-2). She distinctly remembered waking one morning with an itching ear and noticed a drop of blood on her ear lobe and on her pillow. She suspected that she had been bitten by an insect. Her bed was located next to an open, screened window. A biopsy performed in February, 1983, showed amastigotes in dermal macrophages by light and electron microscopy. Patient B had traveled out of the USA as a military dependent more than ten years previously and had visited Chihuahua in northern Mexico in June, 1982. She had one pet dog.

Patient C. The patient was a 5-year-old white male from a suburban neighborhood in northeast San Antonio, Texas, who first noticed an enlarging papule on his left thigh beginning in November

1982 (Fig. 2-3). When the lesion persisted, an excisional biopsy was performed which showed amastigotes in large vesicles within macrophages by both light and electron microscopy. The patient had never traveled outside the USA. He frequently spent the week ends at the ranch of his paternal grandparents in Devine, Texas. The family had one pet dog, and the boy's grandparents had three hunting dogs.

Patient D. The patient was a 10-year-old white male from the rural farming community of D'Hanis, Texas, who developed a lesion near his right eye brow and another on his right cheek in December, 1982 (Fig. 2-4). When the lesions persisted, an excisional biopsy was performed which showed amastigotes in large vesicles within macrophages by light microscopy. Presence of kinetoplasts was confirmed by electron microscopy. Promastigotes were recovered from an aspirate of the eyebrow lesion, but these died before further identification could be performed. The patient had never traveled outside of Texas and had lived all of his life in Medina County with only occasional trips to Uvalde, San Antonio, and Seguin, Texas. He regularly accompanied his father on coyote hunting trips near his home. The family had ten hunting dogs. The patient's mother recalled seeing tiny hopping flies in the house, around the light fixture, and members of the family reported being bitten by tiny gnats. When shown a live female Lu. diabolica the mother said that it was the same as those she had seen in the house.

A suspected case, with history very similar to the others, was reported from Hondo, Medina County, Texas, within 12 miles of the home of Patient D. The patient was a 4-year-old white male who first noticed a lesion on the bridge of his nose beginning in January, 1983.

Figure 2-2. Cutaneous lesion due to Leishmania mexicana on ear lobe of patient B. Photo courtesy Dr. T. Gustafson.

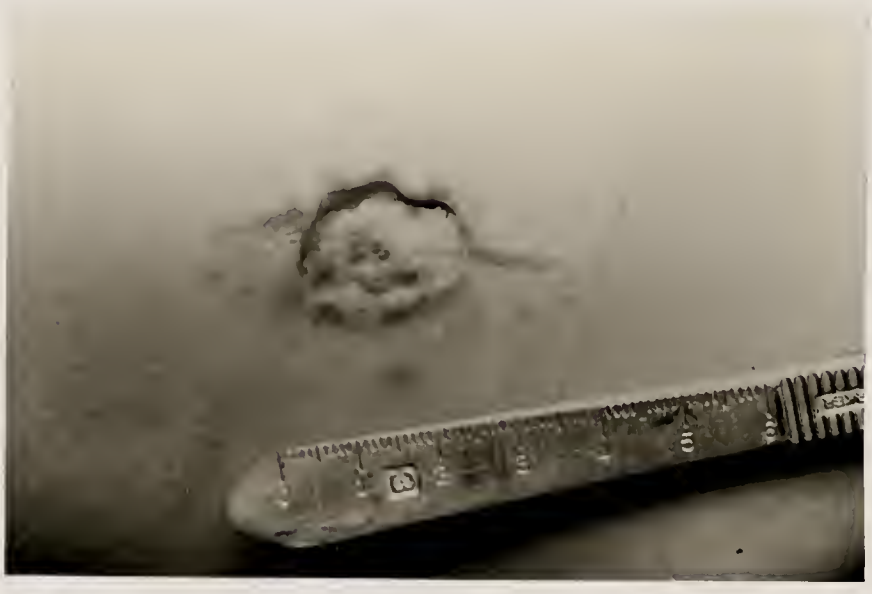


Figure 2-3. Cutaneous lesion due to Leishmania mexicana on thigh of patient C. Photo courtesy of Dr. T. Gustafson.

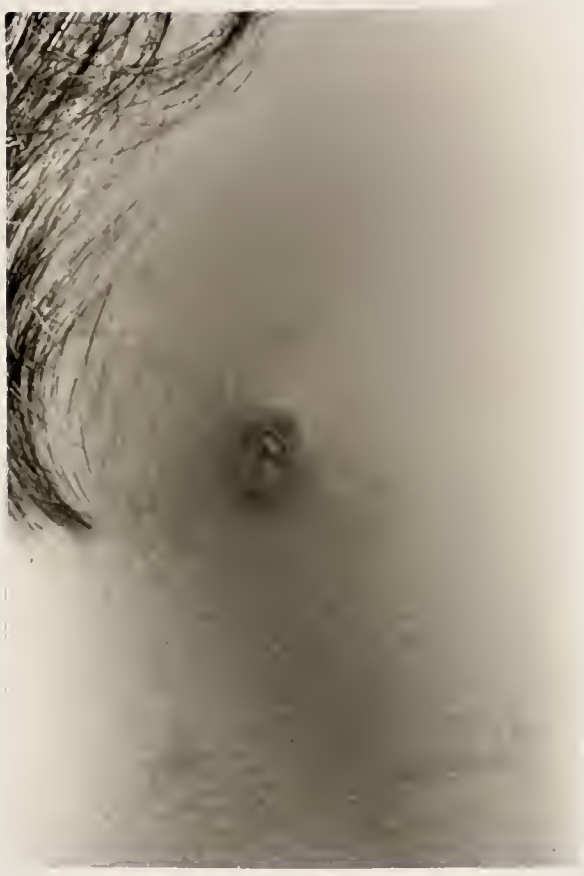


Figure 2-4. Cutaneous lesion due to Leishmania mexicana on cheek of patient D. Photo courtesy of Dr. T. Gustafson.

When the lesion persisted the boy was seen by a physician who biopsied it before a positive diagnosis could be rendered. Tissue samples and saline aspirates taken from the biopsied lesion contained no parasites. The boy had not traveled outside the USA, nor outside the region during the previous year. The family had three pet dogs.

Serologic tests performed for each patient included indirect fluorescent antibody test (IFA) and dot enzyme-linked immunosorbent assays (ELISA) at Walter Reed Army Institute of Research, Wash., DC, and fluorescent immunosorbent assays (FIAX) at Oklahoma State University, Stillwater, Oklahoma (Gustafson et al., 1984). These tests, performed between three and six months after onset, showed that all four confirmed patients had detectable antibody titers to Leishmania. The sister of Patient C and the mother of Patient D had detectable titers by at least two serologic tests. One dog belonging to Patient C and three dogs belonging to Patient D had detectable Leishmania titers by at least two tests. However, all four dogs had antibody titers to Trypanosoma cruzi, and the Leishmania titers may represent cross-reactions (Gustafson et al., 1984).

Description of Study Sites

Eight study sites were selected based on previous collection records of Lutzomyia from south central Texas (Eads et al., 1965; Easton, et al., 1968; Young, 1972; Eads, 1978; Endris, 1982; Perkins, 1982) and on their association with confirmed Leishmania patients (Fig. 2-1). Three of the sites, A, B, and D, were close to the home environs of patient A and he had camped at or near the sites three months prior to onset of symptoms. Lutzomyia species were also known to occur at site

A (Young, 1972; Endris, 1982). Site B was selected on the basis of 1971 light trap collection records of 237 females from a single light trap (Perkins, 1982). The remaining sites E, F, G, and H were the actual home environs of confirmed leishmaniasis patients.

Surveys for potential sand fly vectors were conducted at all sites during the course of the study. Emphasis was placed on qualitative rather than quantitative sampling; no attempt was made to estimate sand fly population densities.

Site A. Garner State Park, Concan, Uvalde County, Texas (4-28 June, 1982; 19-30 September, 1983; Fig. 2-5 and 2-6). This state park is located 50 km north of Uvalde in the Frio River valley. It is situated on the southern edge of the Edwards Plateau, on the Balcones Escarpment, which separates the plateau from the Nueces plains to the south. The habitat is characterized by grassy meadows dotted with cedar (Juniperus sp.), live oak (Quercus virginiana), acacia (Acacia sp.), pecan (Carya illinoensis), mesquite (Prosopis juliflora), and elm (Ulmus crassifolia), with bald cypress (Cupressus arizonica) bordering the Frio river. The park is surrounded by steep rocky hills, the slopes of which are covered with cedar, wild cherry (Prunus serotina), persimmon (Diospyros texana), madrone (Arbutus texana), and other Hill Country shrubs. Potential mammalian hosts for sand flies include white tail deer (Odocoileus virginianus), coyote (Canis latrans), fox (Vulpes sp.), bobcat (Lynx rufus), racoon (Procyon lotor), porcupine (Erethizon dorsatum), skunk (Mephitis sp.), opossum (Didelphus marsupialis), armadillo (Dasypus novemcinctus), jack rabbit (Lepus californica), cottontail rabbit (Sylvilagus floridanus), and a variety of rodents (Texas Parks and Wildlife Department, 1982a; Raisz, 1954; Küchler, 1975).

The nearest weather station to Garner State Park is 50 km south at Uvalde where the climate is fairly representative of the area surrounding the park, characterized by hot, humid summers and pleasantly mild winters (US Department of Commerce, 1965). Annual precipitation averaged 63.1 cm between 1951 and 1960, with a range of 23.3 cm to 98.3 cm. The wettest periods of the year are May-June and September-October. The hottest part of the year is July-August, with extreme temperatures reaching a high of 43°C, and a low around -15°C, reached in January and February. Relative humidity at noon, central standard time, averages between 58% in January to 48% in July. Daily fluctuations in RH occur especially during the warmer months, with a gradual decrease in the afternoon until sunset, then a rapid increase after dark (US Department of Commerce, 1965). Average daily maximum and minimum temperatures for June 1982 were 35°C and 22°C, respectively, and precipitation 14.4 cm (US Department of Commerce, 1982a). Average daily maximum and minimum temperatures for September 1983 were 34°C and 21°C, respectively, with 7.9 cm precipitation (US Department of Commerce, 1983a).

Site B. Rio Frio, Real County, Texas (4-20 June, 1982). This site is located approximately 20 km north of Site A, at the edge of a small gorge carved by the Frio river. The habitat and climate are very similar to that found at Garner State Park.

Site C. Seminole Canyon State Park, Val Verde County, Texas (10-11 June, 1982; Fig. 2-7). This state historical park is located 75 km west of Del Rio, Texas, a short distance downstream from the confluence of the Rio Grande and Pecos Rivers. It is noted for its rugged terrain, sparse vegetation and deep canyons. Situated in the

arid Pecos Shrub Savanna, its flora and fauna are derived from elements of the Texas Hill Country, Tamaulipas Thorn Scrubland, and the Chihuahua Desert (Texas Parks and Wildlife, 1982b). It is considered a true desert habitat, receiving 25 cm or less precipitation annually. The nearest weather station, Amistad Dam, recorded average maximun and minimum temperatures for June 1982 of 36°C and 23°C, respectively, with daily temperatures frequently rising above 38°C during the summer months (US Department of Commerce, 1982a). Relative humidity was not measured, but probably averaged below 30% during the summer. Mammalian inhabitants at the park are limited to coyote, jack rabbit, armadillo, and small rodents.

Site D. Fawcett Boy Scout Camp, Barksdale, Edwards County, Texas (11 and 22 June, 1982; Fig. 2-8). This site is located 7 km north of Barksdale at a bend in the Nueces river. Also at the southern edge of the Edwards plateau, the habitat resembles Garner State Park in most features, but lacks steep, rocky hills. The nearest weather station is about 20 km south at Camp Wood (US Department of Commerce, 1965). Annual precipitation for the ten-year period of 1951-1960 averaged 59.8 cm with a range of 22.3 cm to 106.1 cm. Average daily maximum and minimum temperatures during June 1982 were 33°C and 21°C, respectively (US Department of Commerce, 1982a).

Site E. Myer's Farm, D'Hanis, Medina County, Texas (19-30 September, 1983). This farm, the home of patient D, is in a small rural community 75 km west of San Antonio. The surrounding area is characterized by low rolling farmland with live oak, pecan, and mesquite trees bordering the fields and streams (Fig. 2-9). Uncultivated areas are overgrown with cactus, mesquite and thorny shrubs. One such area, about 50 m west of the farm house, contains a

number of woodrat (Neotoma) nests and armadillo burrows. Roughly 15 m south of the house is a large equipment shed, behind which (to the west) is the kennel in which are kept the family's ten hunting dogs (Fig. 2-10). Between the house and the kennel are a large live oak tree and a wood pile (Fig. 2-11). Approximately 80 m west of the house are the hog pens (Fig. 2-12). Approximately 100 m behind (west of) the house is a dry creek bed lined with mesquite trees, and beyond that, a grove of live oak trees. The closest weather station is in Hondo, 20 km to the east. The climate at this station is representative of Medina County, with hot humid summers and pleasantly mild, dry winters (US Department of Commerce, 1983b). Average annual rainfall is 71.2 cm with heaviest rainfall received during April-June and September-October. Mean length of the warm season is 263 days. Mean dates of last occurrence of freezing temperatures in spring, and first occurence in the fall are March 6 and Nov. 24, respectively. Humidity at noon central standard time averages 58% in January, 56% in April, 48% in July, and 55% in October. Average daily maximum and minimum temperatures for September, 1983, were 32°C and 26°C, respectively, with 13.0 cm precipitation. Temperatures for the year ranged between 43°C and -16°C with ten-year average daily maxima and minima of 27°C and 13°C (US Department of Commerce, 1983b).

Site F. Romer Ranch, Devine, Medina County, Texas (19-30 September, 1983; Fig. 2-13). This is the home of the paternal grandparents of Patient B. Approximately 10 km south east of Devine, it is very similar in general features to Site E in D'Hanis.

Site G. Romer Residence, northwest San Antonio, Bexar County, Texas (22 September, 1983; Fig. 2-14). This is the home of Patient B

Figure 2-5. Habitat typical of that found at Garner State Park, Concan, Uvalde County, Texas.

Figure 2-6. Habitat at Garner Stake Park, Concan, Uvalde County, Texas, showing public latrine resting station.

Figure 2-7. Habitat typical of that found at Seminole Canyon State Park, Val Verde County, Texas.

Figure 2-8. Habitat at Fawcett Boy Scout Camp, Barksdale, Edwards County, Texas, showing open latrine resting station.

Figure 2-9. Farmland surrounding the home of patient D in the rural community of D'Hanis, Medina County, Texas.

Figure 2-10. Hunting dogs in a kennel behind the home of patient D in D'Hanis, Medina County, Texas.

Figure 2-11. Wood pile located behind the home of patient D in D'Hanis, Medina County, Texas. Six female and one male Lutzomyia diabolica were collected in the CO_2-baited CDC light trap hanging in the foreground.

Figure 2-12. Hog pen located about 80 m west of the home of patient D in D'Hanis, Medina County, Texas. Rock pile in foreground offered many potential resting places for sand flies.

Figure 2-13. Romer ranch, near Devine, Medina County, Texas, home of the paternal grandparents of patient B.

Figure 2-14. Neighborhood of patient B in northwest San Antonio, Bexar County, Texas.

and is in a typical suburban neighborhood on the northwest limits of San Antonio. About three blocks south of the residence are some open fields, heavily overgrown with weeds and small shrubs. An equal distance to the north is a small creek basin lined with a variety of large shade trees and shrubs.

Site H. Kelly Residence, San Antonio, Bexar County, Texas (23 and 30 September, 1983; Fig. 2-15). Similar to site G, this site is in a suburban neighborhood in southeast San Antonio. Three large pecan trees shade the backyard. Adjoining the property to the southeast is a large vacant lot, overgrown with tall weeds.

San Antonio climate is about the same as that of Medina County (US Department of Commerce, 1982b) with hot humid summers and pleasantly mild, dry winters. Average annual precipitation is 74.6 cm. Average daily maximum and minimum temperatures during September 1983 were 32°C and 20°C, respectively, with an overall mean of 26°C. Precipitation during September, 1983, measured 13 cm (US Department of Commerce, 1983a).

Materials and Methods

Determination of Sand Fly Fauna

Several useful field methods for sampling sand flies have been used with varying degrees of success and are reviewed by Young (1979) and Killick-Kendrick (1981a). The diversity of potential sand fly habitats at each study site dictated the selection of methods that would sample the broadest cross section. As far as practicable, all representative habitats within a 200 m radius of each site were

Figure 2-15. Neighborhood of patient A in southeast San Antonio, Bexar County, Texas.

surveyed to determine species diversity and relative abundance of sand flies. Collection methods employed included the following:

Resting collections. An extensive search was made at each site for diurnal and nocturnal resting places of adult sand flies. Particular attention was directed to dark protected cavities in rocks, trees, buildings and other man-made structures, as well as in animal burrows, ground nests and brush piles where sand flies might seek shelter. A flashlight was used to enhance visibility. Live specimens were captured with a simple tube aspirator and placed in a 120 ml feeding/rearing chamber (Endris et al., 1982).

Biting collections. Biting collections (Young, 1979) were made at night, usually between 2100 and 2400 hrs. The collector sat in an open area under an incandescent light or Coleman® lantern and captured sand flies with an aspirator as they attempted to feed.

Light traps. Battery powered or CDC miniature light traps (Sudia and Chamberlain, 1962) were secured to tree branches about 2 m above the ground at edges of clearings and near human habitations. Care was taken to insure their placement was away from competing light. In some instances they were placed adjacent to or directly over suspected resting sites such as wood or rock piles (Fig. 2-11).

A New Jersey mechanical light trap (Mulhern, 1942), powered by 110-volt house current, was placed in the back yard, adjacent to the dog kennel at the D'Hanis site. The trap will be operated there 2-4 nights per week for the next year.

A Shannon trap (Shannon, 1939; Fig. 2-16), made from white cotton bedsheets, was erected in open areas near human and animal dwellings. This apparatus does not actually trap insects, but the lantern or

Figure 2-16. Shannon trap erected behind the home of patient D, D'Hanis, Medina County, Texas.

incandescent light inside provides a light source, enabling the collector to aspirate flies which land on the illuminated cloth. Flies are attracted to the light, the collector, or a combination or both (Young, 1979).

Baited traps. Pieces of dry ice wrapped in newspaper and suspended next to the CDC light traps provided CO_2 as an adjunct attractant for hematophagus insects (Fig. 2-11). A caged hamster, suspended in a similar manner, also served as an attractant.

A Disney trap (Disney, 1966) baited with a hamster or wild cotton rat (Sigmodon sp.) was also used. This trap consists of a shallow tray filled with mineral oil and a caged animal supported on slats just above the surface of the oil. After feeding, blood engorged sand flies do not fly away, but hop to the "ground" to rest, becoming entrapped in the mineral oil.

Sticky traps. Sheets of card stock, coated on one side with oil, were placed directly in front of or over burrow entrances and crevices in rocks and trees to catch sand flies emerging from such places at night (Bütticker, 1979).

Processing and Maintenance of Wild-caught Sand Flies and Recovery of Eggs

Wild-caught female sand flies showing evidence of a blood meal (abdomen distended and dark-red or black in color) were transferred to individual 7-dram oviposition vials fitted with screen lids (Endris et al., 1982). Water was added to the plaster of Paris in the bottom of each vial to maintain a high relative humidity, and a drop of Karo® syrup in water (1:1) was placed on the screen lid to serve as an

energy source. Unfed flies were offered a blood meal, through the screen lid of the holding vial (Fig. 3-1, p. 98, Chap. 3) on a human forearm, a hamster or a lizard. Those that fed immediately were transferred to individual oviposition vials, as previously described, and held until they oviposited and/or died. Females that did not accept the initial offer of a blood meal were placed in a holding cage (Endris et al., 1982) with males and offered a blood meal each succeeding day until they either fed or died. Those that fed were retained in oviposition vials until they oviposited and/or died. Dead females were removed and, if they had deposited eggs in the vials, the screen lids were replaced with solid plastic lids that had been perforated with a dissecting needle to permit limited gas exchange. During June 1982, the field laboratory consisted of a permanent, screened camping shelter. To protect the captured flies from the heat of the Texas summer, their containers were kept in a polystyrene cooler. By placing ice in the cooler, removing or replacing the lid as necessary, the temperature within the chambers and vials could be maintained at or near 27°C. During the September, 1983, trip the field laboratory was in an air-conditioned motel room where the temperature was maintained at 23°C.

Preliminary identification of live, wild-caught specimens was performed macroscopically. At death they were more reliably identified by microscopic examination and comparison with appropriate taxonomic keys (Young and Perkins, 1984). Field identifications were later confirmed as necessary in the laboratory by Dr. D. G. Young, after consulting appropriate keys and available type materials. Specimens were prepared and cleared for permanent slide mounts according to the technique of Young (1979).

Post mortem dissections of female sand flies were performed as soon after death as possible (usually within 12 hours) according to the technique of Johnson et al. (1963). Data recorded for each specimen included information regarding collection, sex, identification, blood meals, oviposition, longevity, condition of accessory glands, and presence or absence of natural parasites.

Results

Species of Sand Flies Present

An estimated 2400 sand fly specimens representing five species of Lutzomyia, including one new species, were collected and eight new county records established during the two years of the study (Table 2-1). Sand flies were taken from five of eight case sites; none were collected at sites F, G, and H (San Antonio and Devine).

Of the sampling methods used, only the Disney trap and the oil traps failed to collect sand flies. Approximately 2000 flies, mostly females, were taken in resting collections at Garner State Park and Fawcett Boy Scout Camp. Other trapping methods also yielded mostly females (Table 2-2). The ratio of male to female Lu. diabolica in resting, biting and light trap collections ranged from a high of about 6:10 in resting collections at Fawcett Boy Scout Camp, to around 4:10 in resting collections at Garner State Park. More than 350 female and no male Lu. diabolica were taken in biting collections at Garner State Park. This method was not successful at other sites. At locations where flies were not taken resting, CDC light traps were most effective.

Table 2-1. Summary of Lutzomyia sand fly surveys conducted in vicinities of human case sites of cutaneous leishmaniasis in south central Texas (4-28 June, 1982; 19 Sept. thru 4 Dec., 1983)

Site	Species									
	Lu. anthophora		Lu. diabolica		Lu. texana		Lu. vexator		Lu. new species	
	No.	Method	No.	Method	No.	Method	No.	Method	No.	Method
A Garner State Park	6	resting--latrine walls (night and morning), armadillo burrows (day)	≅2350	resting--latrine walls (night and morning), biting--night, CDC lt trp, Shannon trp	6	resting--latrine walls (night and morning), armadillo burrows (day)	2	resting--latrine walls (night)	1*	resting--latrine walls (morning)
B Rio Frio	0		11*	CDC lt trp	0		0		0	
C Seminole Canyon State Park	0		10	CDC lt trp	0		0		0	
D Fawcett Boy Scout Camp	3*	resting--latrine walls (night)	29*	resting--latrine walls (night)	2*	resting--latrine walls (night)	0		0	
E Myers' Farm	1*	resting--woorat nest (day)	8*	CDC lt trp, New Jersey lt trp	8*	New Jersey lt trp	0		0	
Totals	10		≅2408		16		2		1	

* = new county record

Table 2-2. Number of male and female Lutzomyia diabolica in selected collections from three sites using six trapping methods (4-28 June, 1982; 19-30 Sept., 1983)

Site	Sex	Collection Method: Resting No. (%) (rate)	Biting No. (%) (rate)	CDC Lt Trap No. (%) (rate)	CDC Lt Trp+CO_2[2] No. (%) (rate)	NJ Lt Trp[2] No. (%) (rate)	Shannon Trp No. (%) (rate)
A[1]	♂	84 (29) (8/night)	0 (0) (0/night)	12 (24) (4/night)	- -	- -	0 (0) (0/night)
	♀	223 (73) (22/night)	350 (100) (39/hr)	37 (76) (12/night)	- -	- -	1 (100)[3] (1/3 hr)
D[1]	♂	14 (39) (7/night)	0 (0) (0/night)	0 (0) (0/night)	- -	- -	0 (0) (0/night)
	♀	22 (61) (11/night)	0 (0) (0/hr)	0 (0) (0/night	- -	- -	0 (0) (0/hr)
E[1]	♂	0 (0) (0/night)	0 (0) (0/night)	0 (0) (0/night)	1 (14) (0.5/night)	0 (0) (0/night)	0 (0) (0/night)
	♀	0 (0) (0/night)	0 (0) (0/hr)	0 (0) (0/night)	6 (86) (3.0/night)	1 (100) (0.02/night)	0 (0) (0/hr)
Totals	♂	98 (29)	0 (0)	12 (24)	1 (14)	0 (0)	0 (0)
	♀	245 (71)	350 (100)	37 (76)	6 (86)	1 (100)	1 (100)

[1]A = Garner State Park, Concan, Uvalde Co., Tx.
D = Fawcett Boy Scout Camp, Barksdale, Edwards Co., Tx.
E = Myer's Farm, D'Hanis, Medina Co., Tx.

[2]These methods used only at site E.

[3]Due to inclement weather, the trap was operational for only 3 hr.

Lutzomyia diabolica. Lu. diabolica (Fig. 2-17), the only anthropophilic sand fly found, was collected at all positive sites and accounted for 99% of the total catch (Table 2-1). They were collected in greatest abundance at Garner State Park where they were captured while resting on the tile walls of the public latrines (Fig. 2-6). There were a total of 12 latrines at the park, 8 of which were situated in the upland meadow areas and 4 along the Frio River flood plain, shaded by large pecan, oak, and cypress trees. Up to 150 Lu. diabolica per day (nights and mornings) were taken in resting collections from the 8 latrines in the more elevated portions of the park. Many were engorged with fresh blood, possibly from the unprotected parts of unsuspecting campers, or from deer and jack rabbits that frequent the surrounding meadows by night. No sand flies were collected from the latrines near the river. Although a few sand flies could usually be found in positive latrines at all hours of the day, they typically appeared about one hour after sunset (2200 hrs in June, 2100 hrs in September) and remained until mid morning (about 1000 hrs), their activity coinciding with the daily bathing ritual of the campers. They were observed resting on the interior tile walls between the level of the floor and a height of about 2.5 m and seemed to prefer dark humid corners, such as in shower stalls and under sink counters. Their resting attitude was almost always vertical, with head pointing up. They were frequently observed hopping deliberately toward the collector, presumably seeking a blood meal. If by chance the lights in a latrine had not been turned on, the catch was greatly reduced. Greatest numbers appeared on hot, humid nights (27^{o}C and 80% or higher RH) with little or no air movement. On such nights sand flies

Figure 2-17. Lutzomyia diabolica female (approx. magn. x 10).

were also seen resting on the exterior walls of the latrines under the outside lights and around the entrance. The number of flies collected decreased dramatically with increased wind velocity. At Fawcett Boy Scout Camp, sand flies were also taken in resting collections in a lighted, open-air latrine (Fig. 2-8).

An extensive search for natural diurnal and nocturnal resting sites of Lu. diabolica in rock crevices, under bark of trees, in animal nests and burrows, in leaf litter, and other potential hiding places was fruitless at all case sites.

Female Lu. diabolica were often taken in biting collections during the evening hours at Garner State Park as they came in search of a blood meal. Routine biting collections netted from one to about twenty female sand flies per hour. One biting collection, however, was worthy of special note: The night of 9 June, 1982, was hot and humid (27°C and 80-90% RH) and air movement was about nil. The author and his son had returned to the field laboratory (a screen tent) about 2330 hrs after searching the latrines for resting sand flies. They sat under an incandescent light at a table inside the tent and began to count and feed the evening's catch. Shortly the author's son began to complain of being bitten by sand flies, and five were collected that were biting him on the legs. Lu. diabolica females flew unobstructed through the screen sides of the tent in waves of ten or twenty to feed, biting the occupants on the face, neck, hands, ankles and other exposed skin. These foraging sorties were of about five minutes' duration, with a brief respite interval between. They continued for several hours and about 150 female Lu. diabolica were collected before 0100 hrs, when the author finally retired for the

night. Even with the light turned out, the flies continued their attack, but in lesser numbers.

On several occasions, foraging females were seen flying through the standard mesh screen of the permanent camping shelter (field laboratory) to attack the author and his eight-year-old son. They seemed to exhibit a preference for the son, biting him even after the lights had been turned off. Although most of this biting activity occurred at night, one female Lu. diabolica bit him at about 1400 hours in the heat of the day and in full sunlight (temperature about 38°C, RH about 60%, air calm).

Lu. diabolica were collected in unbaited CDC light traps at Garner State Park, Rio Frio, and Seminole Canyon State Park in June 1982. They were also collected in CO_2 baited CDC light traps (with light on) and in a New Jersey light trap at the site in D'Hanis in September, 1983. Unbaited CDC light traps (light only) were unsuccessful at the D'Hanis site.

One female Lu. diabolica was collected in a Shannon trap at Garner State Park. The trap was set up near the camping shelter about an hour before dark (1800 hrs), but was only operational for about three hours due to inclement weather. Other attempts to attract sand flies to a Shannon trap at D'Hanis and Devine failed.

Disney traps set out near rodent burrows at Garner State Park failed to collect sand flies as did oil traps set out in similar places at D'Hanis and Devine.

Lutzomyia anthophora. Lutzomyia anthophora were taken with Lu. diabolica in resting collections in latrines at Garner State Park and Fawcett Boy Scout Camp. They were also taken from armadillo (Dasypus novemcinctus) burrows at the former site.

One male Lu. anthophora was taken from an active, carefully dismantled woodrat (Neotoma) nest located approximately 75 m east of the farm house at the D'Hanis site (Fig. 2-18). Four other Lu. anthophora (males and females) were seen in the same nest, but escaped capture.

Lutzomyia texana. Lutzomyia texana, a rather large sand fly that inhabits mammal burrows, was taken in resting collections from latrines at Garner State Park and Fawcett Boy Scout Camp. They were also collected from the entrances of armadillo burrows at the former site and in CO_2 baited CDC light traps and in an unbaited New Jersey light trap at the farm in D'Hanis. They were not found in armadillo burrows at the latter site. Efforts to feed Lu. texana on a human, a hamster and a lizard were unsuccessful.

Lutzomyia vexator. This species, which feeds on cold-blooded vertebrates, was collected on two occasions while resting on latrine walls at Garner State Park.

Lutzomyia new species. In September, 1983, a single female of an undescribed species was taken in a resting collection from a latrine at Garner State Park. Efforts to feed the fly on human, hamster and amphibian (toad) blood were futile and she died without depositing eggs. The fly was prepared and examined by Dr. D. G. Young who stated that it belongs in the Cruciata group, but was unlike any other species collected in Texas.

Processing and Maintenance of Wild-caught Sand Flies and Recovery of Eggs

An estimated 1925 female Lu. diabolica were segregated and maintained in individual or group oviposition containers and 7940 ova

Figure 2-18. Dismantled woodrat (Neotoma) nest near the home of patient D in D'Hanis, Medina County, Texas, where specimens of Lutzomyia anthophora were found.

were recovered for laboratory colony stock. Six hundred and one females were dissected post mortem and examined for retention of eggs, condition of accessory glands and natural parasite infections.

Table 2-3 summarizes fecundity for 360 wild-caught females (263 from the first trip and 97 from the second). Regardless of efforts to perform dissections within 24 hrs after death, many flies were in such poor condition that dissection provided little usable information. These and 54 females that died from overheating in a locked car in June 1982 were excluded from the table. The mean number of eggs deposited and the gross number of eggs produced (the number deposited plus the number retained) remained fairly constant from one year to the next. From 31 to 37% of the females retained some or all of their eggs. Females neither depositing nor retaining eggs (in other words maturing none) ranged from 3% in June 1982 to 10% in September 1983. No evidence of autogenous behavior was observed. Females that were denied a blood meal neither laid nor developed mature eggs.

Significant differences were noted between year groups in preoviposition interval, postcapture longevity and postoviposition longevity, all three being longer for flies captured in the fall (Table 2-4). A smaller percentage of females survived oviposition in the fall than in the spring.

Accessory Glands and Parity

Sixty-four wild-caught female Lu. diabolica, which had not been offered a blood meal, were dissected to study the paired accessory glands (Fig. 2-19). The glands of only 36 flies were clearly seen. Of these 36, only three showed evidence of having had a blood meal;

Table 2-3 Fecundity summary for wild-caught Lutzomyia diabolica females from south central Texas (4-23 June, 1982; 19-30 Sept., 1983)

Oviposition Category	June 1982			September 1983		
	Sample		Eggs	Sample		Eggs
	n/263	%	$\bar{x}$ ± 1 SD (range)	n/97	%	$\bar{x}$ ± 1 SD (range)
Laying 1-84 eggs	148/263	56	34.8 ± 20.4 (1-84)	73/97	75	33.3 ± 25.5 (1-62)
Laying no eggs	115/263	44	- -	24/97	25	- -
Laying all their eggs	52/263	20	48.4 ± 15.9 (3-84)	42/97	43	38.3 ± 11.8 (15-61)
Retaining 1-76 eggs	81/263	31	27.0 ± 18.1 (1-60)	36/97	37	22.9 ± 19.5 (1-76)
Retaining all their eggs	25/263	10	37.7 ± 14.3 (1-57)	8/97	8	45.3 ± 18.4 (15-76)
Maturing no eggs	7/263	3	- -	10/97	10	- -
Total eggs produced per female	-	-	43.1 ± 16.4 (1-84)	-	-	41.4 ± 12.8 (15.76)
Total eggs recovered			5506			2434

Table 2-4. Summary of preoviposition intervals, postcapture and postoviposition longevities in wild-caught Lutzomyia diabolica females from south central Texas (based on a sample of 100 females per year group) (4-28 June, 1982; 19-30 Sept., 1983)

Category	June 1982 n/100	June 1982 Days $\bar{x}$ ± 1 SD (range)	September 1983 n/100	September 1983 Days $\bar{x}$ ± 1 SD (range)
Preoviposition Interval				
Captured w/ Blood Meals	31/100	4.5 ± 2.9 (1-10)	46/100	8.6 ± 3.7 (1-16)
Captured w/o Blood Meals	69/100	4.7 ± 2.1 (1-11)	54/100	8.5 ± 3.8 (1-16)
Postcapture Longevity				
Overall	-	5.8 ± 3.0 (1-19)	-	9.7 ± 4.2 (1-19)
Laying Some Eggs	56/100	6.5 ± 6.5 (1-16)	75/100	9.6 ± 3.79 (3-19)
Maturing No Eggs	3/100	5.6 ± 2.1 (3-9)	10/100	10.4 ± 5.4 (1-19)
Postoviposition Longevity				
Taking One Blood Meal	56/100	2.2 ± 1.7 (1-8)	29/100	3.2 ± 3.2 (1-11)
Taking Two Blood Meals	0/100	- -	4/100	4.7 ± 2.3 (2-6)

all three had either mature or developing ova and the accessory glands were full of dark granules. Of the remaining 33 females, 17 had developing ova (most were about half developed) and all of these had either full or partially full accessory glands. Sixteen had undeveloped ovaries, and of these, five had no granules in the accessory glands; one had a very few granules; nine had accessory glands half full of granules; and two had completely full accessory glands.

Observations on the condition of the accessory glands (granule formation) in another 94 dissected wild-caught females are presented in Fig. 2-20. All of these females were blood-fed at the time of capture or were given a blood meal immediately after capture. These were compared with dissections of laboratory-reared, fed and unfed females of known ages. In general, granule formation in accessory glands of wild-caught sand flies paralleled ovarian development, and when the ova were mature, the accessory glands were dark and full of granules. Partial or complete emptying of the accessory glands often occurred as eggs were deposited, but many females that deposited most or all of their eggs maintained full accessory glands. Blood-fed laboratory-reared flies showed basically the same trend as blood-fed wild-caught flies. Unfed laboratory-reared flies either did not develop granules in the accessory glands or, if they did, they developed them within 24 hrs, but to a lesser degree, even after six days, than seen in blood-fed females.

Natural Parasite Infections

The incidence of natural parasite infections found in 341 female sand flies taken from south central Texas, dissected during the two years of the study, is presented in Table 2-5.

Figure 2-19. Paired accessory glands of a gravid female Lutzomyia diabolica showing dense granular material (magn. x 160).

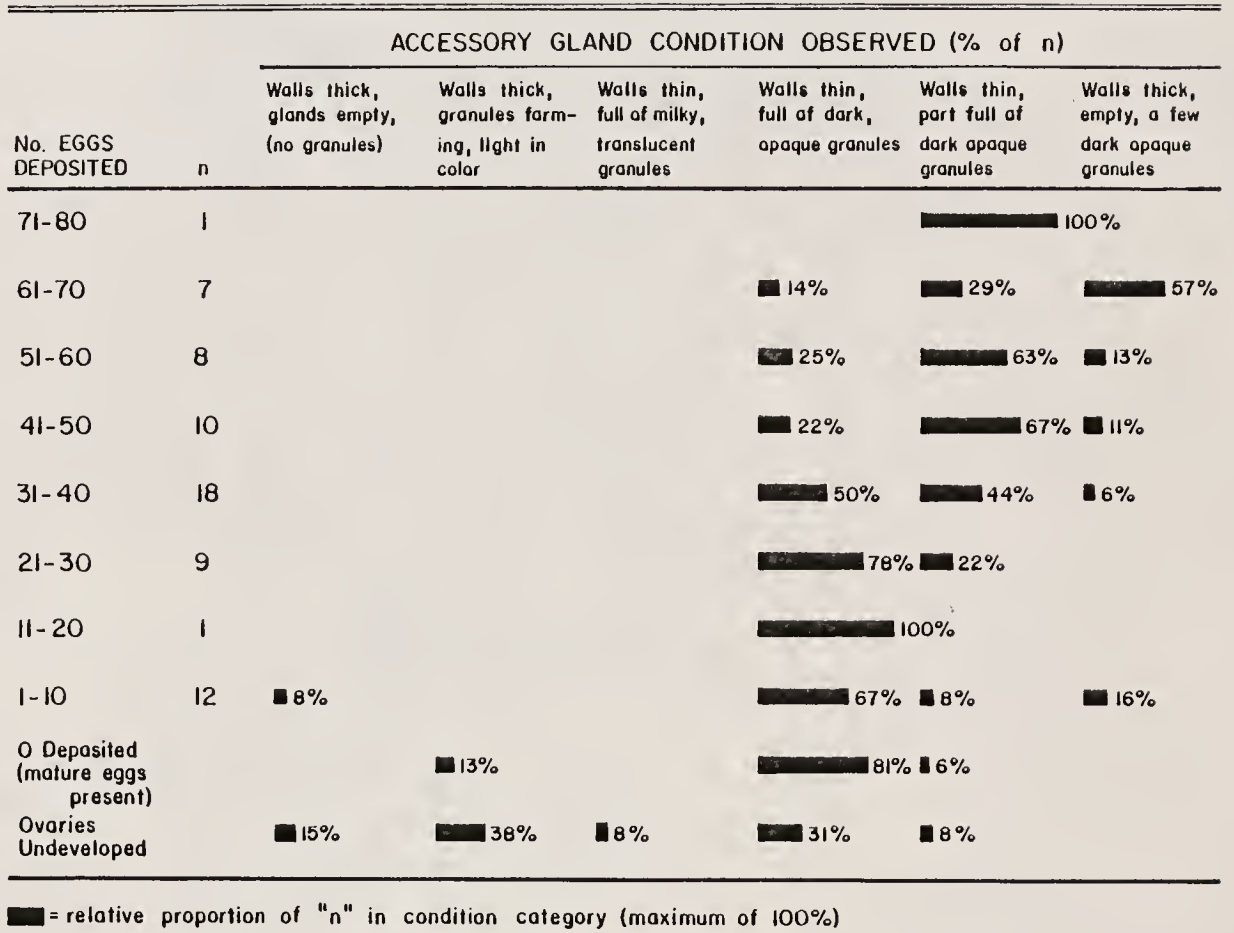


Figure 2-20. Condition of accessory glands in relation to number of eggs deposited by 94 wild-caught female Lutzomyia diabolica.

Table 2-5. Incidence of natural parasite infections in 341 female *Lutzomyia diabolica* collected in south central Texas (4-28 June, 1982; 19-30 Sept., 1983)

	June 1982 (N = 243)[1]		September 1983 (N = 98)[2]	
Parasite	# infected	% infected	# infected	% infected
Small, round, fast moving flagellates	146	60	26	27
Epimastigote-like flagellates	1	<.05	2	2
Aseptate gregarines	12	7	9	9
Microsporidians	8	3	3	3
Mites	14	6	0	0

1. Specimens collected primarily from Garner State Park, Uvalde County, Texas, with small numbers collected from Fawcett Boy Scout Camp, Edwards County, Texas, and Rio Frio, Real County, Texas.

2. All specimens collected from Garner State Park, Uvalde County, Texas, with the exception of six specimens collected from D'Hanis, Medina County, Texas.

Flagellates. Small, rounded, highly motile flagellates were found swimming free in the hemocoel of 27 to 60% of the specimens dissected. They were most often seen in the hemocoel of the anterior thoracic region and immediately posterior to the head. They numbered from one to several, but rarely more than 25. Efforts to photograph these tiny parasites were unsuccessful.

Two females collected in the fall of 1983 contained what appeared to be small thin flagellates in their midguts. The organisms did not move and appeared to be dead. They were stained with Giemsa and mounted on microslides with Euparal, but were unidentifiable.

An infection of unknown epimastigote-like flagellates was discovered in the midgut of one of the females dissected in the spring of 1982 (Fig. 2-21). Several hundred of these promastigotes were observed freely swimming in the lumen of the gut; some attached to the midgut epithelium by their flagella; others formed rossettes with their flagella directed toward the center much like L. mexicana parasites seen in laboratory infections and in cultures in vitro. Their general appearance, however, was not like Leishmania. The infected fly was unfed when captured and was given a blood meal on the author's forearm. Upon dissection (six days later), the blood meal remnant was evident in the midgut. Midgut contents containing the flagellates were inoculated subcutaneously into the foot pad of a hamster. No lesions or other signs of infection developed in either the author or in the hamster.

Gregarines. Aseptate gregarines, possibly Ascocystis chagasi (Adler and Mayrink), were found in 7-9% of dissected females. Up to five gamonts were observed in the abdominal cavity of some infected

flies (Fig. 2-22). These could be seen slowly changing shape and moving in amoeboid fashion. When present, one to three gametocysts could be seen attached to the accessory glands (Fig. 2-23). The eliptical oocysts were most frequently seen spilling from the accessory glands or from ruptured, mature gametocysts (Fig. 2-24). Oocysts were seen glued by the accessory gland material to the chorion of eggs deposited by gregarine infected females.

Microsporidians. Massive microsporidian infections were discovered in the hemocoels of 3-8% of the dissected females (Fig. 2-25). Germination of the spores was stimulated in vitro by adding a few drops of 0.2m KCl (pH 9) to the dissecting medium (insect Ringers solution) (Fig. 2-26). Ungerminated spores washed onto the larval medium were fed to uninfected larvae. No infections were acquired.

Mites. Mites were found attached to the venter and dorsum of the abdomen of 14 females collected in the spring of 1982. None were found on females collected in the fall of 1983. The mites were tentatively identified as Eustigmaeus sp. (Berlese) (Family Stigmaeidae). Scanning electron micrographs of one mite specimen are presented as Figures 2-27 and 2-28.

Other. Fungal and bacterial infections were commonly found in dissections of wild-caught Lu. diabolica.

Discussion and Conclusions

Determination of Sand Fly Fauna

Many methods have been used for sampling sand fly populations. Some were devised for sampling particular habitats (e.g., resting

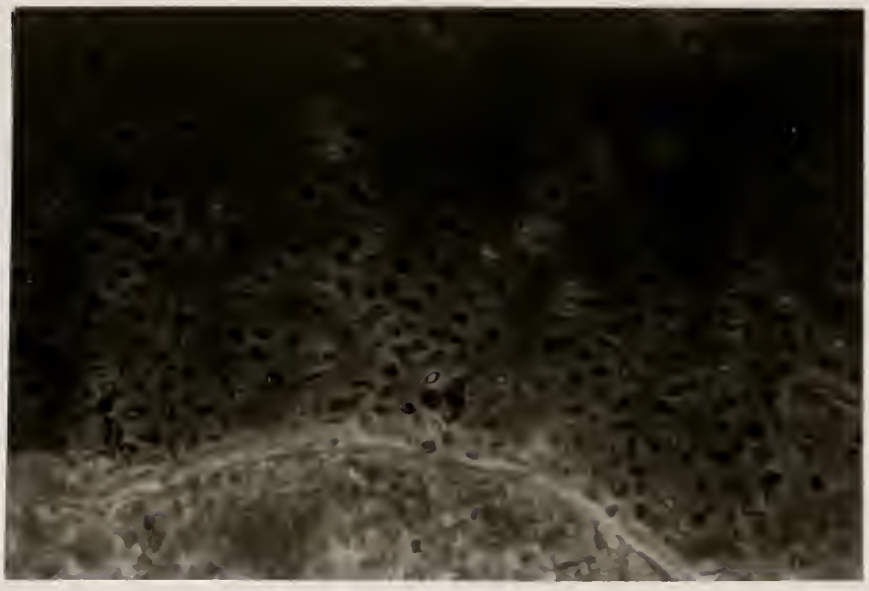


Figure 2-21. Unidentified flagellates in the midgut of a Lutzomyia diabolica female collected at Garner State Park, Uvalde County, Texas, June 1982 (approx. magn. 2100).

Figure 2-22. Gamont of aseptate gregarine in the abdominal cavity of a female Lutzomyia diabolica collected at Garner State Park, Uvalde County, Texas, June 1982 (approx. magn. x 850).

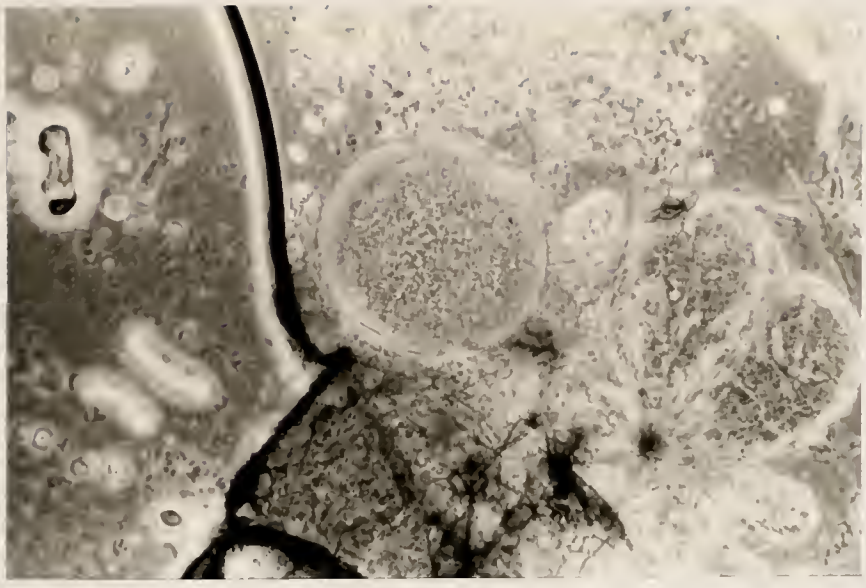


Figure 2-23. Gametocyst of an aseptate gregarine (center) attached to the accessory gland of a female Lutzomyia diabolica collected at Garner State Park, Uvalde County, Texas, June 1982 (approx. magn. x 850).

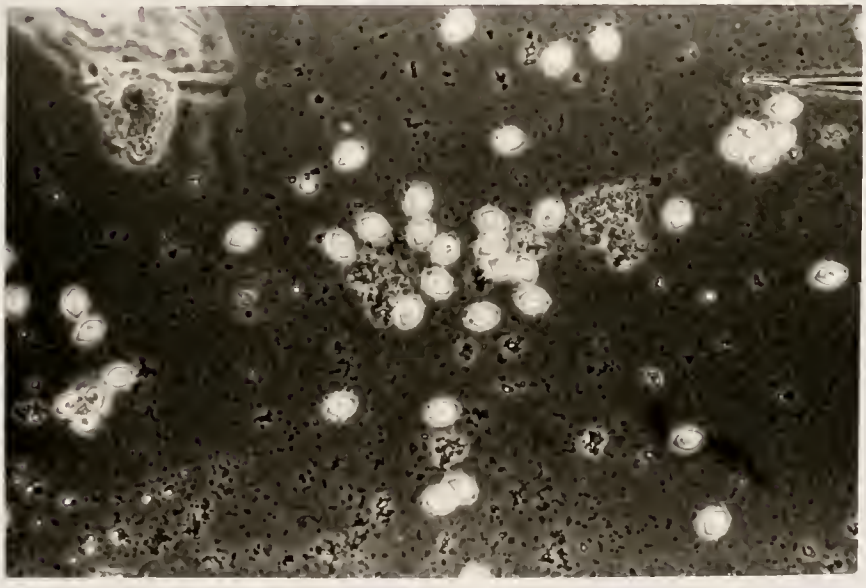


Figure 2-24. Gregarine oocysts spilling from the accessory glands of a female Lutzomyia diabolica collected at Garner State Park, Uvalde County, Texas, June 1982 (approx. magn. x 2100).

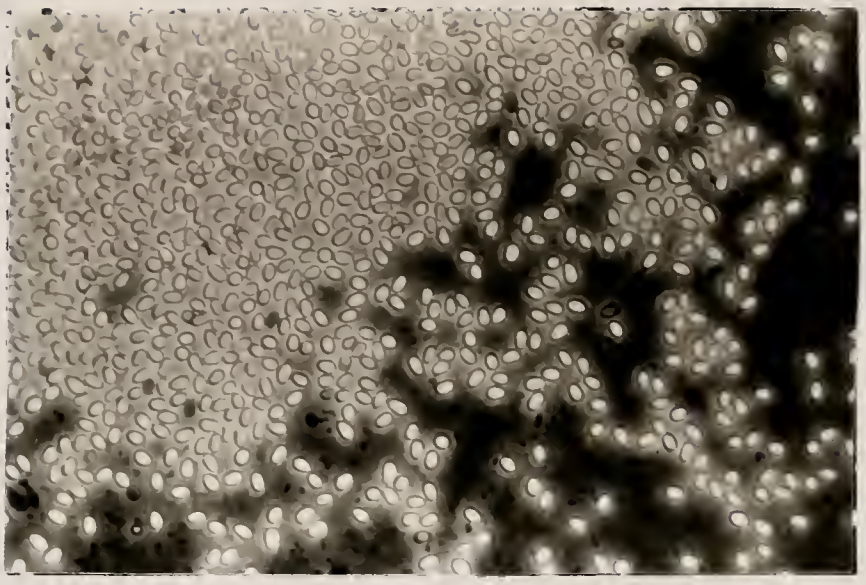


Figure 2-25. Microsporidian spores in the hemocoel of a female Lutzomyia diabolica collected at Garner State Park, Uvalde County, Texas, June 1982 (approx. magn. x 2100).

Figure 2-26. Microsporidian spore germinated in vitro by addition of 0.2 M KCl (pH 9) to dissecting medium (insect Ringer's solution, pH 7.2) (approx. magn. x 2640).

Figure 2-27. Scanning electron micrograph of mite (Eustigmaeus sp.) found on the abdomen of a female Lutzomyia diabolica collected at Garner State Park, Uvalde County, Texas, June 1982 (dorsal aspect; magn. x 1704).

Figure 2-28. Scanning electron micrograph of mite (Eustigmaeus sp.) found on the abdomen of a female Lutzomyia diabolica collected at Garner State Park, Uvalde County, Texas. June 1982 (ventral aspect; magn. x 1700).

collections, sticky traps, Disney traps) and are of little value elsewhere; others have broader utility (light traps) and can be used regardless of the availability of suitable habitat. From the data in Table 2-1 it appears that the latrines at Garner State Park and at Fawcett Boy Scout Camp were universally attractive structures. All species taken at these two sites were taken in resting collections in the latrines. In other localities, where resting sites were not discovered, even after extensive searching, light traps proved most effective. Biting collections were effective as a selective means of sampling anthropophilic species at Garner State Park, and probably would have been useful at other sites as well. Baiting of CDC light traps with dry ice also selected for man biters and was the only way Lu. diabolica were collected at the D'Hanis site.

Due to the success of collections in latrine resting stations at Garner State Park and Fawcett Boy Scout Camp, other methods such as light trapping and bait trapping were not routinely used.

Most sampling methods collected predominantly females, which is consistent with findings of other authors (Young, 1972; Endris, 1982). No males were taken in biting collections, although some would be expected to mate with females on the host as she feeds. The figures in Table 2-2 should not be interpreted to represent the true sex ratios in the natural population. Most trapping methods, especially those using light or bait attractants, select for females. This is especially true if the female's movement is part of a hunting strategy (Killick-Kendrick and Rioux, 1981). Chaniotis et al. (1972) stated that the true sex ratio could only be properly assessed by studying

populations from each habitat using a variety of sampling methods (especially those that do not select one sex over the other).

Lutzomyia diabolica. This species was discovered in Uvalde, Texas, in 1915 and was said to attack man freely (Parman, 1919). It was subsequently collected by other workers in areas limited almost exclusively to the south central part of the state (Young and Perkins, 1984; Fig. 2-29). Disney (1968) held that Lu. diabolica was conspecific with Lu. cruciata, a species widely distributed through Central America, and a suspected vector of leishmaniasis in Belize (Williams, 1966a, 1966b). Young and Perkins (1984) showed that Lu. diabolica from Texas represents a distinct species, separate from Lu. cruciata.

Parman (1919) found Lu. diabolica hiding in dark places during the day, one or two specimens to a place. He offered few clues as to where these "dark hiding places" were, except to say there was evidence that the breeding places are in neglected poultry houses, since the flies were observed in abundance around such places in the late twilight hours. Lindquist (1936) captured male and female Lu. diabolica on walls and curtains in lighted rooms on first and second floors of dwellings. Endris (1982) collected Lu. diabolica resting in latrines at Garner State Park. All resting collections of Lu. diabolica have been made in or on human dwellings or outbuildings. To date, a "natural" resting habitat of this species has not been found, indicating that it may be a peridomestic sand fly. This underscores the need for future searches to locate natural resting places and pinpoint breeding sites. Resting Lu. diabolica were collected in latrines only in the upland portions of Garner State Park, an

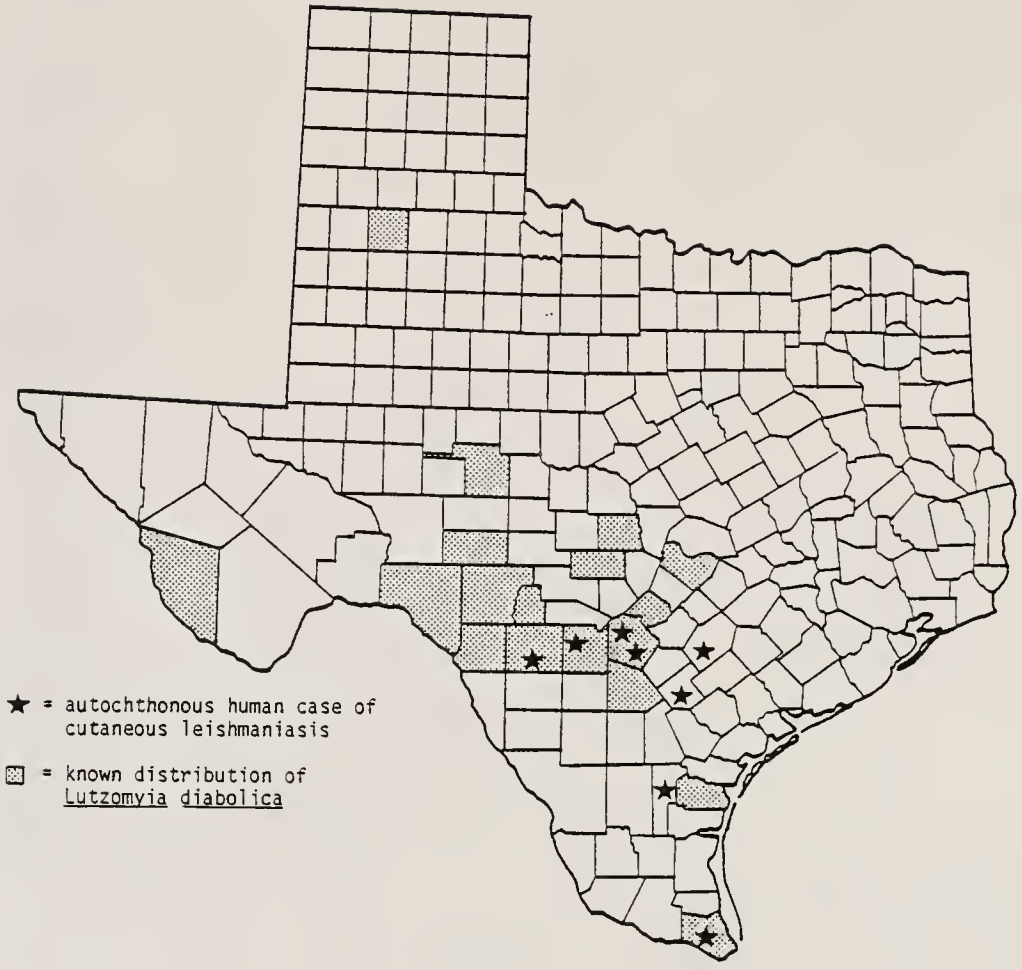


Figure 2-29. Known geographical distribution of the sand fly Lutzomyia diabolica in Texas.

observation consistent with Parman's statement (1919) that the sand fly was found in Uvalde in the more elevated parts of the city. Perhaps this is a clue as to where other resting sites are to be found.

Parman (1919) reported that the earliest authentic record of appearance of Lu. diabolica in Uvalde was 3 September and the latest was 24 November. Lindquist (1936) reported collections between 3 May and 16 November from Uvalde. Although no collecting was done in Uvalde during this study, Lu. diabolica were taken in resting and biting collections at nearby Garner State Park (50 km north of Uvalde) as early as 4 June and in light trap collections at the D'Hanis site (50 km east of Uvalde) as early as 5 May and as late as 4 December. Specimens of this species were taken at Garner State Park in human biting collections by Young (1972) as early as May 17. Adults are probably present in these locations throughout the frost free season, with populations increasing over the summer months and reaching a peak in the fall when they reach a nuisance level and are noticed by the public. Parman (1919) apparently based appearance records on biting activity. If so, this is consistent with the idea of a gradually expanding population over the summer months. Surveys throughout the year in and around Uvalde will be essential to confirm this idea.

Parman (1919) believed that Lu. diabolica never venture out of hiding until well after sunset and never attack earlier than one hour after sunset. Wilkerson (pers. comm., 1984) collected a female Lu. diabolica biting him at 1400 hrs in full sunlight in mid July at Canyon Lake, Comal County, Texas. During the 1982 research trip one female was collected while biting the author's son at 1400 hrs in full sunlight (temperature 38°C, RH approximately 60%). These may be

isolated instances, but they indicate that perhaps the daily feeding activity of Lu. diabolica is not as restricted as previously reported. Lindquist (1936) reported the feeding period to be from 2000 hrs to 2400 hrs and Endris (1982) reported Lu. diabolica feeding during all hours of darkness. Williams (1966b), in studies of biting rhythms of ten anthropophilic sand flies in Belize, found the greatest period of activity was between 0600 and 0659 hrs. After this small burst of activity, the number of flies decreased. He said that the flies were least active between 1400 and 1459 hrs (the hottest part of the day), but that biting activity increased gradually from 1500 hrs onward. Flies were not collected in appreciable numbers, however, until dusk (1800-1859 hrs). He noted that numbers increased still further during the early hours of darkness, reaching the peak of greatest activity between 2100 and 2159 hrs. Thereafter, biting density diminished. Consistent with these later reports, the peak feeding period of Lu. diabolica in June 1982 was observed between one hour after sunset and midnight (2130 to 2400 hrs). Killick-Kendrick and Rioux (1981) described similar activity for Phlebotomus ariasi Tonnoir in the Cevennes, France, and said it was probably triggered, at least partially, by the rise in relative humidity as the temperature falls at sunset. Parman (1919) said Lu. diabolica would not bite in total darkness or in full moonlight. Although most biting collections in June 1982 were taken in the presence of artificial light, sand flies were also collected in lesser numbers while biting in total darkness, having no apparent difficulty finding their host. They were also observed biting outdoors in full moonlight.

Parman (1919) noted that the abundance of Lu. diabolica in the fall was extremely variable, ranging from only one specimen attacking in several nights, to 25 to 30 attacking each night for a short period. This variability in numbers and peak time of attack was observed both in the spring and fall during this study, and appears to be strongly influenced by ambient temperatures, relative humidity and air movement. Sand flies were observed in greatest numbers on hot humid nights (27°C or above and 70% or greater RH) with little or no air movement. Not surprisingly, air movement seemed to affect their presence the most, as numbers decreased dramatically with an increase in wind velocity above 8 kph (5 mph). Foraging sorties or wave attacks such as described previously did not occur except on hot, humid and windless nights. Killick-Kendrick and Rioux (1981) reported the peak biting activity of P. ariasi may be delayed because of wind or suppressed by storms or a fall in temperature below about 16°C. Some workers have studied the effects of wind on the movement and activity of sand flies but probably overestimated the wind speed at which activity ceased (Killick-Kendrick and Rioux, 1981).

Blood-fed females have greater difficulty flying than unfed females and apparently rest in protected sites for up to 24 hrs to allow for diuresis and partial digestion of the blood-meal. This accounts for the large number (31 to 46%) of blood-fed females taken in latrine resting stations.

Both male and female Lu. diabolica are assumed to disperse from their breeding sites, possibly in search of sugar or a blood-meal. Their attraction to light draws them close to human dwellings where other shorter range attractants, such as exhaled CO_2

or body warmth, may aid them in finding a blood meal. This may also be a genetically selected, triggered-sequence response, i.e., lights = people = food. For whatever the reason, their response to light contributes to the success of light-trapping, and several workers have collected Lu. diabolica by this method (Young and Perkins, 1984). Indeed the lighted latrines at Garner State Park and Fawcett Boy Scout Camp functioned as giant light traps. The exposed positions of the positive latrines was somehow important, since those at lower elevations in more protected positions yielded nothing. Perhaps they were more visible and attracted flies from considerable distances. It may also be that the sand flies were seeking something other than a blood meal, such as shelter. As was the case at the farm in D'Hanis, CDC light traps may not be bright enough to attract sand flies and must be augmented with CO_2 (dry ice). The brighter lights of the Shannon trap and the New Jersey light traps were apparently sufficient to attract foraging flies.

The distance traveled by a sand fly to its host may depend upon the habitat and the species. Estimates by other workers of distance traveled to light ranges from 200 m in a neotropical forest (Chaniotis et al., 1974), to as far as 2300 m in open habitat (Killick-Kendrick and Rioux, 1981). These authors also suggested that the movement of females is a hunting strategy and hence the difference in dispersal distance between males and females. Since Lu. diabolica occurs in a rather open habitat, it is likely that they disperse a considerable distance (1000 m or more) from the unknown breeding site. Males probably have a more limited flight range. The greater number of males found in resting collections at Fawcett Boy Scout Camp may

have been an indication that the breeding site was close by. Mark-release and recapture experiments will be necessary to determine the actual extent of dispersal from the breeding site.

Lutzomyia anthophora. This species was first collected while feeding on rabbits in Uvalde, Texas, in the type locality of Lu. diabolica (Addis, 1945a). Easton (1968) collected them in Malaise traps in Kinney and Presidio Counties, Texas. Young (1972) found Lu. anthophora in the nest of the plains woodrat (Neotoma micropus) in San Antonio, along the Rio Grande near Brownsville, Texas, and at Welder Wildlife Refuge near Sinton, Texas. Endris (1982) also collected Lu. anthophora from woodrat nests near Brownsville, Texas. Finding this species at Garner State Park, Fawcett Boy Scout Camp, and D'Hanis expands its known geographic distribution (Fig. 2-30). The close association of Lu. anthophora with the woodrat, and the finding of recently engorged females in the soft inner nest of the main Neotoma den strongly suggest the preferred host to be the woodrat (Young, 1972). Endris (1982) fed females of this species on the following anesthetized animals: woodrat, white footed mouse (Peromyscus leucopus), Syrian hamster (Mesocricetus auretus), grey squirrel (Sciurus carolinensis), white mouse (Mus musculus), guinea pig (Cavia porcellus), domestic rabbit (Oryctolagus cuniculus), and opossum (Didelphis marsupialus). Rodent reservoirs of leishmaniasis in the Neotropics, but not in Texas, have been reported by several authors (Bray, 1974a; Lainson and Shaw, 1979). Still, this possibility must not be overlooked and should be investigated in future studies. Lu. anthophora is not known to be anthropophilic, but Perkins (pers.

comm., 1984) reported that females in flourishing laboratory colonies will occasionally bite humans.

Lutzomyia texana. Lutzomyia texana was described from specimens collected in the nest of the leaf-cutting ant, Atta texana (Buckley), in San Antonio, Texas. This species has been collected in light traps at several sites throughout south central Texas (Fig. 2-31). Young (1972) reported that Lu. texana frequently inhabit mammal burrows, especially those dug by armadillos, and that they were collected from such places throughout most of the year. Efforts to feed specimens on a variety of hosts in June 1982 and September were unsuccessful. Young (pers. comm., 1984) believes that armadillos may be the principal hosts. Lainson et al. (1979) isolated Leishmania from armadillos in Brazil. There is no evidence of armadillo reservoirs of cutaneous leishmaniasis in Texas; however, the matter has never been investigated and should be given further attention.

Lutzomyia vexator (Coquillet). Lutzomyia vexator is the most widely distributed sand fly in the USA (Young and Perkins, 1984). It was collected previously by Young (1972) at Garner State Park and Fredricksburg, Texas, in light traps. These flies are reptile feeders and are of no known medical or economic importance.

Lutzomyia new species. This new species, known from a single female, raises to seven the number of sand fly species collected from Texas. Future field surveys will be essential to collect more specimens on which to base a valid description of the species and to study its biology and host associations.

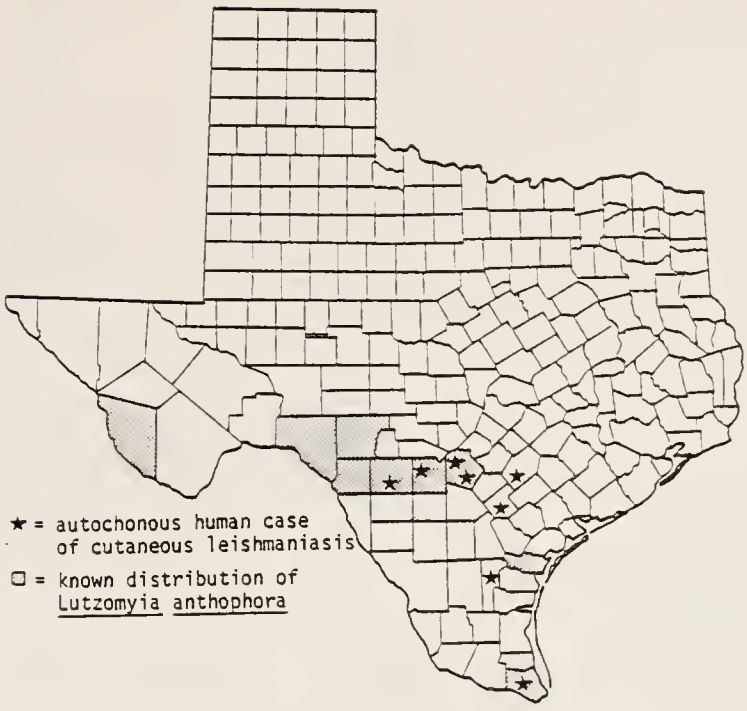


Figure 2-30. Known geographical distribution of the sand fly Lutzomyia anthophora in Texas.

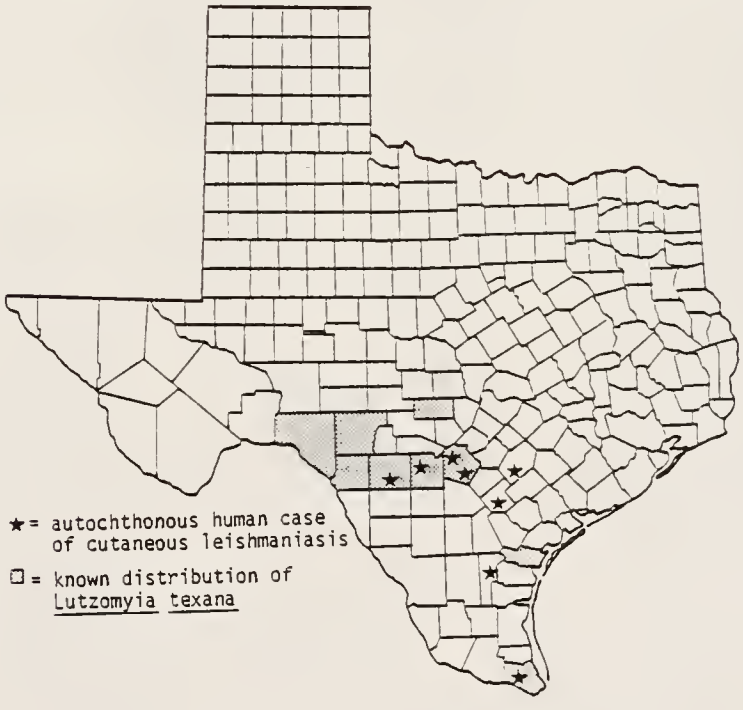


Figure 2-31. Known geographical distribution of the sand fly Lutzomyia texana in Texas.

Potential Vectors

Based on the results of this field study and the records of other workers, it can be said that Lu. diabolica is an abundant, widely distributed sand fly species in south central Texas. As the only known anthropophilic species known in the state, with peridomestic habits, and having a distribution that roughly coincides with areas of Leishmania endemicity, it is strongly implicated as the probable vector of human cutaneous lesishmaniasis in Texas (Fig. 2-29). Further evidence to support this is provided by experiments in which laboratory-fed Lu. diabolica were shown capable of transmitting L. mexicana from infected to uninfected hamsters by bite. These experiments are discussed in detail in Chapter 4.

Endris et al. (1984) transmitted L. mexicana from infected to uninfected hamsters with laboratory-bred Lu. anthophora. This led them to suggest that L. mexicana could be maintained in wild rodent populations by a non-anthropophilic species, such as Lu. anthophora, and secondarily transmitted to man by a sympatric anthropophilic sand fly, such as Lu. diabolica, thus implcating Lu. anthophora as a possible accomplice in the transmission of human cutaneous leishmaniasis in Texas. A further possibility that can not be ruled out is that Lu. texana may likewise be implicated as an accomplice vector when knowledge of its hosts and feeding behavior are revealed.

Processing and Maintenance of Wild-Caught Sand Flies and Recovery of Eggs

The advantage of recovering eggs from wild-caught females in the field is clear. They are much easier to handle than adults and

without significant mortality, allowing for field transportation of F_1 genetic strains for laboratory studies.

It was found that gravid females could be transported long distances in oviposition vials in a vehicle when they were protected from excessive heat and provided moisture periodically. One batch of about 40 adult females was hand carried 1,000 miles on an airplane with no mortality.

Oviposition records (Table 2-3) indicate that most of the females did not deposit all their eggs and that the number of eggs laid per female was quite variable (1-84 in June 1982; 15-76 in September 1983). Less variability in number of eggs deposited was observed in 1982. The higher percentage of females depositing ova during the fall 1983 trip as compared to spring 1982 (Table 2-3) is probably a reflection of improved handling techniques and "laboratory" facilities, and cooler temperatures. During June, 1982, the "laboratory" was a screened camping shelter in which temperatures remained about ambient. Mean temperatures at Garner State Park and surrounding areas during that month were about 28°C with average daily maximum temperatures of about 35°C. Relative humidity ranged between about 50 and 95%. Special care such as adding ice to the polystyrene cooler and addition of water to the plaster in the vials kept the specimens alive until oviposition. In September, 1983, ambient temperatures were lower (mean, 25°C; maximum, 32°C; RH about 65%) and the "laboratory" facility consisted of an air-conditioned room that was maintained at approximately 24°C.

The mean gross egg production (eggs laid plus eggs retained) of captured females was about the same for both trips. The high

percentage of females retaining eggs may simply reflect the stress of captivity. The number of females retaining eggs in the laboratory colony (after 13 generations) was much less and presumably approximates what would be found in nature. Females that neither deposited nor retained ova amounted to 3% of the catch in 1982 and 10% in 1983. Whether this is evidence of possible gonotrophic disassociation is only conjecture. Occurrence of the phenomenon to a higher degree in the fall of the year is consistent with what has been observed in other insects.

Differences in preoviposition interval, postcapture longevity, and postoviposition longevity were also probably due to improved handling techniques in the second year when it was cooler (Table 2-4). Comparison of preoviposition longevity of females captured with and without blood meals (Table 2-4) reveals no significant differences, suggesting that females captured with blood meals had recently fed and that blood-fed females do not linger at the latrine resting stations more than a few hours.

At least 57% of the egg batches laid by wild-caught, blood-fed females hatched. Since these females were not placed with males after capture, it is obvious that at least this percentage of females were inseminated prior to capture. Sperm were observed in the spermathecae of many of the dissected females, but spermathecae were not always visible in dissections.

Accessory Glands and Parity

According to Chapman (1971), accessory glands in most insects arise from the genital chamber or vagina. Their function varies in

different insects, but they commonly produce a substance for attaching the eggs to the substrate. This material may also serve as a protective covering for the eggs. There is considerable confusion, even controversy, in the literature over the value of accessory gland examination as an indicator for determing parity in sand fly populations. Adler and Theodor (1935) stated that it was possible to distinguish between blood-fed and unfed females of the palearctic sand fly, P. perniciosis Newstead, by examining the accessory glands since granules never appeared in the accessory glands unless the female had had a blood meal. A few days after a small blood meal they were full of granules. They further stated that during egg laying most of the granules were passed out with the eggs, but some granules remained and could be seen in dissections. The presence of granules in the accessory glands of female P. perniciosus with an empty alimentary tract was the only morphological feature that distinguished parous females from newly hatched ones. They also stated that the granules were formed independent of copulation, depending only on a blood-meal in P. perniciosus.

Adler and Theodor (1957) referred to the indicative value of the accessory glands for sand flies in general. Garnham and Lewis (1959) noted high proportions of dissected sand flies with granules in the accessory glands and concluded that some of the flies might be nullipars secreting granules. They suggested studying the value of these organs as indicators of nulliparous flies in Belize. Lewis and Minter (1960) examined ovaries and accessory glands of some tropical African sand flies, in Kenya, and found that when the ovaries were small, residual secretions in the accessory glands were useful in

recognizing most parous females. Johnson and Hertig (1961) found that some Panamanian sand flies secreted accessory gland granules before taking blood and discharged them at various times afterwards. Adler and Mayrink (1961) observed dark brown fluid in the accessory glands of blood-fed Lu. longipalpis (Lutz and Neiva) and noted changes in glands of five other species. Johnson et al. (1963) found that in laboratory-reared Panamanian sand flies, granules may be present or absent without regard to parous or nulliparous condition of the female. They concluded that examination of the accessory glands does not aid in determining whether females of Panamanian sand flies are parous or nulliparous. Minter (1964) believed that the accessory glands were useful in estimating parous rates in Kenya. Lewis (1965) examined accessory glands of five species of Lutzomyia in Belize and concluded that the glands were unreliable for indicating whether or not a fly was parous. Chaniotis and Anderson (1968) studied laboratory-bred females of three California species and found no granules in accessory glands of 150 nullipars. Scorza et al. (1968), on the other hand, concluded that accessory gland granules were unreliable for recognizing parous females in Venezuela. Lewis et al. (1970) reconsidered a statement made earlier by Lewis (1965) regarding the indicative value of accessory glands by stating that the glands are quite useful for recognizing parous females of many Old World and some New World species. They reported that errors in interpretation are caused by irregular secretions, loss of secretion, the effect of blood-meals, and the parasite Monocystis (Ascocystis). Ward (1974) found that 82.05% of laboratory-reared Lu. longipalpis developed granules, but no eggs when kept for seven days without blood or sugar. He questioned the value of accessory glands in determining parity in

wild populations. Finally, Perkins (1982) believed that the examination of accessory glands for determining parity is open to question. He suggested, however, that the presence of accessory gland granules in apparently nulliparous Lu. shannoni females from Florida was evidence of a high probability of autogeny. The presence of accessory gland granules in nulliparous, anautogenous Lu. diabolica may tend to invalidate this suggestion.

Dissections of wild-caught and laboratory-bred female Lu. diabolica showed a wide range of variation in accessory gland condition. Based on presence or absence of accessory gland granules, little can be concluded from the dissections described in Fig. 2-20 except that those flies that died without developing ova and with no evidence of granules were probably nulliparous. Based on observations of laboratory-bred females, it would be virtually impossible to distinguish between 4-6 day old nullipars and uni or multipars that had voided the accessory glands during oviposition. One must conclude that examination of accessory glands as an indicator of parity in Lu. diabolica is unreliable at best.

Natural Parasite Infections

Young and Lewis (1977) compiled an extensive list of accounts in the literature of natural and laboratory infections of parasites in Psychodidae. In their review, no records of natural parasites were reported from Lu. diabolica. This study gives the first indication of several parasite infections in the species.

Flagellates. Keithly (1984, pers. comm.) believes that the small, rounded, highly-motile flagellates found swimming in the hemocoel of

50% of dissected Lu. diabolica are cilliated protozoans. She based this judgment on their rapid movement and sudden directional change. These parasites do not appear to have any adverse effect on the sand fly and are most likely true commensals. Lewis (1965) found probable ciliates in 10% of Lu. shannoni taken in resting collections in Panama. The decrease in infection rate from June 1982 (60%) to September (27%) remains unexplained. It is possibly due to changes in meteorological conditions or in seasonal changes in the availability of some food source from which the organism is acquired. Further studies on this parasite are needed to identify their interaction with Lu. diabolica.

It is possible that the small thin "flagellates" found in two females in the fall of 1983 were merely artifacts. Upon close re-examination of the stained microscope slide mounts, no such objects could be seen.

The flagellates found in the one female collected at Garner State Park during June of 1982 remain unidentified. A permanent slide mount of rather poor quality was made of these protozoans and is in the author's possession. Chaniotis and Anderson (1968) described similar flagellates, trypanosomes, in Lu. vexator (Fairchild and Hertig) collected at Capay, Yolo County, California. They reported a 20% infection rate in females and no infection in males. Other authors, too numerous to list here, have reported a variety of leptomonad and herpetomonad flagellates in different sand fly species.

Gregarines. Gregarine parasites have been reported from 24 sand fly species (Young and Lewis, 1977, 1980). These protozoans, belonging to the subphylum Apicomplexa, subclass Gregarina Oufour,

inhabit the digestive tract or body cavity of many invertebrates. Levine (1977) provided a taxonomic revision and checklist of the aseptate gregarine family Lecudinidae, which contains gregarine parasites of insects. Ascocystis chagasi is the only gregarine identified so far from New World sand flies (Scorza and Carnevali, 1981).

According to Levine (1977), sporozoites in oocysts (spores) infect the new host probably by being ingested. These enlarge, becoming gamonts, and attach to the intestinal or coelomic wall where they grow. Two gamonts become joined to one another in an association known as syzygy and encyst together to form a gametocyst. One gamont produces numerous male and the other numerous female gametes. Fertilization (by fusion) occurs, forming the oocyst. Within each oocyst form eight (rarely four) naked sporozoites. The oocysts or gametocysts are passed out into the environment and remain there until ingested by a new host.

Early instar larvae become infected by ingesting oocysts passed out in the feces of other larvae, or by eating oocysts adhering to eggs deposited by infected females (Coelho and Falcão, 1964). Liberated sporozoites penetrate the digestive tract and enter the hemocoel and later enter the abdominal cavity of the fly. Gametocysts full of oocysts are found attached to the accessory glands of females. Mature oocysts pass into the accessory gland material, and attach to the eggs deposited by the females.

Gregarine infection rates reported in sand fly species other than Lu. diabolica range from less than one to 26% (Young and Lewis, 1977).

Lien and Levine (1980) reviewed evidence that gregarines are host specific and that they will only develop to the oocyst stage in their

specific host. If this is true, the species of gregarine found in Lu. diabolica during the present study is undescribed, since it has not been reported by other workers.

Microsporidians. Natural infections with microsporidians have been reported in Lu. lainsoni (Fraiha and Ward), Lu. complexa (Mangabeira), and Lu. maripaensis (Flock and Abonnenc) in the New World, and in P. perniciosus in the Old World (Young and Lewis, 1977; Canning, 1977). Infection rates were about 1% or less (Young and Lewis, 1977).

Microsporidia are obligate intracellular parasites of invertebrates. Their life cycle comprises a period of asexual proliferation by binary fission (schizogony) and sporulation in which stages called sporonts undergo further division into sporoblasts, which transform into spores. The thick-walled spore contains a coiled polar filament, which under certain conditions is extruded and attaches to a host cell (Fig. 2-26). The sarcoplasm is then conveyed along the filament and invades the host cell (Canning, 1977). Some microsporidian species cause high mortalities in their hosts under laboratory conditions and show limited promise in biological control (Canning, 1977).

Mites. McConnell and Correa (1964) found mites of Ledermulleria spp. (Stigmaeidae) attached to the abdomen and thorax of several species of sand flies. Lewis and MacFarlane (1981) reported that mites of 14 families, particularly Stigmaeidae, and at least 16 genera and 21 species have been found on 39 species of sand flies. Infestation rates were 0.1-9% or more (Lewis and MacFarlane, 1981). According to these authors, mites may be phoretic, parasitic or both.

They added that scars left by mites may be harmful to the host and further suggested that identification of the mites and a knowledge of their habitat or mammalian host preference may provide information about the life cycles of the sand flies, which are difficult to study owing to their small size and nocturnal habits. The absence of these phoretic mites on sand flies collected in September 1983 may reflect a seasonal change in the population density or behavior of the mite, perhaps in response to cooler weather. It may also relate to a seasonal change in resting behavior of the sand fly, such as a move from where the mites are present to where they are not.

Other. Fungal and bacterial infections in wild-caught sand flies were reported by several authors (Young and Lewis, 1977, 1980). The significance of infections by these organisms is uncertain. Many may have resulted in premature death of the fly. Some infections may have been acquired as a result of retention in holding vials and chambers provided with apple slices and Karo® syrup.

CHAPTER 3
LABORATORY COLONIZATION OF *Lutzomyia diabolica* WITH NOTES ON ITS BIOLOGY IN THE LABORATORY (DIPTERA: PSYCHODIDAE)

Introduction

The importance of maintaining large laboratory colonies of sand flies was summarized by Safyanova (1964) as "necessary for the experimental study of their biology, behavior and mutual relations with disease agents and for the testing of new methods of vector control" (p. 573). These aspects, as well as genetic studies, were considered by the WHO Scientific Working Group on the Leishmaniases (WHO, 1977) as "neglected subjects of high priority" (p. 26). Inspite of recent advances in the laboratory colonization of vector species, few functional colonies exist, due to persistent problems of high larval mortality, death of females at oviposition, and intensive labor requirements (Killick-Kendrick, 1978).

Grassi (1907) was the first to rear a sand fly species, *Phlebotomus mascitti* Grassi, in the laboratory and Bayma (1923) cultured *Lutzomyia intermedia* (Lutz and Neiva) through one generation--the earliest recorded rearing of a New World sand fly species. Subsequent to these earliest efforts, fewer than 25 of the approximately 600 known sand fly species have been colonized in large numbers for more than ten generations (Ward, 1977; Killick-Kendrick, 1978; Young *et al.*, 1981; Endris *et al.*, 1982). Killick-Kendrick (1978) distinguished between rearing sand flies for only a few

generations, and establishing colonies that regularly produce enough flies for experimental work. Most colonies established since 1978 are in this second category, with studies on the biology of colonized flies just beginning (Killick-Kendrick, 1978).

The establishment of a productive colony of Lutzomyia diabolica was essential to studying its biology and vector potential for leishmaniasis. Lindquist (1936) collected adults of this species at Uvalde, Texas, recovered their eggs and reared the progeny through one generation, but did not establish a laboratory colony. Endris (1982), in connection with studies of the ecology of Rio Grande virus, established the first laboratory colony of Lu. diabolica from stock material obtained at Garner State Park, Texas. He described certain aspects of the biology of the species, but due to insufficient numbers, did not adequately study its life cycle and vector capacity. The colony survived for only seven generations. The objectives of the current study were

1. to establish a productive laboratory colony of Lu. diabolica; and

2. to study the biology and life history of the species under laboratory conditions.

Materials and Methods

General

Field sites and methods used for collecting wild Lu. diabolica with which to stock a laboratory colony were discussed in Chapter 2. The materials and methods used for handling, feeding, and rearing

Lu. diabolica are basically those described by Endris et al. (1982). Any departures from these techniques are explained under the subheadings below.

Immature Stages

Recovery and maintenance of eggs. Blood-fed females were kept in individual or group-oviposition/rearing vials with screen-covered lids until they deposited eggs and/or died. Most of the eggs were deposited on the moist plaster of Paris in the bottom of the vials, but occasionally a few were deposited on the plastic sides of the container. These were knocked off onto the surface of the plaster with a dissecting needle to prevent them from dessicating. Dead adults were removed from the vials to prevent mold growth. Water was added as needed to insure adequate relative humidtiy (RH) in the vials during incubation. The screen lids were replaced with solid plastic lids that had been perforated with a dissecting needle to allow for limited gas exchange. For the first three generations, incubated eggs were routinely maintained in a Hotpack® environmental chamber (Hotpack Inc., Philadelphia, PA) at 24 ± 1°C, 70 ± 10% RH, 16:8 LD photoperiod, but later rearing temperature was changed to 27 ± 1°C.

Maintenance of larvae and pupae. Individuals from a single egg batch were usually reared together in the same vial. Group-rearing chambers (120 ml) were also used to accomodate progeny of up to ten females. Containers for rearing individual larvae were made from 24-well (one larva/well) plastic tissue-culture trays with about 1/2 cm plaster of Paris in each well (Perkins, 1982). A solid Plexiglas®

cover, held in place with an elastic band, was used to prevent dessication of the larval food and movement of larvae from one well to another. Four-well tissue-culture trays with similar modifications and 7-dram oviposition/rearing vials were also used for individual rearing. The tissue culture trays were placed in Nalge® (19 x 16 x 4 cm) utility boxes (Nalge Co., Rochester, NY), to maintain constant RH.

When the eggs hatched, moist larval diet (Young et al., 1981) was sprinkled sparingly on the surface of the plaster of Paris in the bottom of each container. The containers were examined at least every other day to monitor immature development and to replenish food and moisture. Particular care was taken to insure that 1st-instar larvae did not dessicate from too little moisture or drown from too much. Less attention was required for later instars and pupae.

Slow larval development and excessive mortality, especially during the 1st stadium, prompted experiments aimed at accelerating the former and decreasing the latter parameter to enhance net productivity of the colony. The effects of different temperatures and of different larval diets were tested.

Rearing temperature experiments. The objectives of these experiments were to compare the effects of two environmental temperatures (24°C and 27°C) on immature development time and to ascertain the best temperature for rearing Lu. diabolica. Egg batches (less than 24-hrs-old) from laboratory-reared females were divided equally into two groups and the eggs were placed individually in wells of modified tissue-culture dishes (Perkins, 1982) or 7-dram oviposition/rearing containers. Eggs that were deposited on the sides of the vials, or that looked abnormal, were not used. A total of 295

eggs from 13 egg batches were set up in this manner and incubated at either 24 ± 1°C or at 27 ± 1°C.

Each vial was checked daily (AM) and the developmental progress of the immatures was recorded through adult emergence. The data were analyzed and compared statistically for significant differences using analysis of variance (ANOVA) procedures in conjunction with the student's t (z) test (Marks, 1982).

Larval diet experiments. These experiments were designed to evaluate the efficacy of various larval diets on development time and survival rate. Four larval diets, listed below, were compared with standard sand fly diet (Young et al., 1981).

A. Standard sand fly diet, ground fine and applied dry;

B. Standard sand fly diet mixed and incubated with liver powder in a ratio of 10:1, respectively, ground fine and applied dry;

C. Horn fly [Haematobia irritans (Linn.)] diet (Greer and Butler, 1973), consisting of sugar cane bagasse, dry mix (48 g wheat flour, 36 g fish meal, 6 g sodium bicarbonate, 20 g alfalfa meal), fresh cattle manure and tap water mixed in an approximate ratio of 1:1:5:5 by weight respectively, incubated at 27°C for 45 days, ground fine and applied dry;

D. Horn fly diet, unincubated, ground fine and applied dry; and

E. Standard sand fly diet, coarsely ground and applied moist.

Diets A, B, C, and D were ground in a household coffee mill (Krups type 280, Robert Krups, North America, Allandale, NJ) and sifted through a 40-mesh soil sieve. The life-cycle parameters of larvae fed on these test diets were compared with those of individuals fed on diet E, which was the same diet used in all previous colony

generations. Diet E was ground in a hand-operated bread crumber (L., F. & G., New Brittain, CN) and sifted through an 18-mesh wire screen.

A total of 784 eggs from 22 batches of the 12th colony generation were set up in the same manner as in the rearing-temperature experiments, except that all were held at 27 ± 1°C and 87 ± 5% RH. Each vial was checked daily (AM) and the developmental progress of the immature stages was recorded through adult emergence. The diets were applied with small shakers fashioned from 10-dram plastic medicine vials fitted with screen (18-mesh bridal veil) lids. Data were statistically analyzed and compared using ANOVA procedures in conjunction with Duncan's multiple range test (Marks, 1982).

Diapause and quiescence. To investigate the possibility of winter diapause or quiescence in the immature stages, 249 gravid laboratory-reared (27°C, 70% RH, 16:8 LD photoperiod) females, held in individual vials, were placed in an outdoor, screen cage at one to five-day intervals from May to early December during 1982 and 1983. The cage was situated in a heavily shaded area adjacent to the east side of the laboratory building. It received virtually no direct sunlight but was subject to ambient factors such as wind, rain, temperature and photoperiod. The vials were kept in a tray that was enveloped in a plastic bag to maintain constant RH. The tray rested about 30 cm off the ground on a pedestal of concrete blocks. It was shielded from the rain by a metal cover. The females were allowed to oviposit and/or die, then were removed. The eggs were monitored daily for hatch; in some instances for nearly 300 days. Hatchlings were also monitored daily for signs of diapause development. Daily climatological data were obtained from the Agronomy Department-NOAA weather station, University of Florida, Gainesville, FL.

Adults

Maintenance. Newly emerged adults were released into a mating chamber (Endris et al., 1982) supplied with apple slices or wedges leaned against the back wall or placed on the floor of the chamber. A 100-ml bottle, filled with tap water and plugged with a sponge wick, was placed in a corner of the chamber to help maintain the RH at or above 80%. The mating chamber was kept in an environmental cabinet at 27 ± 1°C, 70 ± 10% RH, and 16:8 LD photoperiod.

Females were usually held in the chamber for 48 to 72 hours, to allow time for mating before being offered a blood meal by one of the following methods:

1. Females were captured with a tube aspirator and transfered to 120-ml oviposition/rearing vials provided with screen feeding lids (Endris et al., 1982). The exterior surface of the feeding lid was held against the host's skin until the flies fed to repletion (Fig. 3-1).

2. A mouse or hamster, anesthetized (IM) with ketamine chloride (0.07 to 0.01 cc for mice; 0.18 to 0.20 cc for hamsters), was placed in the mating chamber for up to an hour at a time.

Hosts used for blood feeding laboratory-reared females also included human, dog and rabbit. Duration of individual feedings was timed by a stop watch.

For the first two generations, males were placed with blood-fed females in group or individual oviposition/rearing vials. In subsequent generations, replete females were released into the mating chamber with males for another 24-hr period before being placed, without males, in group or individual containers. A drop of Karo®

Figure 3-1. Bloodfeeding female sand flies, Lutzomyia diabolica.

syrup and water (1:1) was placed daily on the screen lid of each vial, as an energy source.

During the first five colony generations, the screen-lid vials containing gravid females were kept in an open tray inside the environmental chamber. Because the air inside the chamber was continuously circulated by a fan, the plaster in the vials dried out within one or two days, resulting in an unsatisfactory surface for oviposition. To prevent this excessive drying in subsequent generations, moist paper towels were placed in the bottom of the open tray and the entire tray was enveloped in a clear plastic bag. This innovation maintained the RH in the holding environment for gravid females at or near 100%.

About 10% of the females that survived oviposition were offered a second blood meal to see if they would complete a second gonotrophic cycle.

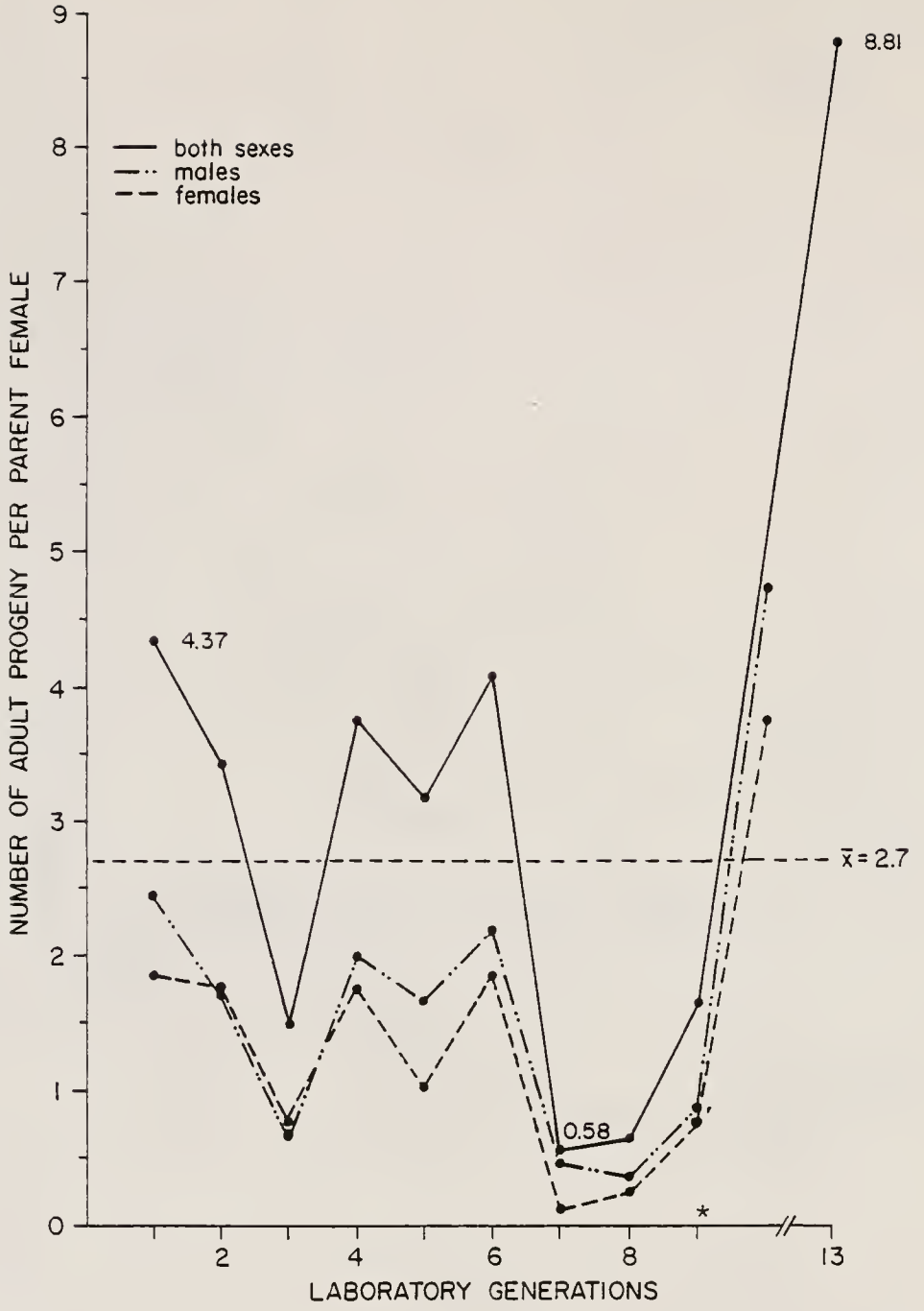
Longevity experiments. Newly emerged individuals (14th generation) of each sex, from vials in which no adults of the opposite sex had eclosed, were held in clean oviposition/rearing vials at 27°C and near 100% RH until death. A drop of Karo® syrup, placed daily on the surface of each screen lid, provided the sole source of nutrient.

Autogeny experiment. Thirty newly emerged, 14th-generation females were paired with newly emerged males in 7-dram oviposition/rearing vials and held at 27°C and near 100% RH until death. They were checked daily for signs of autogenous egg production. Each day a drop of Karo® syrup was placed on the screen lid of each vial as the sole source of nutrient. The females were dissected post mortem to determine if mature, undeposited eggs had developed.

Results

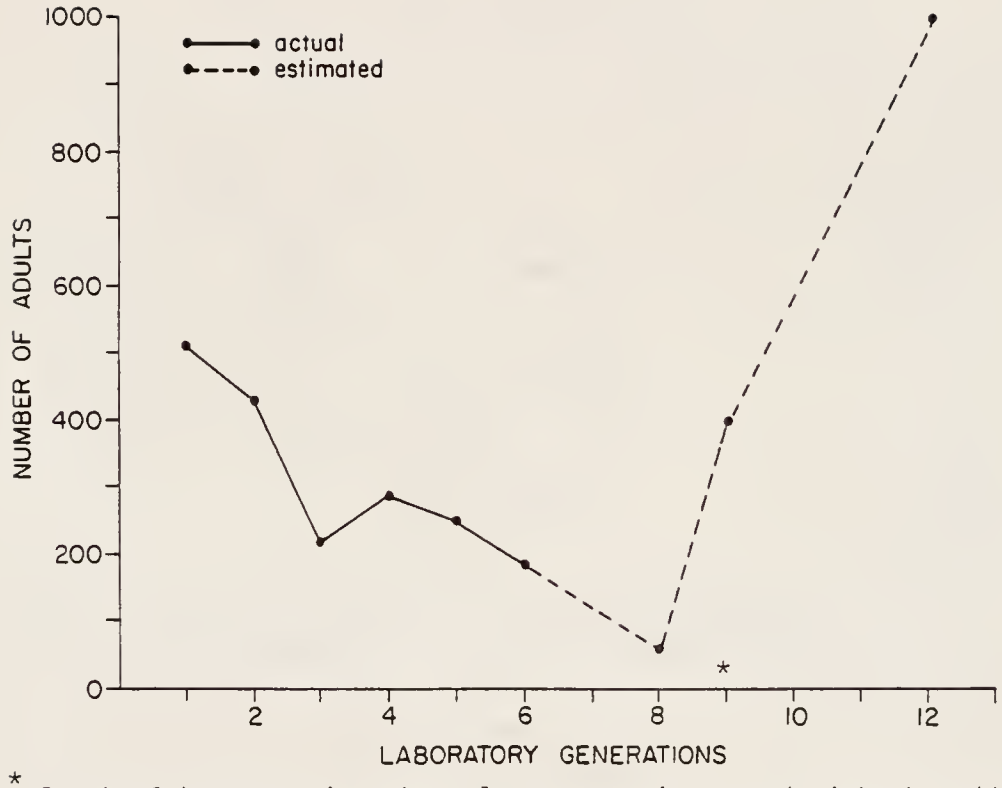
General Observations

A laboratory colony of Lu. diabolica was established from 164 wild-caught females that laid 6012 eggs. From these, 262 male and 252 female 1st-generation progeny were successfully reared. During the first six generations (from July of 1982 through June of 1983) 1694 flies were reared from 14,407 eggs laid by 524 females. Productivity through the first nine generations was rather low, with a mean number of adult progeny per female of only 2.7 (1.4 males and 1.3 females) (Fig. 3-2). During the 7th and 8th generations, critically low numbers of adults were produced, with fewer than one adult per parent female emerging. Whereas the number of adults emerging during the 1st generation was 514, by the 8th only about 60 emerged (Fig. 3-3). In the 9th generation, the colony was rejuvenated with an additional 76 Lu. diabolica egg batches recovered from wild-caught females collected in September 1983 from the same sites as the original colony stock (Chap. 2). The new material was assummed to be representative of the same genetic source from which the original colony stock was derived. This infusion, combined with changes in handling procedures and rearing conditions in the 9th and subsequent generations, increased adult numbers to roughly 1,000 individuals in the 12th generation (Fig. 3-3). From the 8th to the 13th generation, productivity increased steadily from an average of less than one adult progeny per female to nearly nine (Fig. 3-2). In the 14th generation it was necessary to provide a second mating chamber to accomodate the burgeoning adult population.



* In the 9th generation the colony was rejuvenated with the addition of 76 egg batches from wild-caught females.

Figure 3-2. Number of adult progeny per parent female of Lutzomyia diabolica reared during the first nine generations of a laboratory colony.



* In the 9th generation the colony was rejuvenated with the addition of 76 egg batches from wild-caught females.

Figure 3-3. Total number of Lutzomyia diabolica adults per generation in a laboratory colony.

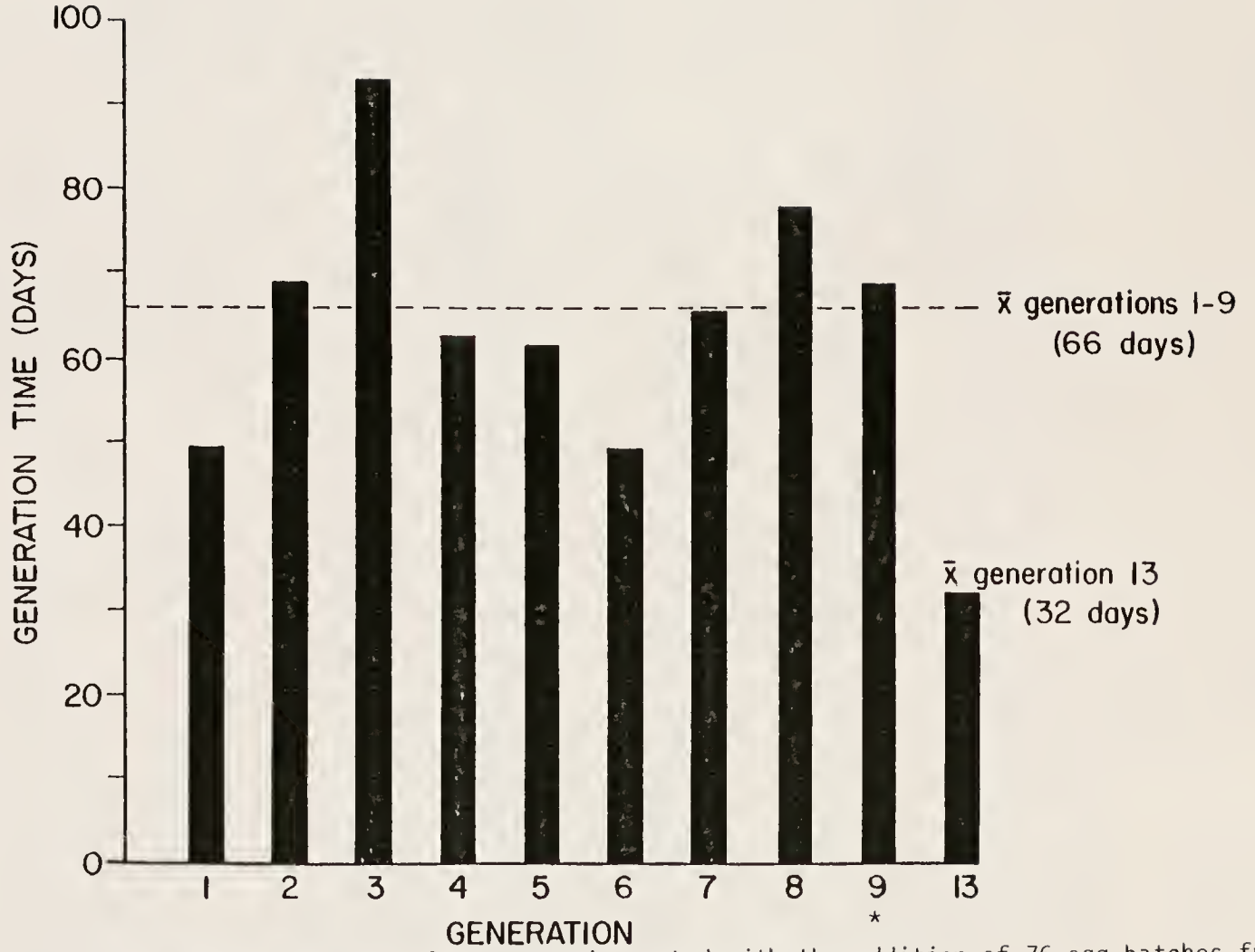
The time of development from engorgement to first emergence of adults of the succeeding generation (generation time) averaged 66 days for generations 1 through 9, with a range of 49 to 92 days (Fig. 3-4). With changes in handling procedures and rearing conditions, and possibly some genetic adaptation of the colony, the mean generation time dropped to about 32 days in the 13th generation.

Immature Stages

Eggs. The eggs of Lu. diabolica have a characteristic morphology which distinguishes them from other Lutzomyia eggs (Endris, 1982). At oviposition they are white to gray in color, but turn shiny dark-brown or black within a few hours. They are banana-shaped and measure about 0.35 x 0.1 mm. The thick shells retain their shape after hatching, often with little more than a faint slit to betray the first instar's emergence.

Table 3-1 summarizes fecundity and hatching observed in generations 1 through 9 and 13. Egg batches ranged in size from one to 76, with a mean batch size of about 27 eggs. One wild-caught female laid 84 eggs and one F_{10} female laid 88 eggs. Neither of these was included in the table. The largest mean number of eggs per batch in a given generation was 36.1 (5th generation) and the smallest was 24.9 (3rd generation).

Incubation time ranged from 5 to 113 days in the laboratory, with an overall mean of 8 days. First generation eggs hatched in 1-1/2 to 3 days less time than those of the 2nd and 3rd generations. At the beginning of the 4th generation the colony rearing temperature was raised from 24°C to 27°C, resulting in an average reduction in



*In the 9th generation the colony was rejuvenated with the addition of 76 egg batches from wild-caught females.

Figure 3-4. Generation times for generations 1 through 9 and 13 in a laboratory colony of Lutzomyia diabolica maintained at 24 ± 1°C and 70 ± 10% RH for the first three generations and 27 ± 1°C and 70% RH for all subsequent generations.

Table 3-1. Summary of fecundity, incubation time, and hatching for generations 1 through 9 and 13 in a laboratory colony of Lutzomyia diabolica maintained at 24 ± 1°C and 70 ± 10% RH for the first three generations and 27 ± 1°C and 70 ± 10% RH for all subsequent generations.

Generation	Number of Egg Batches Observed	Eggs per Batch $\bar{x}$±1SD (range)	Incubation Time in Days $\bar{x}$±1SD (range)	Fertile Egg Batches # (%)	Hatch Per Fertile Egg Batch: Number Hatch $\bar{x}$±1SD (range)	Hatch Per Fertile Egg Batch: Percent Hatch % (range)
1	87	26.9±13.9(2-59)	9.2± 3.3(7-26)[1]	79 (91)	13.1±10.1(1-41)	50.4 (2-100)
2	95	26.9±15.3(1-75)	10.8± 7.6(7-66)	69 (73)	9.2±10.7(1-42)	38.4 (2-100)
3	82	24.9±15.3(2-55)	12.0±12.8(7-113)	67 (82)	10.3±10.1(1-40)	45.7 (3-100)
4	64	26.6±14.5(4-57)	9.1± 1.6(7-16)[2]	47 (73)	10.6±11.1(1-52)	49.8 (5-100)
5	72	36.1±17.9(1-76)	8.5± 1.6(5-14)	58 (81)	13.6±11.8(1-36)	51.3 (2-100)
6	36	25.8±14.3(1-60)	8.2± 1.4(6-12)	27 (75)	9.5± 9.6(1-41)	37.2 (3-95)
7	29	32.3±18.1(1-65)	7.8± 1.0(7-10)	8 (28)	4.7± 9.6(7-38)	28.6 (16-100)
8	77	28.9±12.2(1-53)	7.7± 1.1(5-12)	35 (45)	5.4± 8.1(1-36)	18.5 (5-85)
9[3]	24	26.5±12.4(1-62)	8.4± 3.8(6-22)	15 (63)	8.8± 8.5(2-30)	30.1 (6-97)
13	32	28.5± 8.8(6-42)	8.7± 3.8(6-26)	28 (88)[4]	11.2± 9.8(1-35)	36.3 (1-100)
Overall	595	26.9 -- (1-76)	8.2 -- (5-113)	455 (76)	8.0 -- (1-52)	28.9 (1-100)

1. Parent generation = wild-caught females; egg batches incubated under near ambient field temperatures; significantly shorter incubation time than generations 2 and 3 (ANOVA, PR > F = 0.0001; Duncan's multiple range test, α = 0.05).
2. Colony rearing temperature in 4th generation raised from 24°C to 27°C; significant decrease in incubation time as compared to generations 2 and 3 (ANOVA, PR > F = 0.0001; Duncan's multiple range test, α = 0.05).
3. Colony rejuvenated in 9th generation by addition of 76 egg batches from wild-caught females.
4. Significantly higher percentage of fertile egg batches than in all preceding generations except 1st (ANOVA, PR > F = 0.0001; Duncan's multiple range test, α = 0.05).

incubation time of at least 3 days. Incubation times determined at 24°C and 27°C for individual eggs are given in Table 3-2. A highly significant decrease in incubation time of nearly two days was observed from the lower to the higher temperature (ANOVA procedures, PR>F = 0.0001; student's t (z) test, a = 0.01).

Of a total of 595 egg batches, 455 (76%) were fertile (had at least one egg hatch) (Table 3-1). The percent fertile egg batches ranged from a high of 91% in the 1st generation (progeny of wild-caught females) to a low of 28% in the 7th. By the 13th generation, the percent fertile egg batches had recovered to 88%, only slightly less than that observed in egg batches from wild-caught females. Hatching within fertile batches was roughly 40 to 50% for the first five generations, then declined to 18.5% in the 8th. A reversal of this trend was observed in subsequent generations, and by the 13th generation, the percent hatch had doubled from the corresponding 8th generation figure. The estimated hatch for the 16th generation was around 50%. The percent hatch for individual eggs at 24°C and 27°C was nearly the same at both temperatures (Table 3-2).

Larvae and pupae. There are four larval instars of Lu. diabolica, each of which can be distinguished with the aid of a stereo dissecting microscope (Fig. 3-5). Newly hatched, 1st instars are about 0.5 mm long and 0.1 mm wide and grow to about twice their original length and two to three times their original width before molting. They are characterized by a conspicuous egg burster on the vertex of the head capsule, and two long caudal bristles on the last abdominal segment. Lateral and dorsal segmental setae are small and inconspicuous. Prior to molting, the larva (all instars) becomes

Table 3-2. Comparison of life-cycle parameters of immature Lutzomyia diabolica reared at 24°C and 27°C. (3rd colony generation).

	Rearing Temperature			
	24°C		27°C	
Life Stage	Duration in Days $\bar{x}$±1SD(range)	Surviving[1] n/146 (%)	Duration in Days $\bar{x}$±1SD(range)	Surviving[1] n/149 (%)
Egg[2]	10.2±2.7(6-33)	82/146 (56)	8.4±0.8(7-10)	79/149 (53)
1st Stadium[2]	8.2±2.6(4-22)	70/146 (48)	6.9±1.4(4-12)	71/149 (48)
2nd Stadium[2]	6.3±1.5(3-11)	67/146 (46)	4.8±1.2(2-8)	70/149 (47)
3rd Stadium[2]	6.2±1.8(3-16)	64/146 (44)	4.8±1.2(5-13)	70/149 (47)
4th Stadium[2]	10.9±2.7(5-23)	64/146 (44)	8.2±1.5(5-14)	70/149 (47)
Larva[2] (1st+2nd+3rd+4th)	31.4±5.2(21-49)	64/146 (44)	24.7±2.8(18-30)	69/149 (46)
Pupa[2]	9.3±0.8(8-11)	64/146 (44)	7.8±1.8(6-26)	69/149 (46)
Immature[2] (All Stages)	50.8±5.2(37-69)	64/146 (44)	40.8±3.4(34-54)	69/149 (46)

1 = Number of individuals alive at end of life stage.

2 = Significant difference in duration between temperatures (ANOVA, PR > F = 0.0001; Student's t(z) test, α = 0.01).

somewhat inflated, causing the skin to stretch and take on a shiny appearance. Molting is always preceded by evacuation of the gut contents and cessation of movement. At the initiation of the molt, the old skin, which is glued posteriorly to the substrate by the excretion (secretion) of a substance, splits in the region of the head capsule and the new instar pulls out of it by anteriorly directed peristaltic movements. The egg burster is absent on the 2nd instar, having been shed with the old skin, and four, instead of two, caudal bristles are present. Slender lateral and dorsal segmental setae are clearly visible. The 2nd instar grows to about twice the maximum length of the 1st before molting. The 3rd instar also has four long caudal bristles but lateral and dorsal segmental setae are stouter and conspicuously more spatulate than on the 2nd instar. It grows to about twice the maximum length of the 2nd instar before molting. The 4th instar is robust and easily distinguishable from the others with the unaided eye. It has four long caudal bristles; stout, spatulate lateral and dorsal segmental setae; and, unlike the other instars, bears a heavily sclerotized, saddle-shaped anal plate. Its maximum dimensions just prior to pupation are about 3.2 mm long and 0.6 mm wide.

Larvae required a moist diet medium, not saturated and not too dry. When it was too moist, larvae were observed climbing the sides of the vial to escape the moisture; when it was too dry, development was retarded. If the plaster in the bottoms of the rearing vials was saturated with water at the time of oviposition, replenishment of moisture was usually not necessary until the 4th instar.

Larvae fed on the surface of the medium, clearing tiny furrows; rarely did they burrow under the food. Individuals approaching a colony of mold remained in one spot for several hours while sweeping the anterior half of their bodies from side to side, eating a semicircular swath. They exhibited gregarious habits, and in vials containing only a few larvae, would usually all be found together. Groups of larvae kept the medium churned and loose, and mold growth minimal. In single rearings, the diet medium often became packed and moldy.

Experiments using the modified 24 and 4-well tissue-culture trays for individual rearing were sometimes complicated by migrations of wandering larvae from one well to another, in spite of tight-fitting covers designed to preclude this. Seven-dram rearing vials proved to be better containers for individual rearing because the larvae could not migrate from one to the other. Also, high RH was more easily maintained due to the larger volume of plaster in the bottom of each vial.

Just prior to pupation, 4th instar larvae moved to the periphery of the medium where they attached their posterior ends to the side of the vial; they rarely attached to the surface of the food material. At the onset of pupation, the body swelled anteriorly and the old larval skin split. The pupa emerged, still attached to the substrate and with the larval skin retained at its posterior end. Newly formed pupae were whitish in color and soon turned golden brown. About 24 to 36 hours prior to adult eclosion, the pupa turned dark with the formation of eyes, wings, legs and antennae. Pupae were able to survive under much dryer conditions than larvae.

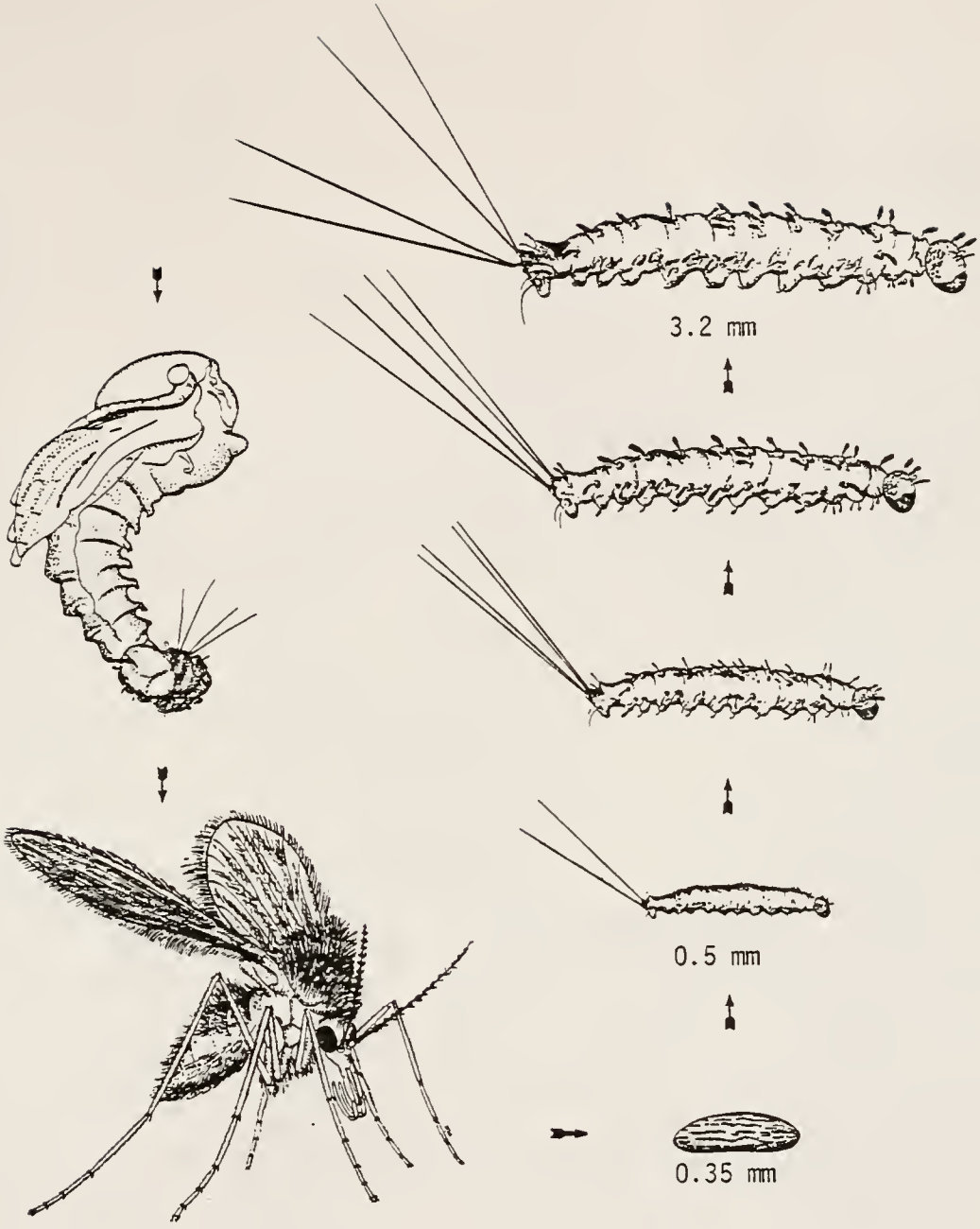


Figure 3-5. Life cycle of the phlebotomine sand fly Lutzomyia diabolica (Hall). Not drawn to scale. Artwork by Margo Duncan and Hilda Muñoz.

Development. The overall mean durations of larval and pupal development periods for all generations was 35.9 and 8.4 days respectively (Table 3-3). Statistically significant decreases in development times between generations were brought about by an increase in rearing temperature (24°C to 27°C between the 3rd and 4th generations) and by improvements in larval diet (13th generation).

Mean development times for individually reared Lu. diabolica were significantly shorter for each immature life stage at 27°C than at 24°C (Table 3-2). Total immature development time was, on the average, ten days shorter at the higher temperature than at the lower one. At both temperatures, the order of immature stages from longest to shortest duration was egg, 4th stadium, pupa, 1st stadium, 2nd stadium and 3rd stadium. The 2nd and 3rd stadia were of nearly equal duration.

Survivorship curves for immatures reared at the two temperatures are presented in Figure 3-6. Highest mortality occurred in the egg stage (46% at 24 C; 44% at 27 C), followed by the 1st stadium (12% at 24 C; 10% at 27 C). Mortality beyond the 1st instar at both temperatures was rather low.

Table 3-4 and Figure 3-7 compare immature development times of F_3 males and females at 24°C and 27°C, respectively. Males developed two days faster than females at 24°C and one day faster at 27°C. The duration of the 4th stadium was significantly greater in females than in males, at both temperatures (student's t (z) test, a = 0.01).

Twelfth-generation larvae were reared individually at 27°C on five larval diets (Table 3-5). The shortest development times were observed under diet regimen C (incubated horn fly diet, finely ground,

Table 3-3. Duration of larval and pupal stages in generations 1 thru 9 and 13 of a laboratory colony of Lutzomyia diabolica maintained at 24 ± 1°C and 70 ± 10%RH for the first three generations and 27 ± 1°C and 70 ± 10%RH for all subsequent generations.

Generation	Number of Broods	Larval Stage in Days (1st thru 4th instar) $\bar{x}$± SD (range)	Pupal Stage in Days $\bar{x}$± SD (range)
1	60	27.7± 6.4(18-50)	8.2±2.3 (5-22)
2	55	37.7±24.4(17-163)[1]	9.5±8.1 (5-67)
3	45	49.4±29.3(23-151)	10.2±4.3 (4-28)
4	40	31.9± 6.5(21-47)[1]	8.0±2.4 (3-16)[1]
5	42	31.5± 7.4(23-49)	7.1±2.2 (4-14)
6	23	21.3± 4.4(15-33)	6.7±1.1 (5-8)
7	6	38.8±20.7(20-65)	9.0±5.6 (6-19)
8	27	53.4± 8.9(35-71)	8.0±2.5 (4-20)
9[2]	13	45.1± 9.4(35-72)	7.6±4.0 (4-20)
13	25	17.3± 2.3(14-23)[1]	6.2±0.51(5-7)[1]
Overall	336	35.9 (14-163)	8.4 (3-67)

1 = Significantly different from preceding value (ANOVA procedures, PR > F = 0.0001; Duncan's multiple range test, α = 0.05).

2 = In the 9th generation the colony was rejuvenated by the addition of 76 egg batches from wild-caught females.

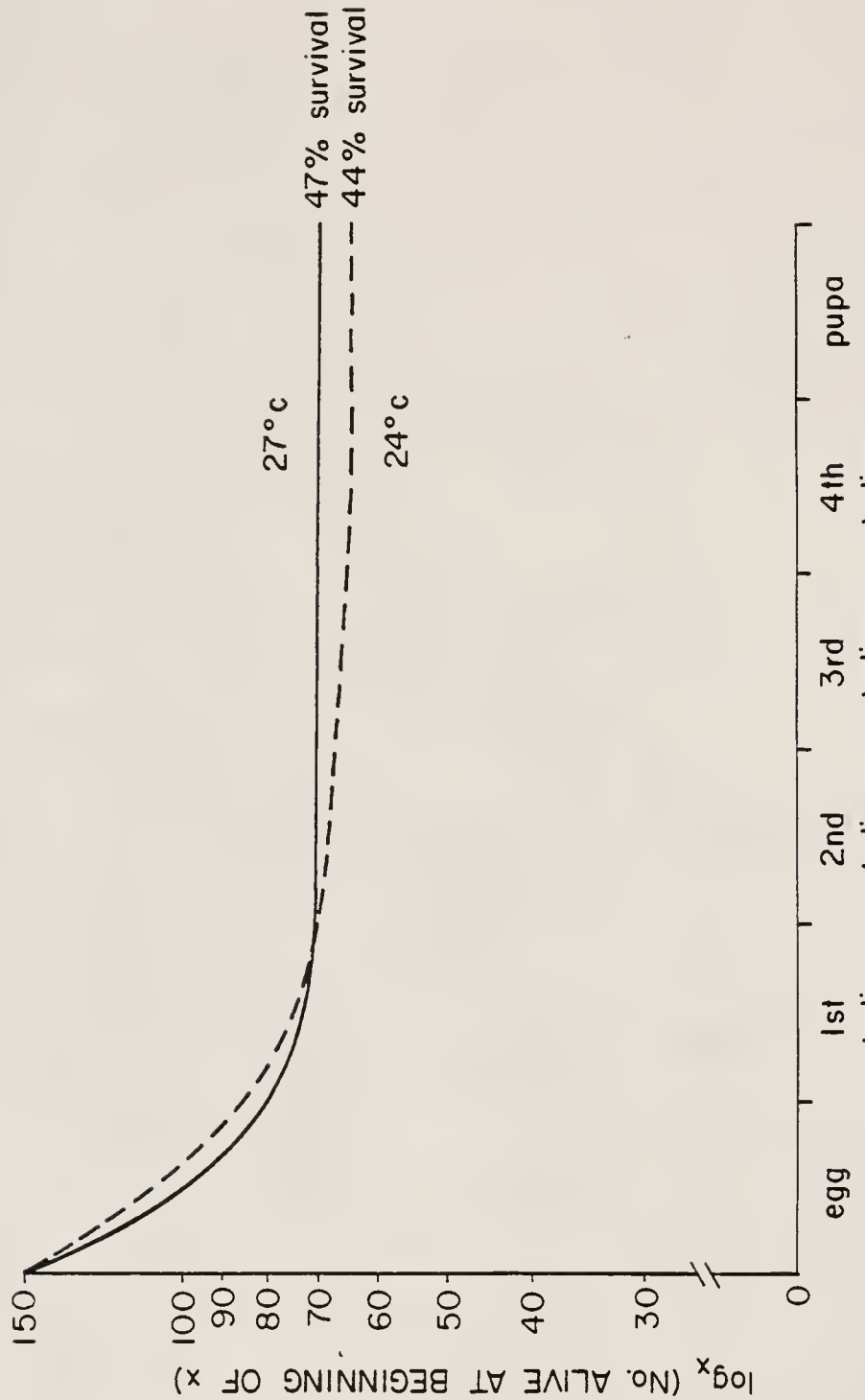


Figure 3-6. Survivorship curves for immature Lutzomyia diabolica reared at 24 ± 1 °C and at 27 ± 1°C (RH for both groups was 90%).

Table 3-4. Comparison of immature development times for male and female Lutzomyia diabolica reared individually to adult stage at 24 and 27°C (3rd colony generation).

	Sex	
	Males	Females
Life Stage	Duration in Days $\bar{x}$±1SD(range)	Duration in Days $\bar{x}$±1SD(range)
	24°C	
	n = 30	n = 20
Egg	10.5±0.9 --	10.4±0.8 --
1st Stadium	7.8±2.1 --	7.5±1.4 --
2nd Stadium	6.0±1.1 --	6.0±1.4 --
3rd Stadium	5.5±1.2 --	6.3±1.2 --
4th Stadium[1]	9.6±1.3 --	11.3±1.4 --
Pupa	9.2±0.7 --	9.4±0.8 --
Egg to Adult[2]	48.9±3.3(44-57)	50.8±3.4(47-62)
	27°C	
	n = 31	n = 38
Egg	8.3±0.7 --	8.5±0.8 --
1st Stadium	7.1±1.5 --	6.6±0.9 --
2nd Stadium	4.7±1.2 --	4.7±1.1 --
3rd Stadium	4.7±1.6 --	4.8±1.2 --
4th Stadium[1]	7.7±1.2 --	8.6±1.7 --
Pupa	7.6±0.7 --	7.9±0.5 --
Egg to Adult[2]	40.4±3.4(34-46)	41.2±2.5(34-46)

1. Significant difference in duration between males and females at both temperatures (Student's t test, α = 0.01).

2. Significant difference between flies reared at 24°C and 27°C (Student's t test, α = 0.01).

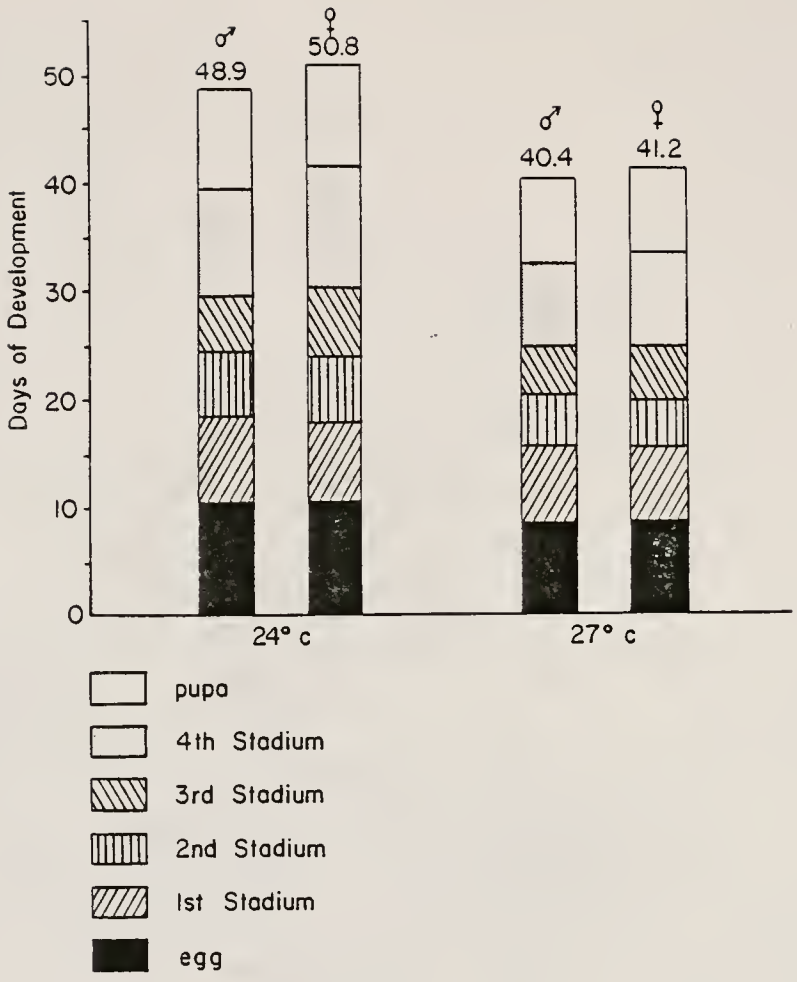


Figure 3-7. Graphic comparison of immature development times for male and female Lutzomyia diabolica reared individually to the adult stage at 24 and 27° C (3rd colony generation).

applied dry), followed in ascending order by D (unincubated horn fly diet, finely ground, applied dry), A (standard sand fly diet, finely ground, applied dry), B (standard sand fly diet incubated with liver powder, finely ground, applied dry), and E (standard sand fly diet, coarsely ground, applied moist). Development times under C and D were not significantly different from each other but were 48 to 52% shorter than under A and B, and 76 to 82% shorter than under E. There were no significant differences in development times observed between diets A and B; both produced shorter times than diet E. Figure 3-8 shows the development times of immature stages on each diet regimen. On diets A, B, and E, the 4th stadium was longer than either of the nonfeeding stages (egg and pupa). On diets C and D, the 4th stadium was slightly shorter than either nonfeeding stage. Very little difference was observed in duration of egg and pupal stages under diets A through D. Unexpectedly, the durations of the nonfeeding egg and pupal stages, as well as each feeding stage, were significantly longer under diet E. Under this regimen, the 4th stadium exceeded both nonfeeding stages and the pupal stage exceeded the egg stage. Percent survival to the adult stage varied from 30% on diet E, to 94% on diet D (Table 3-5).

Diapause and quiescence. Most eggs in batches laid within a two-day period hatched in five to ten days regardless of generation. In at least 3% of the cultures, when the majority of larvae were in the 4th stadium, already pupae or adults, newly hatched 1st-instar larvae were also observed, at least 30 days after oviposition. Delayed hatch in one laboratory culture was observed 113 days after oviposition. This was usually, but not exclusively, observed under adverse culture

Table 3-5. Comparison of life cycle parameters of sand flies (Lutzomyia diabolica) reared on 5 larval diets at 27 ± 1°C and 87 ± 5% RH (12th colony generation).

	Diet Regimens[1,2]				
Life Cycle Parameter	A n = 142	B n = 142	C n = 142	D n = 142	E n = 216
Number Eggs Hatching	94 = 66%	91 = 64%	90 = 63%	80 = 56%	151 = 70%
	Days $\bar{x}$±1SD(range)	Days $\bar{x}$±1SD(range)	Days $\bar{x}$±1SD(range)	Days $\bar{x}$±1SD(range)	Days $\bar{x}$±1SD(range)
Incubation Time	7.6±2.9(5-27)	7.4±4.5(5-49)	7.4±1.7(5-14)	7.5±2.6(5-24)	8.5±2.8(5-17)
Duration Stadium 1	5.8±2.0(3-17)	5.5±1.5(3-9)	4.7±0.9(3-7)	4.7±1.2(2-9)	10.2±3.0(3-17)
Duration Stadium 2	3.8±1.2(2-7)	4.2±1.5(2-10)	3.2±0.9(1-6)	3.5±1.2(2-9)	7.1±3.0(3-19)
Duration Stadium 3	4.7±1.4(2-11)	5.3±1.7(2-11)	3.6±1.3(1-10)	3.8±1.4(1-10)	8.2±3.2(4-25)
Duration Stadium 4	9.3±1.8(5-13)	10.1±3.0(5-23)	6.6±1.3(4-10)	6.5±1.6(3-13)	12.8±2.6(5-19)
Larval Development Time	23.9±4.6(18-58)	25.2±5.8(16-46)	18.1±2.3(14-26)	18.5±3.8(14-37)	37.2±9.0(21-75)
Duration of Pupal Stage	6.6±0.8(5-10)	6.9±1.7(5-17)	6.7±0.9(4-12)	6.9±1.5(4-16)	12.4±3.5(4-22)
Total Immature Development Time	38.9±6.3(31-64)	40.1±9.0(30-80)	32.5±3.7(27-46)	33.7±2.3(26-66)	59.2±13.3(33-95)
Number Surviving to Adult	73 = 78%	51 = 56%	73 = 81%	75 = 94%	65 = 30%

1. Diet regimens: A = standard larval diet, finely ground, applied dry; B = standard larval diet, incubated with liver powder, finely ground, applied dry; C = incubated horn fly diet, finely ground, applied dry; D = unincubated horn fly diet, finely ground, applied dry; E = standard larval diet, coarsely ground, applied moist.
2. A and B are significantly different from C and D, but not from each other; C and D are not significantly different from each other; E is significantly different from all other sets (ANOVA, PR > F = 0.0001; Duncan's multiple range test, α = 0.05).

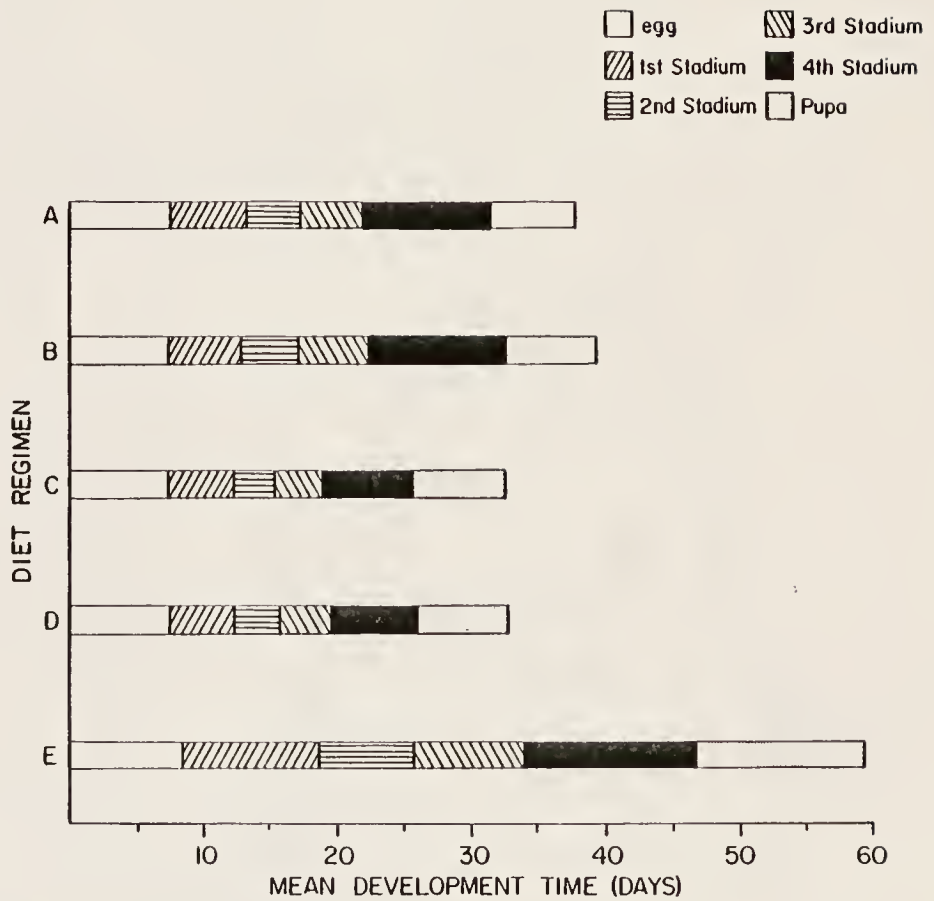


Figure 3-8. Graphic comparison of immature development times for Lutzomyia diabolica reared on five diet regimens: A = standard sand fly diet, ground fine, applied dry; B = standard sand fly diet with liver powder, ground fine, applied dry; C = incubated horn fly diet, ground fine, applied dry; D = unincubated horn fly diet, ground fine, applied dry; E = standard sand fly diet, ground coarse, applied moist (12th laboratory generation). Rearing conditions = 27°C and 70% RH.

conditions, such as too much or too little moisture, or lowered atmospheric temperature. In some cultures in which conditions appeared to be optimal the same phenomenon was observed. The actual frequency of this sort of quiescence was not determined, since, for the sake of efficiency, unhatched egg batches were routinely discarded after 30 days.

Of 249 gravid females placed in the outside colony, 119 (48%) deposited eggs. The latest date of ovoposition was 16 December. Of the 119 egg batches, 73 (61%) were fertile (had at least one egg hatch), and of those, 33 (45%) showed evidence of diapause. Egg batches contained either 100% fast-developing eggs (nondiapause), 100% slow-developing eggs (diapause), or a mixture of both types, depending on the time of year they were laid (Fig. 3-9). In June and July, all egg batches contained exclusively nondiapause eggs and hatched within 5 to 18 days after oviposition. In a batch of 27 eggs laid on the 27th of August, 1982, 9 (33%) hatched within 7 days (5 September, 1982), one (4%) hatched in 93 days (4 December, 1982), and 4 (15%) hatched in 270 days (26 May, 1983). With the exception of this batch, no diapause eggs were laid before 16 October (18 October, 1982, and 16 October, 1983). By November, all egg batches contained at least some diapause eggs and by December, 100% were diapause eggs. At the top of Figure 3-9 are listed the average maximum and minimum temperatures for the months of interest. Duration of the egg stages as a function of month of oviposition is depicted in Figure 3-10. A range of duration of 90 to 270 days was observed for diapause eggs as compared with 5 to 27 days for nondiapause eggs. Mean duration of diapause egg stages was 160 days, compared to about ten days for nondiapause eggs. A few

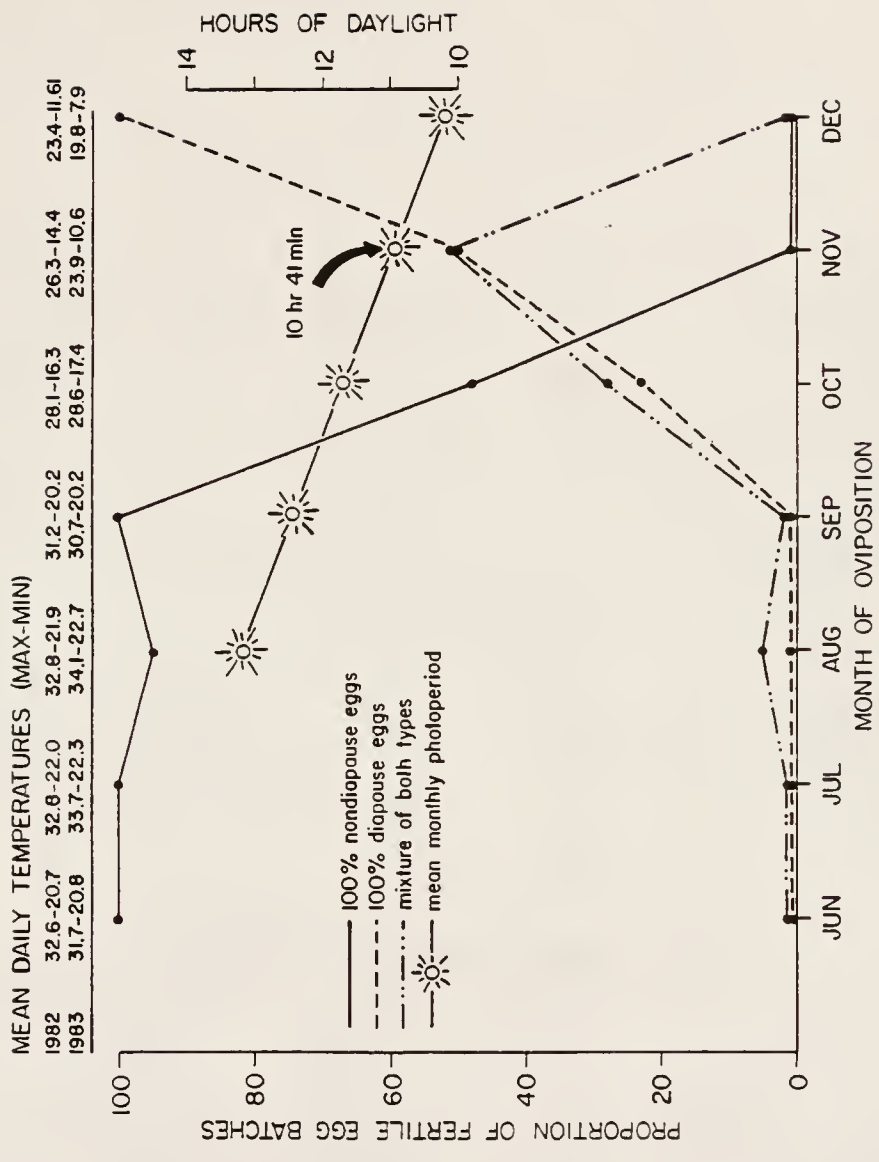


Figure 3-9. Proportion of diapausing eggs in batches laid outdoors by Lutzomyia diabolica females between June and December at Gainesville, Florida (29°39.6' N).

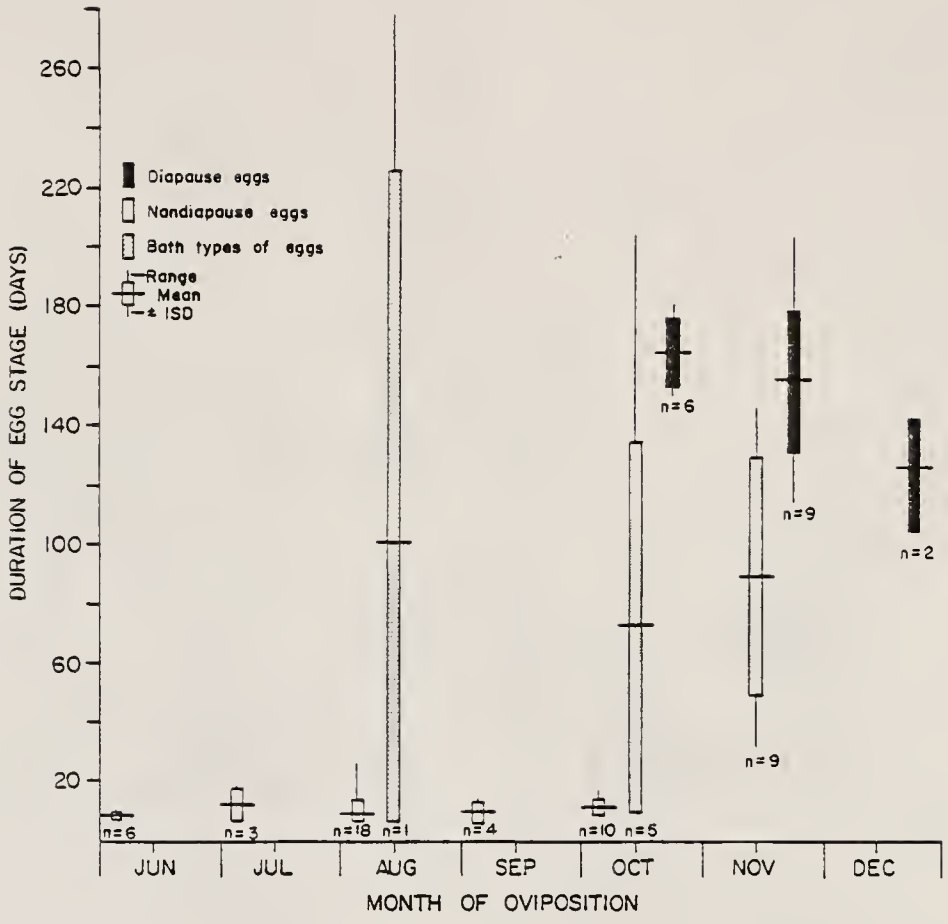


Figure 3-10. Duration of the egg stage in outdoor-reared Lutzomyia diabolica according to month of oviposition at Gainesville, Florida (29°39.6' N).

egg batches showed three hatching peaks, the first after 8 to 10 days, the second after 30 to 60 days, and the third after about 160 days. Figure 3-11 shows the development of 33 diapausing egg batches from time of oviposition to adult emergence. Adults of the overwintering generations emerged from late May to early July during 1983 and from late March to mid July in 1984. The commencement of the 1984 emergence was approximately one month earlier than the first appearance of adult Lu. diabolica in light trap collections from the D'Hanis, Texas, study site (1 May, 1984).

Quiescent 3rd and 4th stage larvae were observed in cultures, usually associated with adverse conditions, such as excessive moisture, extreme temperatures, or poor diet. These larvae continued to feed and move about rather sluggishly for as long as three or four weeks without any apparent development. Addition of fresh diet usually, but not always, resulted in resumption of development and pupation occurred within a few days. Some larvae lingered in their quiescent state for a few more weeks and then resumed development spontaneously, in the absence of apparent stimuli. Others never resumed development and perished after several weeks.

In the outside colony, eggs deposited in July hatched within 10 days, but larval development was arrested for long periods, presumably due to hot summer temperatures (lows, 22°C; highs, 36°C). During the quiescent period, food and moisture were replenished periodically and the larvae continued to feed but did not resume development for weeks, sometimes months. One batch of eggs was laid on 10 July 1983 by a female that had emerged from a batch laid 20 October 1982. The eggs hatched 20 July 1983, but the larvae did not pupate until 2 November

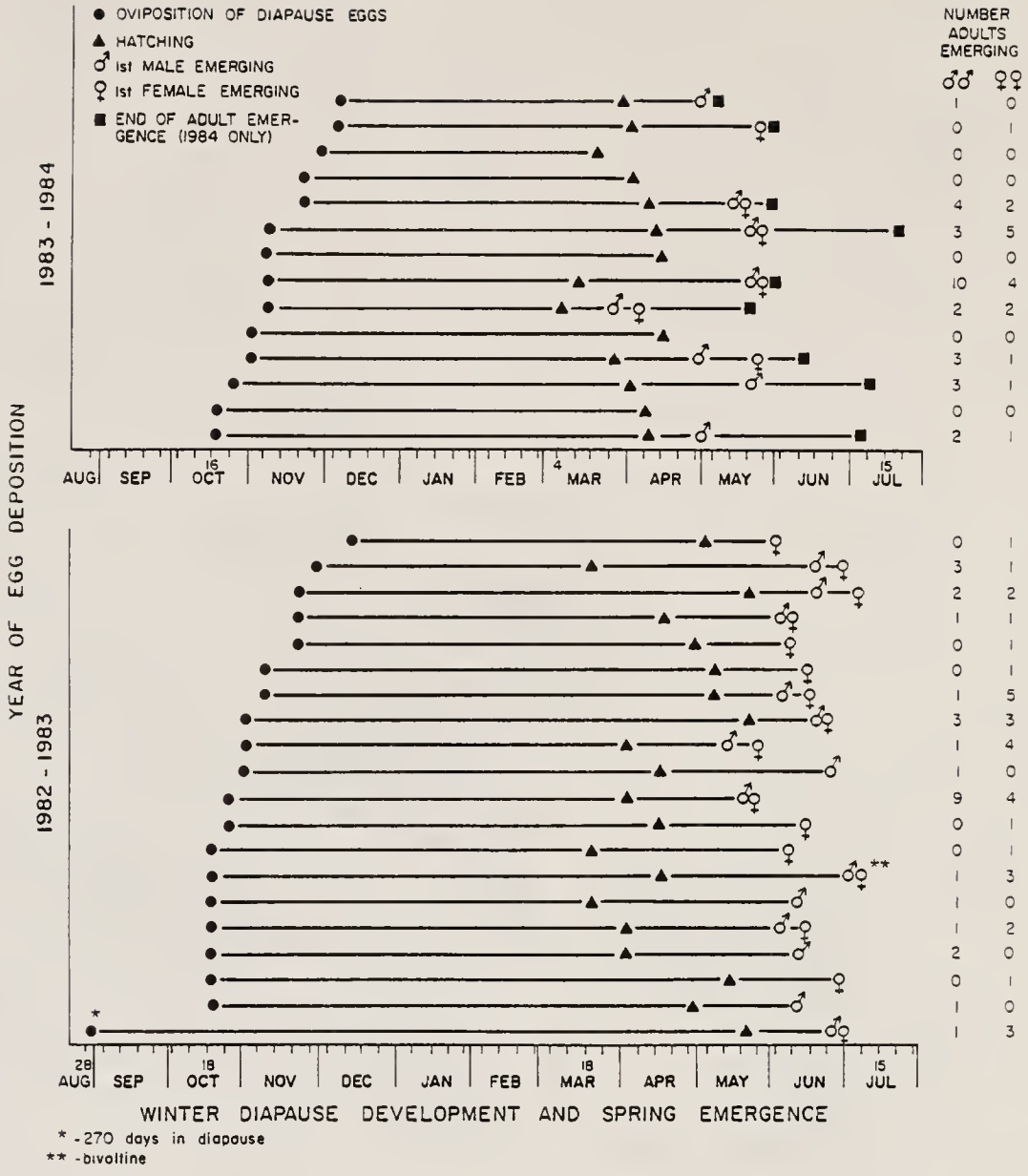


Figure 3-11. Winter diapause development and spring emergence in 33 Lutzomyia diabolica egg batches deposited outdoors at Gainesville, Florida (29°39.6' N), between August and December in 1982 and in 1983.

1983. The first adult (female) did not appear until 21 November 1983 (134 days after oviposition). This female mated, fed and deposited a batch of 100% diapause eggs.

Larvae hatching in October and November experienced cooler temperatures than larvae hatching during the summer. These individuals developed normally until about the 3rd and 4th instars and then ceased development. On warm days they would move sluggishly about and continue to feed, but on cold days (near freezing) activity ceased and did not resume until the next warm day. Several 4th instars survived freezing temperatures, but all succumbed to temperatures below -2°C, and neither pupae nor larvae endured the winter.

Adults

Emergence patterns. The degree of synchrony and time of adult emergence varied depending on rearing temperature, moisture factors, and larval diet. The top portion of Figure 3-12 depicts the adult emergence pattern of the first colony generation (76 egg batches from wild-caught females). It typifies the prolonged adult emergence observed during the first three laboratory generations. The immature stages were reared at 24°C and fed standard sand fly diet, coarsely ground and applied moist. The range of emergence was 29 to 80 days postoviposition, with a mean emergence time of 45 days. Males emerged an average of five days earlier than females, with a mean time to eclosion of 43 days, compared to 48 days for females. Eighty-two percent of adults emerging during the first five days were males and 83% emerging during the last 20 days were females. In contrast,

the lower portion of Figure 3-12 shows the emergence pattern of adults from 32 egg batches of the 13th generation. The immature stages were reared at 27°C and fed unincubated horn fly diet, finely ground and applied dry. Emergence times ranged from 26 to 52 days post oviposition, with an average of 32 days, 13 days shorter than in the 1st generation and 30 days shorter than in the 6th. Males emerged an average of 1.5 days earlier than females. Eighty-one percent of adults emerging in the first five days were males and eighty percent during the last 15 days were females.

The adult emergence patterns of colonies reared at 24°C and 27°C on regular sand fly diet (coarsely ground and applied moist) are presented in Figure 3-13. The time to adult emergence was ten days shorter, and the range of emergence was 20 days shorter at 27°C than at 24°C, producing a more synchronous pattern. Protandry, or the average time that male eclosion preceded female eclosion, was reduced an average of 3 days.

Emergence patterns of adults reared on five larval diets are compared in Figure 3-14. Diets C and D produced adults one week earlier than diets A and B, and almost a month earlier than diet E.

Sex ratios. The overall ratio of males to females (based on 100 females) for nine generations was 114:100 and ranged from 87:100 in the 3rd generation to 427:100 in the 7th generation. The results of larval diet experiments provide evidence that sex ratios may be modified by nutrition. Male to female ratios were 128:100, 58:100, 103:100, 103:100, and 236:100 for diets A, B, C, D, and E, respectively. Significantly fewer males were produced on diet regimen B (standard sand fly diet with liver powder) than on the others, and

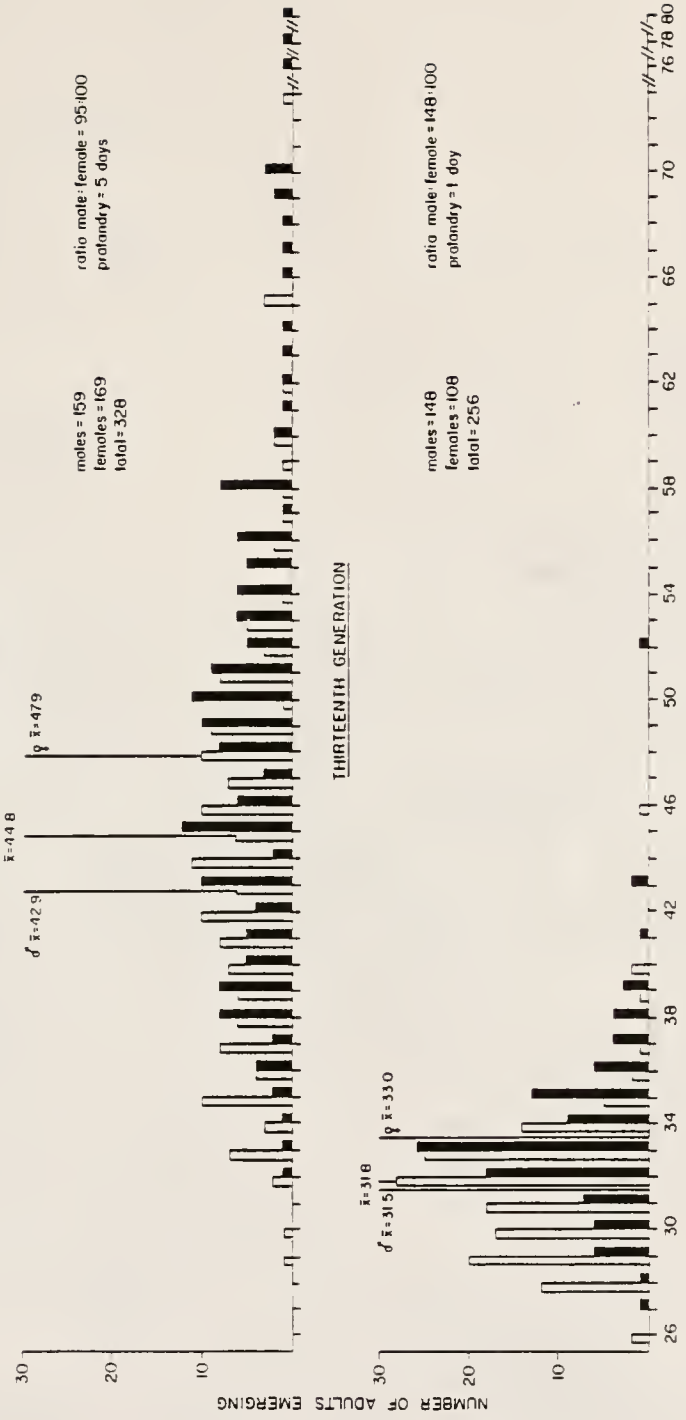


Figure 3-12. Adult emergence patterns of Lutzomyia diabolica in a laboratory colony: 1st generation reared at 24°C, 70% RH, and fed standard sand fly diet, coarsely ground and applied moist; 13th generation reared at 27°C, 70% RH, and fed unincubated horn fly diet, finely ground and applied dry.

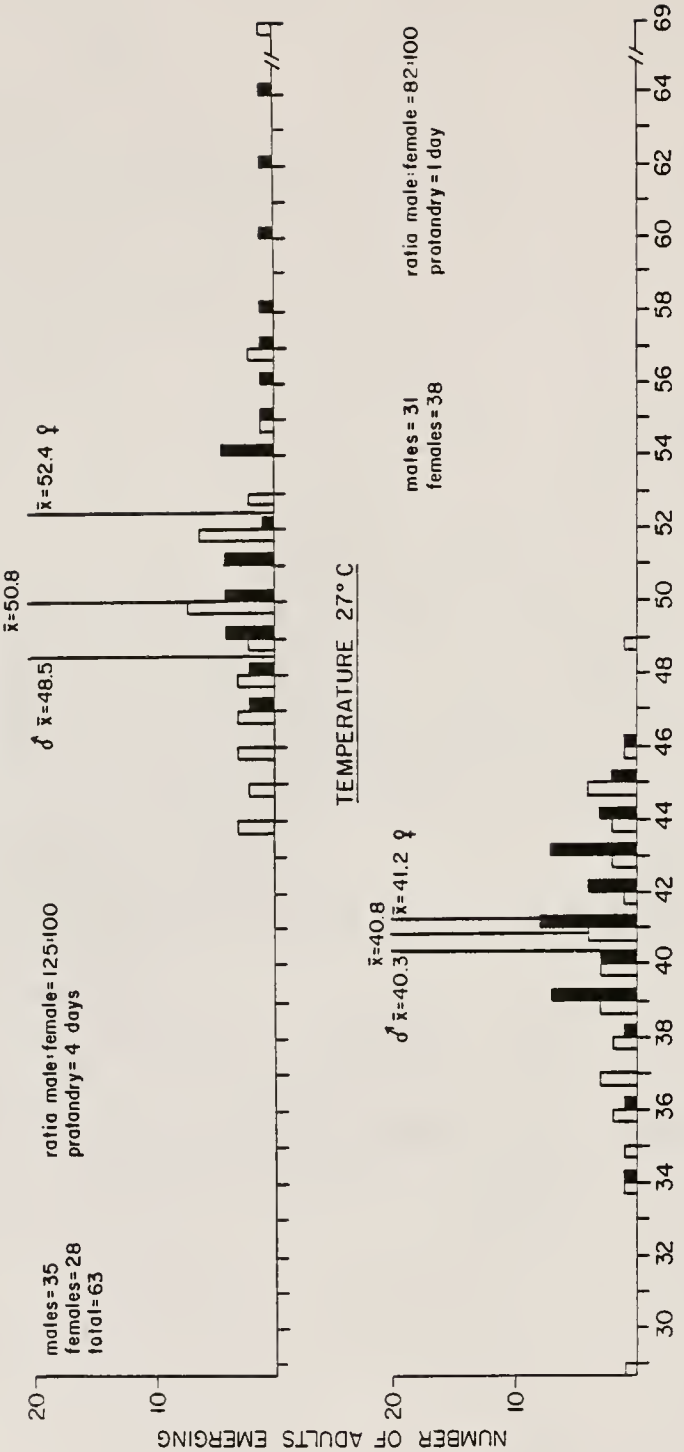


Figure 3-13. Adult emergence patterns of Lutzomyia diabolica reared individually at 24°C and 27°C. Both groups were maintained at 87% RH and were fed standard sand fly diet, coarsely ground and applied moist (3rd colony generation).

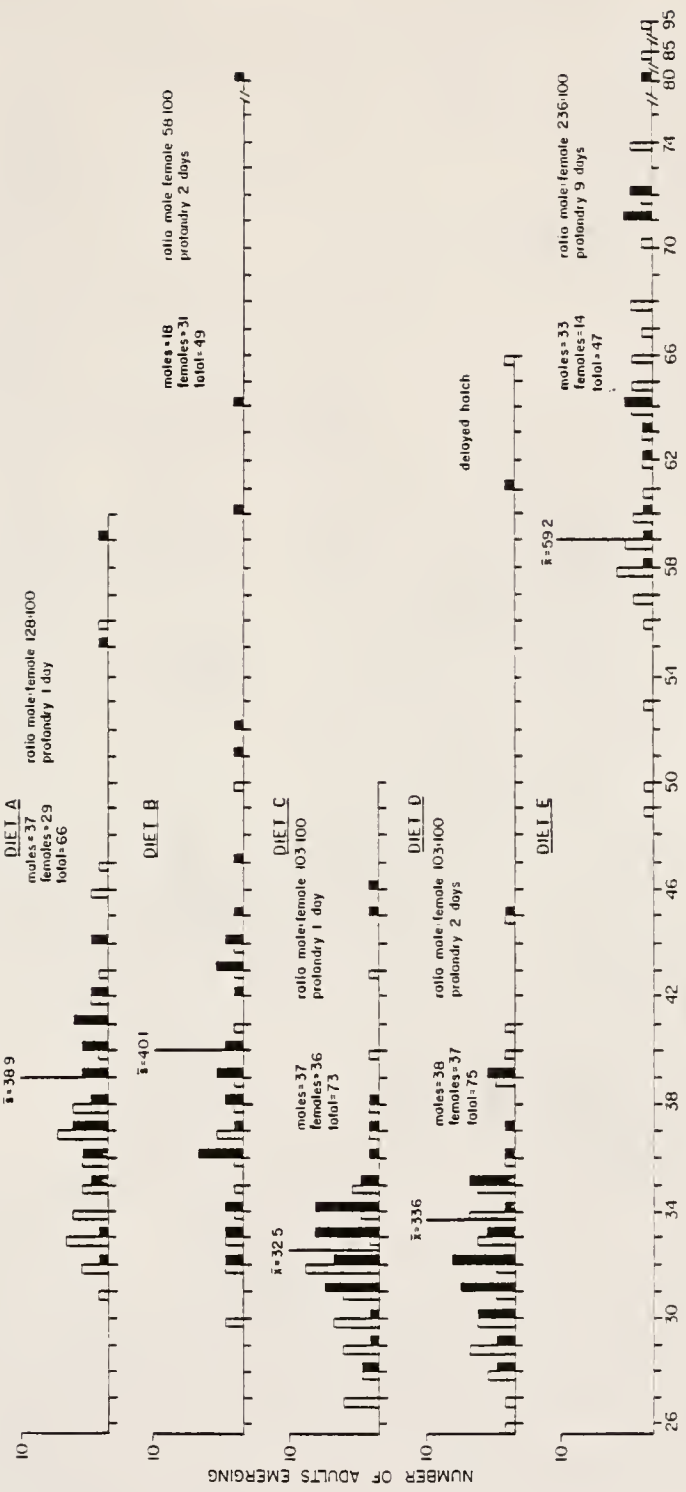


Figure 3-14. Emergence patterns of 12th generation Lutzomyia diabolica adults reared at 27°C and 87% RH on five larval diets: diet A = standard sand fly diet, ground fine, applied dry; diet B = standard sand fly diet, mixed and incubated with liver powder, ground fine, applied dry; diet C = incubated horn fly diet, ground fine, applied dry; diet D = unincubated horn fly diet, ground fine, applied dry; diet E = standard sand fly diet, coarsely ground, applied moist.

significantly more males were produced on diet E (standard sand fly diet, coarsely ground, applied moist) (ANOVA procedures, PR>F = 0.0001; Duncan's multiple range test, a = 0.05).

Longevity. The mean longevity of 30 virgin males of the 13th generation, held in individual oviposition/rearing vials at 27°C and near 100% RH, was 14.9 days (range = 4 to 20 days). The mean longevity of virgin females of the same generation, held under the same conditions, was 11.7 days (range = 4 to 21 days). Longevity of females that took a single blood meal was 10.1 days for all generations and ranged from 5 to 38 days (Table 3-6). Postoviposition longevity averaged about 1 day for all generations and ranged from 0 to 29 days (Table 3-6). Females that survived oviposition and which took a second blood meal lived an average of 12.3 days (n = 47), or about two days longer than singly fed females.

Mating. Males emerged with genitalia inverted 180° from the normal position and were not reproductively mature until their genitalia had rotated, about 12 to 24 hrs posteclosion. Females seemed to be reproductively mature at the time of eclosion and were seen mating immediately after being released into the mating chamber. Mating was observed at all hours of the day, before, during, and after blood feeding, but usually after. Copulation lasted from three to five minutes, but was as short as 47 sec and as long as 14 min 23 sec (n = 16). During the early part of the 1st generation, males were paired with blood-fed females for life in individual oviposition/rearing containers to insure maximum opportunity for insemination. It was later discovered that equivalent mating success could be achieved by releasing blood-fed females into the mating chamber for an additional

Table 3-6. Summary of oviposition and longevity data for generations 2 through 9 and 13 in a laboratory colony of Lutzomyia diabolica maintained at 24 ± 1°C and 70 ± 10% RH for the first three generations and 27 ± 1°C and 70 ± 10% RH for all subsequent generations.

Generation	Number of Females	Percent Ovipositing	Preoviposition Period $\bar{x}$±1SD(range)	Number of Eggs per Batch $\bar{x}$±1SD (range)	Postoviposition Longevity $\bar{x}$±1SD(range)	Longevity $\bar{x}$±1SD(range)
2	121	76%	5.5±1.4(3-10)	26.9±15.3(1-75)	0.7±1.4(0-6)	9.3±2.3(5-17)
3	112	73%	6.2±1.7(3-13)	24.9±15.3(2-55)	1.0±1.5(0-6)	10.3±1.9(6-18)
4	74	86%[1]	6.6±1.5(3-11)	26.6±14.5(4-57)	1.0±1.5(0-7)	10.6±2.1(6-17)
5	78	92%	6.8±2.0(3-12)	36.1±17.9(1-76)	1.9±4.9(0-29)	11.9±5.1(7-38)
6	45	80%	5.9±2.2(3-12)	25.8±14.3(1-60)	1.0±1.4(0-5)	10.0±2.4(6-16)
7	35	83%	8.2±3.6(4-17)	32.3±18.1(1-65)	0.4±0.7(0-2)	11.6±3.9(6-20)
8	91	85%	4.5±1.3(3-8)[2]	28.9±12.2(1-53)	0.9±1.2(0-4)	8.1±1.8(5-13)
g[3]	29	83%	3.7±1.0(2-6)	26.5±12.4(1-62)	1.4±1.8(0-7)	7.6±2.0(4-12)
13	32	100%	5.3±2.2(3-11)	28.5±8.8(6-42)	1.7±1.8(0-5)	10.0±2.7(6-15)
Overall	617	82%	5.3 (3-17)	28.1 (1-76)	1.0 (0-29)	10.1 (4-38)

1. Significant increase in % ovipositing attributed to increase in colony temperature from 24°C to 27°C (ANOVA > F = 0.0001; Duncan's multiple range test, α = 0.05).

2. Significant decrease in preoviposition period attributed to change in adult holding procedure after feeding (ANOVA, PR > F = 0.0001; Duncan's multiple range test, α = 0.05).

3. Colony rejuvenated in 9th generation by addition of 76 egg batches from wild-caught females.

24 hrs after feeding. Matings with blood-fed females were observed almost immediately after they were released into the chamber.

Mating behavior of laboratory-reared Lu. diabolica almost invariably follows a 6-step sequence:

1. Orientation. The male, upon noticing the female, aligns his body parallel with hers and faces in the same direction.

2. Pursuit. The male does not touch the female, but pursues her if she moves to another location.

3. Wing fluttering. The male flutters his wings repeatedly over his body. This may occur during all steps, whether he is stationary or in pursuit.

4. Abdomen waving. The male waves his abdomen from side to side or curls it 180° in a J-shape, left and right. This may be performed when the female is not in the immediate vicinity.

5. Precoupling. The male closely approaches the female and adopts a stance parallel to hers and faces in the same direction for several seconds.

6. Coupling. While still in the precoupling stance, the male curves the tip of his abdomen 180° in the female's direction and grasps the tip of her abdomen with his genital claspers (gonostyles). He then abruptly straightens his abdomen and realigns his body end-to-end with the female. Vigorous wing fluttering follows intromission of the male genitalia and continues periodically throughout the copulatory period, particularly when the female attempts to move or uncouple. During copulation the pair usually remains stationary but may move about and fly in copula.

Feeding. Although some females accepted a blood meal within 24 hrs of eclosion, most fed between 72 and 96 hours posteclosion. In the laboratory, feeding occurred at all hours of the day and night, at temperatures ranging from 23°C to 27°C, and with no noticeable periodicity. Maximum feeding occurred when 3 to 4-day-old females were restrained in a 120-ml feeding vial and the screen feeding lid placed against the skin surface of the host (man, rabbit or dog). Hungry females usually fed to repletion in ten minutes when restrained in this manner. By offering a blood meal three times per week, an estimated 97% of the females fed. Females fed but with less avidity on anesthetized hamsters and mice, or on the hand of the author, when these were placed inside the mating chamber. They bit the hamsters and mice on the ears, nose, around the eyes, on the foot pads and on the tail.

Hungry females hopped nervously about the feeding vial or mating chamber when a host was offered. This activity increased when the author exhaled lightly into the feeding vial or mating chamber, presumably in response to increased levels of CO_2. Upon contacting the surface of the host's skin, the female stroked it once or twice with her palpi, probed with the fascicle until she located a suitable site, then plunged it into the skin in one quick, crouching motion. Once inserted, the mouthparts usually remained in place until the female was replete. On occasion, feeding was interrupted and the female moved to a new location and resumed feeding. During the feeding period the female's abdomen swelled tremendously and turned bright red. Feedings lasted an average of about 6 minutes and ranged from one to 18 minutes. After withdrawing her mouthparts, the replete

female often attempted to feed a second time, usually only for a few seconds, before hopping to the floor of the feeding vial to rest and digest the blood meal. Partially replete females, interrupted during the first feeding, would feed on consecutive days until replete. Droplets of clear liquid were excreted from the tip of the abdomen during or shortly after feeding. Within about 24 hrs, the blood meal turned from bright red to dark brown or black and dark spots of excreted pigment could be seen on the white-plaster floor of the container. In a few instances, feeding females took only clear fluid (serum without red blood cells), having evidently pierced a lymph vessel.

Oviposition. Oviposition data for laboratory generations 1 through 9 and 13 are summarized in Table 3-6. A significant increase in oviposition was observed in the 4th and subsequent generations, coinciding with a change in rearing temperature from 24°C to 27°C (ANOVA procedures, PR>F = 0.0001; Duncan's multiple range test, a = 0.05). The mean preoviposition period was 5.3 days and ranged from 3.7 days in the 9th generation to 8.2 days in the 7th. Midway through the 8th generation a change was initiated in the holding procedure for blood-fed females. Instead of placing the oviposition/rearing vials in an open tray inside the environmental chamber, wet paper towels were placed in the bottom of the tray and it was enveloped in a plastic bag. This change increased the relative humidity in the vials to near saturation and significantly decreased the preoviposition period by as much as four days (ANOVA PR>F = 0.0001; Duncan's multiple range test, a = 0.05).

Most females completed oviposition in 24 hrs or less, but some deposited several eggs a day for up to five days. The mean number of eggs deposited for all laboratory generations was 28.1, and ranged from 24.9 in the 3rd generation to 36.1 in the 5th. Individuals that took meals of serum laid only about half the usual number of eggs. Eggs were laid singly or in groups of two or three, rarely in clumps of ten or more. If they were laid in clumps, the female was moribund and died during or immediately after oviposition. Eggs were glued in place, presumably with accessory-gland material, to the surface of the moist plaster in the bottom of the oviposition vial. If the plaster was allowed to dry, the females were more prone to glue their eggs to the vertical walls of the containers. Eggs were often deposited carefully in cracks, holes and other protected places that were available. They could not be dislodged by shaking the vial, or washed away with a syringe full of water.

Autogeny. No autogenous oviposition was observed in thirty newly emerged 14th generation females paired with newly emerged males. Nor was it observed in selected pairs from previous generations or in wild-caught flies. Post mortem dissections of these females revealed undeveloped ovaries and accessory glands full of granules.

Age-Specific Life Table

Table 3-7 is an age-specific life table patterned after one constructed by Morris and Miller (1954). It does not represent any particular colony generation but represents what might be expected in a generation of Lu. diabolica reared under current colony conditions and procedures (27 $\pm$ 1°C; 70 $\pm$ 10% RH; 16:8 LD photoperiod). It is

Table 3-7. Age-specific life table for laboratory-reared Lutzomyia diabolica based on observations of several generations. [1]

x	l_x	t_x	$d_x f$	d_x	$100q_x$
Age interval	No. at beginning of x	Time (days) spent in age interval x $\bar{x}\pm 1SD$(range)	Factors responsible for d_x	No. dying during age interval x	d_x as percentage of l_x
Egg	1000[2]	8.1±3.8(6-26)	Not fertilized Excessive moisture Dessication Mold	641	64%
1st Stadium	359	4.7±1.2(2-9)	Entrapped in moisture Entrapped in mold Dessication	48	5%
2nd Stadium	311	3.5±1.2(2-9)	Entrapped in moisture Entrapped in mold Cannibalism? Fungus?	3	0.3%
3rd Stadium	308	3.8±1.4(2-10)	Cannibalism? Fungus?	3	0.3%
4th Stadium	305	6.5±1.6(3-13)	Cannibalism? Fungus?	3	0.3%
Pupa	302	6.9±1.5(4-16)	Fungus?	3	0.3%
Adult					
♂♂	164	14.9±4.2(4-20)	--	--	--
♀♀	135	11.7±4.5(4-21)	--	--	--
Totals	299	45-48 days	--	701	70%

Rate of increase = adult female progeny (137) divided by parent female (35), or

$$\frac{137}{35} = 3.9x \text{ per generation}$$

1. Rearing conditions = 27 ± 1°C; 70 ± 10% RH; 16:8 LD photoperiod; diet of incubated or unincubated horn fly medium ("gator grits").

2. From 35 adult females.

based on data obtained from several generations but principally from a cohort of individuals produced by 32, 12th-generation females that emerged together and fed on the same day.

Discussion and Conclusions

General

Johnson and Hertig (1961) discovered that when sand fly cultures were moved from one room at 25.5°C to another at 26.5°C, certain species, which were not doing well in the first room, began to thrive, and that the "hardier" species fared better at even higher temperatures. Gemetchu (1976) attributed much of the success of a colony of P. longipes Parrot and Martin to changes in the quality of larval diet provided. Killick-Kendrick et al. (1977) observed an approximate 25% reduction in generation time of Lu. longipalpis (Lutz and Neiva) due to a change from an open insectary, with less constant conditions, to environmental cabinets with controlled moisture. Ward (1977) observed a ten-fold increase in numbers of adult Lu. flaviscutellata (Mangabeira) from the 6th to the 9th colony generation as a result of adaptation to exclusive feeding on hamsters and increased refeeding.

The most significant factors contributing to the success of this laboratory colony of Lu. diabolica were 1) a large initiatory stock of eggs and timely rejuvenation with additional wild-stock, insuring adequate numbers of adults for mating and reproduction, in spite of less than optimal rearing conditions; 2) a change in rearing temperature from 24°C to 27°C; 3) strict regulation of moisture in

oviposition/rearing vials; 4) improvements in larval diet; and 5) genetic selection, since each successive generation was set up with flies emerging from the preceding generation (see Killick-Kendrick et al., 1977). It is impossible at this point to identify the specific effects of infusion of the original inbred colony stock with wild material. Two possibilities exist; either the original genetic stock was completely or partially replaced by the new material, or the new material was assimilated into the existing colony. This latter possibility would likely have reversed, at least temporarily, much of the genetic selection that occurred in previous colony generations. The original inbred stock may have selected for low survival, a trend turned around by the infusion with wild stock.

From the colony's inception, frequent changes and adjustments in handling procedures and rearing conditions were essential to meet the specific needs of the subject population. Initial changes amounted to little more than guesswork, but through trial and error the guesswork became educated and fine-tuning of the colony to acceptable levels of productivity was possible.

Immature Stages

Eggs. Lindquist (1936) first described the eggs of Lu. diabolica, saying that they possessed slightlty branched, longitudinal striations on their surfaces. Endris (1982) examined the eggs with an electron microscope and described the surface topography as a series of discontinuous, parallel-longitudinal ridges that are not laterally connected. He proposed a scheme for classification of 41 species of New World sand flies based on the distinctive surface topographies of

their eggs. This ridged pattern is probably formed by accessory gland material as the egg passes through the oviduct. Undeposited eggs, even if they have darkened, do not have ridges (Perkins, 1982). The accessory gland material is presumed to be the glue with which the eggs adhere to the substrate (Chaniotis, 1967). In nature, this adhesive probably prevents the eggs from being blown or washed away by wind or rain, or from being carried away by predators, as well as serving as a barrier against dessication or mold (Johnson and Hertig, 1961). The thick shell of Lu. diabolica eggs, in addition to furnishing protection against drying during the normal prehatching period, probably serves as a protection during periods of egg quiescence or diapause. This is consistent with the observations of Johnson and Hertig (1961) who noted that most surface-feeding Panamanian species have dark, thick-shelled eggs and that at least some, i.e., Lu. gomezi (Nitz.), undergo periods of quiescence in the egg stage.

Reports on fecundity rates in sand flies are often misleading since the number of eggs laid is usually different from the number matured (Table 2-3, p. 59, Chap. 2; Chaniotis, 1967). Thirty-one to thirty-seven percent of wild-caught Lu. diabolica females retained at least some of their eggs and a comparable percentage of laboratory-bred females retained eggs. Lindquist (1936) stated that as many as 40 eggs may be laid by a single female. Endris (1982) reported means between 32.0 and 39.6 eggs per batch and a maximum of 64. The overall mean observed for all generations during this study (26.9 eggs per batch) was lower than that observed by Endris, but 5th and 8th generation means (36.1 and 32.3 eggs per batch) were comparable. The

maximum number of eggs per batch (88) was higher than observed by Endris. Johnson and Hertig (1961) reported fecundity rates for several neotropical sand flies that are consistent with those reported here for Lu. diabolica. Chaniotis (1967), working with California species reported similar fecundity rates for Lu. californica (Fairchild and Hertig) and Lu. stewarti (Mangabeira and Galindo) but much higher rates (mean, 43; range 2-106) for Lu. vexator (Coquillett).

Incubation periods observed in laboratory-reared Lu. diabolica are consistent with Lindquist's (1936) report of 7 to 14 days. The one and one-half to three days shorter incubation period observed in the 1st generation (progeny of wild-caught females collected in June 1982) probably reflects the difference between field and laboratory-rearing temperatures. Most egg batches from these wild-caught females were deposited and incubated in the field at temperatures of 27°C or higher, before they could be transferred to a controlled laboratory environment. When the laboratory rearing temperature was increased from 24°C to 27°C, the average incubation time was immediately reduced to or below the level of the 1st-generation figure (Table 3-1). Similar temperature-dependent decreases in incubation times were observed in several Panamanian species (Johnson and Hertig, 1961), as well as in other nearctic species of sand flies (Chaniotis, 1967; Perkins, 1982; Endris, 1982).

Assuming that the percent fertile egg batches (at least one egg hatching) can be used as an indicator of mating success, the high of 91% observed in the 1st generation (progeny of wild-caught females) indicates that mating success in the field is greater than in the

laboratory (Table 3-1). Johnson and Hertig (1961) reported greater fertility in wild-caught than laboratory-reared Lu. sanguinaria (Fairchild and Hertig) and Lu. gomezi. Perkins (1982) reported that 68% of the egg batches of laboratory-bred Lu. shannoni (Dyar) females were fertile, indicating at least that degree of mating success. He also found that 100% of wild-caught females dissected had sperm in their spermathecae, representing 100% mating success. Endris (1982) reported that up to 58.8% of laboratory-bred Lu. anthophora (Addis) were infertile, based on percent fertile egg batches. In laboratory-reared Lu. diabolica, the percent fertile egg batches per generation remained fairly constant until the 7th through 9th generations (Table 3-1). Low fertility in these generations probably reflects poor mating success due to small adult numbers and long generation times, which produced asynchronous male/female emergence patterns (many males died before the females emerged).

Johnson and Hertig (1961) reported a 23% hatching rate in laboratory-reared Lu. sanguinaria as opposed to 92% in eggs of wild-caught flies. The corresponding rates for laboratory-reared and wild-caught Lu. diabolica were 28.9 and 50.4%, respectively (Table 3-1). These figures are probably low due to the difficulty in assessing hatching rates in egg batches with quiescent eggs. In routine maintenance, egg hatch was checked for the first 20 days only, and unhatched egg batches were discarded after thirty days. These data are, however, consistent with Endris' (1982) description of "partial fertility" in laboratory reared Lu. diabolica eggs in which as many as 70% of the eggs laid by a single female failed to hatch. Perkins

(1982) reported a higher hatching rate of 59% in laboratory-bred Lu. shannoni.

Larvae and pupae. Johnson and Hertig (1961) found that most Panamanian species of sand flies fall roughly into two classes according to their behavior in culture: 1) those that feed on the surface of the food material, and 2) those that burrow in the food mixture. In general, the surface feeders have long caudal bristles, which presumably discourage burrowing, and dark, thick-shelled eggs with a sticky substance that cements them to the substrate. The burrowers usually have short caudal bristles, pale brown or black eggs, often with thin shells and lacking an adhesive substance. Some species such as Lu. gomezi, which has short caudal bristles and feeds on the surface, fit neither category. From the larval behavior, these authors were able to predict that Lu. panamensis (Dyar), Lu. pessoana (Barretto), Lu. trapidoi (Fairchild and Hertig), and Lu. ylephiletor (Fairchild and Hertig) would be found on the surface of objects such as rotting leaves. These predictions were confirmed by Hanson (1961), who found the larvae on dead leaves scattered on the forest floor. Since Lu. diabolica is a surface feeding species, it seems reasonable to predict that it will be found in the field on the surface of its food material. Further field studies will be required to confirm this prediction.

According to Gemetchu (1976) and other workers, an essential feature of a good rearing environment for sand flies is porosity of the substrate to ensure dampness without excess free water. Judgment of dampness is empirical and depends on experience and personal judgment (Eldridge et al., 1963)

Development. The ten-day decrease in total immature development time due to increased rearing temperature (Table 3-2) is consistent with findings by other authors of temperature dependent increases in rates of development in neotropical and nearctic sand fly species (Johnson and Hertig, 1961; Chaniotis, 1967; Killick-Kendrick, 1977; Endris, 1982; Perkins, 1982). Highest mortalities occurred at both 24°C and 27°C in the egg stage. It should be noted that no distinction was made between losses due to egg death, i.e., death of the embryo, and nonhatching due to infertility. Highest mortalities beyond the egg stage occurred in the 1st stadium at both temperatures. These results are consistent with the observations of the previously mentioned authors. Differences in mortality between the two temperatures were not significant (Fig. 3-6).

The observation that females eclose later than males is consistent with the findings of other workers (Gemetchu, 1977; Perkins, 1982). The longer 4th stadium in females may indicate a critical period during which sexual development occurs, or during which the female stores up fat deposits to be invested in egg production. On the other hand, it may also be critical for males to have a shorter 4th stadium than females, thus enabling them to eclose earlier and mature sexually before the females make their debut. A knowledge of how much later female eclosion will be at specific temperatures may facilitate planning of disease transmission experiments requiring large numbers of unfed females.

These findings indicate that rates of development in the field are strongly influenced by ambient temperatures and that the number of generations per year will depend on mean seasonal temperatures in both

macro- and microenvironments. For laboratory colonization, a rearing temperature of 27 ± 1°C appears to be about optimal for Lu. diabolica. Further rearings at temperatures above 27°C and below 24°C should be conducted to determine the critical temeperature range for immature development.

Killick-Kendrick (1978) listed excessive larval mortality, due to fungal growth, improper diet or moisture, disease or other factors, as an outstanding problem inhibiting progress in sand fly rearing. Since larvae live on or in their food material, one must conclude that these mortality factors are directly related to the diet medium.

Lindquist (1936) attempted to rear Lu. diabolica larvae on soil containing considerable organic matter and on chicken and rabbit feces, but on both diets the larvae died before pupation. When he used moistened soil containing less organic matter and supplemented it with the tissue of dead flies (Sarcophaga, Cochliomyia, Lucilia, and Musca) he successfully reared larvae through the adult stage in 16 to 27 days with an average of 18.5 days. These times are considerably shorter than those observed for laboratory generations fed the standard sand fly larval diet (Table 3-3). This diet consists of equal parts of laboratory-rabbit chow and rabbit feces, ground, mixed, moistened, and then cured for about one month. It has been used for rearing at least nine sand fly species (both Old and New World) with good success (Endris et al., 1982).

As the results of the larval diet experiments (Table 3-5) show, diet A was a vast improvement over the standard diet regimen, diet E. These diets differed only in particle size (A, fine; E, coarse) and moisture content (A, dry; E, moist), yet development times under A

were 20 days shorter than under E. This indicates that particle size of the medium is critical. Larvae reared on coarse diets have difficulty chewing the large particles and, consequently, are undernourished and suffer high mortality. The extra moisture in diet E caused packing of the food material, making it difficult to chew, and encouraged excessive mold growth, which entrapped some early instars. It is interesting to note that even development times of the nonfeeding egg and pupal stages were significantly longer under diet E than under any of the other regimens. Excessive moisture may retard development, at least during the egg stage, and undernourishment in the larval stage may result in a prolonged pupal stage.

The addition of liver powder to regular sand fly diet (diet B) resulted in little change in development time but caused a significant increase in mortality (ANOVA PR>F = 0.0001; Duncan's multiple range test, a = 0.05). The liver powder introduced undesireable fungal growth which proved deleterious to the larvae. Killick-Kendrick et al. (1977) fed Lu. longipalpis larvae on dessicated liver powder alone, with good results, but commented that overfeeding with liver powder encouraged growth of fungi, which is a danger to younger larvae.

Diets C and D (incubated and unincubated horn fly medium, respectively) produced about equal results, reducing the total immature development time by 6 to 27 days, with an accompanying increase in the number of individuals surviving to the adult stage. Both of these diets have been used in the laboratory colony since the 13th generation. As a result, the duration of the larval stage has been reduced substantially from a mean of 45 days in the 9th

generation to 17 days in the 13th (Table 3-3). Diets C and D, dubbed "gator grits," are being used in laboratory colonies of four other nearctic sand flies with equally promising results. Since diet C must be incubated, it requires about one month to prepare, while diet D can be prepared in about one day. On the other hand, there is a little more mold growth in diet D and usually more mites. The mold is a problem only with very young larvae and can be easily controlled by adding very small amounts of food at a time. After the first molt, the larvae manage the mold better and the diet can be added in larger quantities. The mite populations can be controlled by autoclaving the diet before use.

Judging from Figure 3-8, improvements in larval diet had their greatest impact on the 4th instar, which is the largest and, hence, the instar that consumes the most food. It is noteworthy that all four larval stages maintained the same order relative to each other but not relative to nonfeeding stages. Under diets C and D, the duration of the 4th stadium was reduced to less than that of either egg or pupa. Under diet E the order of the egg and pupal stages were reversed.

Diapause and quiescence. Johnson and Hertig (1961) observed extremely uneven rates of hatching time and larval development in some Panamanian species. In Lu. gomezi, Lu. panamensis, and Lu. geniculata (Mangabeira) egg batches, they reported hatching of some eggs three or four weeks after the first hatching, and at least 30 days after oviposition. They concluded that since other eggs in the same cultures had hatched at normal times, culture conditions were probably not involved and some eggs were destined to be quiescent. This is apparently the same type of quiescence observed in many indoor

laboratory cultures of Lu. diabolica. Such mixed oviposition of fast and slow-developing eggs in the same batch by a single female has been reported in several insects (Walker, 1980). According to Walker (1980), for many insect species living in habitats where moisture and temperature are unpredictable (such as south central Texas), the optimal strategy is to produce progeny with a frequency of developmental programs reflecting the probability of failure and success. No single program is always a winner, and the payoffs for winning programs vary within a year, from year to year. Females that spread their risks by laying normal-developing and quiescent eggs, avoid genetic catastrophe. This seems to be the case with Lu. diabolica, whose environment is extremely unpredictable in terms of precipitation and temperature. The delayed hatching (obligatory diapause) of a few of the eggs in a batch "spreads the risk" and insures the survival of at least some members of the species.

Lindquist (1936) described another type of quiescence in Lu. diabolica, which has not been reported in any other sand fly species. Eggs deposited by wild-caught females in the laboratory on 14 October 1933 and kept in an indoor room during the winter, hatched on 29 March 1934, 167 days after oviposition. This phenomenon, also observed in the outside colony of Lu. diabolica during this study, probably represents a true winter diapause (hibernation) in the egg stage. The type of egg produced (diapause or nondiapause) is dependent upon the temperature and/or photoperiod experienced by the female during the preoviposition period (Walker, 1980). The first appearances of diapause eggs in the outside colony, 18 October and 16 October for 1982 and 1983, respectively, are consistent with Lindquist's

observation. Since the outside colony and field populations were at virtually the same latitude (Gainesville, Florida, 29°39.6' N; D'Hanis, Texas, 29°20.0'N), they experienced almost identical photoperiods. The photophase for October 16, 1983 was 11 hr 27 min and 11 hr 28 min at D'Hanis and Gainesville, respectively. These observations suggest that Lu. diabolica is a long-day species, depositing diapause eggs in response to shorter day length. The percent diapause eggs increased from zero in early October to 100% in December (Fig. 3-9). The critical day length, at which 50% of the eggs were in diapause, occurred some time in November, which had a mean photophase of 10 hr 41 min. Temperature may act indirectly in long-day insects by modifying the degree of the diapause response or by altering the position of the critical daylength (Saunders, 1976). Therefore, some variation in onset of diapause may be expected from year to year, depending on the mean fall temperatures. Termination of this winter diapause is probably temperature dependent and occurs in the spring after a critical number of day degrees has accumulated (Fig. 3-11).

Quiescence in the larval stage has been reported by several authors, with overwintering occurring in this stage. Most palearctic species of sand flies are subject to a facultative diapause and pass the winter as 4th stage larvae, low temperatures being the main, but not the only factor which induces diapause (Adler and Theodor, 1957). Addis (1945b), working in Texas with Lu. anthophora was unable to obtain pupation of 4th instar larvae over a period of two months when they were fed a mixture of dried rabbit feces and blood. When a few drops of all known components of vitamin B complex were added to the

cultures, the larvae pupated within three days. Johnson and Hertig (1961) stated that in laboratory cultures of neotropical flies (Lu. sanguinaria and panamensis), adverse conditions, mainly too little moisture, result in diapause of the 4th instar. In the indoor Lu. diabolica colony, larval quiescence occurred most often in response to excessive or inadequate moisture, or poor diet, but in a few cases it occurred when rearing conditions appeared to be optimal. Quiescence in the outside colony was mostly temperature related, resulting in summer aestivation and possibly winter hibernation. Larvae usually resumed normal development with the restoration of favorable conditions, but in some cases the quiescence was not readily reversible, indicating a possible true diapause.

Chaniotis (1967) reported facultative diapause in the 4th larval stage of nearctic Lu. californica, Lu. stewarti, and Lu. vexator up to 230 days postoviposition. He concluded that the percent and amount of diapause was related to generation and temperature, believing that the 1st generation of flies emerging from hibernation produced nondiapausing larvae, and the 2nd and 3rd-generation flies produced larvae of which only a portion diapaused. He further stated that the higher the temperature, the less diapause occurred and that low temperature should be regarded as the principal factor inducing diapause in California species. He added that under optimal conditions of temperature, humidity and food, a certain proportion of 2nd and 3rd-generation larvae entered diapause and suggested that factors intrinsic to eggs, larvae or females are also involved.

In contrast to Chaniotis' (1967) observations of California sand flies, hot summer temperatures may also induce quiescence/diapause in

Lu. diabolica. This is consistent with the paucity of adult collection records from July to August. It should be noted that Lindquist (1936) collected the species during July, 1934, near Sonora, Texas, indicating that a summer diapause is not universal throughout the species' geographic range.

Ready and Croset (1980) showed that for two Mediterranean sand fly species, P. ariasi Newstead and P. perniciosus Tonnoir, environmental stimuli (photoperiod and temperature) can induce a growth arrest, even in midsummer, that is not immediately reversible, i.e., a true diapause.

Late-instar Lu. diabolica developing in the outdoor colony probably entered a true winter diapause (hibernation), but since they were unnaturally exposed in rearing vials, and could not protect themselves against the cold, they succumbed to subfreezing temperatures. It is the author's opinion that some would have survived the coldest winter days (-9°C) had they been afforded the opportunity to secrete themselves under a blanket of leaf litter or in a crack in the soil. Soil temperatures (10 cm depth) did not drop below 8°C at any time during the winters of 1982 or 1983 (Agronomy Dept. and NOAA cooperating, Gainesville, FL, 1982, 1983).

Since the outside colony and field populations were located virtually at the same latitudes and experienced similar photoperiods and seasonal temperatures, it is likely that the same patterns of diapause occur in the field. Lu. diabolica probably produce two to four generations per year. In a two generation (bivoltine) strategy, the 1st-generation adults emerge in late May and early June and deposit fast-developing eggs, which hatch in 8 to 10 days to produce

2nd generation larvae that are subject to summer diapause. The intensity of this diapause varies depending on the prevailing temperatures and is apparently terminated by lower fall temperatures. If diapause is broken in late September, the larvae will complete their development and pupate, producing 2nd generation adults by mid-October or later. These adults will then deposit fast-developing eggs, diapause eggs, or a combination of both. If they are diapause eggs, they will eventually produce the 1st generation of adults for the succeeding year. If they are nondiapause eggs, the resulting larvae will be subject to cool fall and winter temperatures and may enter diapause, producing 1st-generation adults for the next season.

In a three-generation (trivoltine) strategy, the 1st-generation adults emerge in late March or early April, early enough to produce a complete, nondiapausing 2nd generation before the summer's heat. Second generation adults then deposit fast-developing eggs that hatch in eight to ten days, producing 3rd-generation larvae that are subject to summer diapause. These do not pupate until fall, and the resulting adults deposit nondiapause eggs, diapause eggs or a mixture of both, as in a bivoltine program. In either case, the eggs or larvae are subject to winter diapause.

A 4th generation is possible if 3rd-generation larvae develop normally and pupate in late summer, producing adults by early September. These adults will most likely lay 100% nondiapause eggs and the resulting 4th generation larvae will be able to complete their development before diapause-inducing fall and winter temperatures set in. Any progeny from this 4th generation will be subject to diapause.

Adults

Emergence patterns. Male Lu. diabolica have a shorter egg-to-adult development period than females, enabling them to mature reproductively (rotate their genitalia) before the females emerge. This is consistent with findings of other workers. Gemetchu (1976) observed that for 402 adult P. longipes from the same pupal generation and eclosing over a 10-day period, 95% of the flies that appeared during the first three days were males and 90% during the last three days were females. Endris (1982) and Perkins (1982) reported similar adult emergence patterns for Lu. anthophora and Lu. shannoni, respectively.

Comparison of emergence patterns of the 1st and 13th-generation adults (Fig. 3-12) reveals that the longer the mean generation time was, the more prolonged the adult-emergence interval, and the more pronounced the protandry. In extreme situations, under less than optimal conditions, the generation time was extended beyond 60 days, resulting in a more asynchronous male:female emergence pattern. This reduced mating success, since some males died before they had a chance to mate with the later-emerging females. During the first nine laboratory generations, and particularly when adult numbers were low (7th, 8th, and 9th), it was not uncommon to have almost exclusively one sex at a time in the mating chamber.

The total period of adult emergence and protandry were contracted significantly by increasing the rearing temperature from 24°C to 27°C and by improving the larval diet. The resulting synchronization increased mating success, as witnessed by an increase in percent fertile egg batches and an accompanying increase in percent hatch per

egg batch in the 13th generation (Table 3-1). In terms of colony productivity, the best combination of rearing temperature and larval diet, was 27°C and "gator grits" (incubated and unincubated horn fly medium).

Sex ratio. Perkins (1982) reported a male:female ratio in Lu. shannoni of approximately 101:100. This is slightly less than the 114:100 male:female ratio observed in the first 13 laboratory generations of Lu. diabolica.

Colonies reared on "gator grits" (diets C and D, incubated and unincubated hornfly diet, respectively) showed less male bias (103:100) (Fig. 3-14). Due to the small sample size under diet regimen E (standard sand fly diet, coarsely ground, applied moist), it is difficult to draw conclusions, but it appears that the diet produced a male biased population by prolonging the immature development period. The longer the period was extended, the higher the probability of mortality in the remaining individuals. Since most of those remaining at the end of the cycle were females, fewer females than males survived to the adult stage. The fact that fewer males were produced on diet regimen B (standard sand fly diet with liver powder, ground fine and applied dry) may indicate that males are more susceptable than females to certain fungi encouraged by the addition of liver powder to the diet. Further tests involving greater sample sizes will be necessary to confirm these hypotheses.

Longevity. Unmated males tended to outlive unfed, unmated females by four to five days at 27°C and near 100% RH. The range of longevity in both sexes was nearly the same (males 4 to 20 days; females, 4-21 days). Blood-fed, mated females lived an average of one

and one-half days less than unfed females, most of their mortality occurring at the time of or shortly after oviposition. Females that survived oviposition and took a second blood meal, lived only slightly longer than unfed females. Death of females at oviposition is one of the most important problems encountered in sand fly colonization. It hampers experiments in which transmission by bite is attempted, and reduces the productivity of the colonies (Killick-Kendrick, 1978). Retention of gravid females in a high humidity environment significantly reduced the preoviposition period but did not increase the postoviposition longevity.

Adult longevity figures for Lu. diabolica are considerably shorter than those observed by Chaniotis (1967) in California sand flies under variable conditions of temperature, humidity and food. He concluded that 1) the mean length of life decreases with rising temperature and constant RH, 2) at constant temperature, rising humidity promotes better survival of flies, and 3) the range of survival is broader at low temperatures or high humidity.

Mating. The epigamic behavior exibited by male Lu. diabolica is very similar to that described by Chaniotis (1967) in Lu. vexator and by Alexander (pers. comm., 1984) for Lu. anthophora. Encounter of the sexes in the laboratory colony usually occurred as they rested on the plaster of Paris wall of the mating chamber. Occasionally they were observed copulating on the host animal while the female fed. In June 1982, wild flies were observed in copula on the tile walls of the public latrines at Garner State Park. Chaniotis (1967) suggested that the host may be the principal site where sexes come to close proximity

in nature. This may explain why males, as well as females, are attracted to light or baited traps.

Exactly what attracts the males to females, or visa versa, remains a mystery. Chaniotis (1967) cited references suggesting that pheromones produced by the male might be involved. Schlein et al. (1984) observed aggregation behavior in feeding P. papatasi and suggested that a pheromone produced in the maxillary palps attracted other females of the same species. Males did not produce the pheromone nor did they respond to it. Sex and courtship pheromones are generally produced by female arthropods (Shorey, 1973). Exceptions include some tephritid and drosophilid fruit flies, in which males produce long-distance chemicals attractive to females (Nation, 1972; Burke, 1981). Workers at the British Museum (Natural History) in London, studying electron micrographs of sand flies, have revealed the presence of small pores on the 3rd and 4th abdominal tergites of male Lu. longipalpis (Lutz and Neiva) which may be the sites of pheromone production; this is borne out by observations of the courtship behavior of this species, in which the male waves his wings in a manner suggestive of "wafting" pheromone towards the female (Alexander, pers. comm., 1984).

Feeding. Observations of highest feeding success in 3 to 4-day-old Lu. diabolica are consistent with findings of Endris (1982) and Perkins (1982) in their studies of Lu. anthophora and Lu. shannoni, respectively.

Once replete, Lu. diabolica females did not feed again until after oviposition. Feedings on consecutive days by partially replete females were also observed in Lu. shannoni (Perkins, 1982). If the

female survived oviposition, she took a second blood meal, but no subsequent feedings were observed in Lu. diabolica females. Other authors have reported multiple feedings in several species of sand flies (Gemetchu, 1976; Endris, 1982; Beach, 1984).

Laboratory-reared Lu. diabolica feed indiscriminantly on a wide variety of hosts including man. Endris (1982) reported feedings on the following: human (Homo sapiens), dog (Canis familiaris), woodrat (Neotoma micropus), Syrian hamster (Mesocricetus auretus), gray squirrel (Sciurus carolinensis), domestic rabbit (Oryctolagus cuniculus), opossum (Didelphis marsupialis), calf (Bos taurus), horse (Equus caballus), and sheep (Ovis aries). With the exception of Syrian hamsters, these hosts occur within the geographic range of Lu. diabolica. Such a broad range of hosts is certain to enhance the vector capacity of the insect.

The diuresis observed in blood-feeding Lu. diabolica has also been observed by other workers (Chaniotis, 1967; Gemetchu, 1976). This phenomenon is common in fluid feeding insects, which at least for a time after feeding, contain an excess of water. This excess is reduced by a rapid diuresis, thus preserving the osmotic concentration of the hemolymph (Chapman, 1971).

There was no significant difference in fecundity due to the type of host offered. Ready (1979) found that mammalian bloods differed in their ability to promote oocyte maturation in Lu. longipalpis. This subject is still under investigation with Lu. diabolica, especially in connection with membrane feeding studies.

Johnson and Hertig (1961) found that when immobilized with drugs, hamsters seem to lose their attractiveness for sand flies, possibly

because of a drop in skin temperature. This may partly explain the poor feeding success on anesthetized hamsters observed in this study. Lu. diabolica females demonstrated a discriminatory behavior when feeding on human volunteers, accepting blood from some individuals and consistently rejecting others. This response may be related to the individual body chemistry of the human host. Investigations to determine the factors associated with the host which serve as phagostimulants are essential to the development of efficient artificial-membrane feeding systems.

Oviposition. In the laboratory colony, 82% of blood-fed females deposited eggs, compared to 56% for wild-caught females from the June 1982 survey trip and 76% for the September 1983 survey trip. This increase reflects improvements in handling procedures, rearing conditions and larval diet. Of the 32 females sampled in the 13th generation, 100% laid eggs. The procedures and conditions used in that generation have been used exclusively in subsequent generations with highly satisfactory results.

Gravid females retained at 100% RH deposit their eggs earlier than at lower humidity, all other factors being the same. This suggests that at higher RH, females sense that conditions are favorable for oviposition and are less inclined to retain their eggs after they mature than they would be at lower RH. Chaniotis (1967), noticed that at RH of less than 60%, a saturation deficiency was created in the environment of the gravid female sand fly, causing a delay in oviposition.

The number of eggs laid per batch varies from species to species. Gemetchu (1976) reported a mean of 52 eggs per batch in P. longipes

from Ethiopia. The mean number of eggs in Lu. diabolica batches (28.1) is low compared to P. longipes but comparable to that reported by Johnson and Hertig (1961) in several Panamanian species and by Endris (1982) and Perkins (1982) in other USA species.

Most Lu. diabolica females completed oviposition in 24 hrs or less at 27°C and 100% RH. The process is apparently very exhausting, since fewer than half survived long enough to complete oviposition and take a second blood meal. Only a few of those that took a second blood meal completed a second gonotrophic cycle; most died within two or three days after the second meal. In contrast, multiple refeedings and up to four gonotrophic cycles have been reported in other sand fly species (Lu. flaviscutellata, Ward, 1977; Lu. anthophora, Endris, 1982; P. dubosqui Neveu-Lemaire, Beach, 1984). Beach (1984) reported that 75% of P. dubosqui females in a laboratory colony survived oviposition, took additional blood meals and completed a 2nd and, in some cases, a 3rd gonotrophic cycle. Further studies designed to increase postoviposition survival and refeeding in Lu. diabolica are planned and will clearly be of interest, particularly as they relate to the success of leishmaniasis transmission studies.

Autogeny. Although it was not observed in Lu. diabolica, autogeny is known to occur in at least two other USA species of sand flies, Lu. shannoni and Lu. cruciata (Perkins, 1982).

Age-Specific Life Table

Life tables are devices that record in a systematic fashion those facts basic to the age distribution of mortality (Morris and Miller, 1954). They were first used by insurance companies for human

populations, where they underlie actuarial studies (Pianka, 1978). Deevey (1947) pointed out three ways in which life table may be developed: 1) by directly observing the age at death (d_x) for a large and reasonably random sample of the population; 2) by following the survival (l_x) of a large cohort (born more are less simultaneously) at fairly close intervals throughout its existence; 3) by obtaining the age structure from a random sample of the population, and inferring dx from shrinkage between successive age classes. Morris and Miller (1954), stated that only the second sort of information is statistically respectable. These authors constructed a life table for the spruce budworm, Choristoneura fumiferana (Clem.), using a modification of Deevey's second method. They used column headings conventionally employed in life tables (Southwood, 1978) to include

x Age interval or life stage;

l_x The number surviving at the beginning of the age interval stated in column x;

d_x The number dying within the age interval stated in column x; and

q_x The number dying in the age interval divided by the number of survivors at the beginning of the interval (the rate of mortality).

In addition, they inserted a new column, "$d_x f$," between l_x and d_x in which to record the factors responsible for death (d_x). This placed the emphasis on the causes of mortality rather than the order of dying (Morris and Miller, 1954).

The life table constructed in Table 3-7 for Lu. diabolica is patterned after that of Morris and Miller (1954) but differs in two respects: 1) It is based on a cohort of laboratory-reared individuals rather than on a naturally occurring population. Although it may have

some application to field situations, it is adapted primarily for use in experimental laboratory studies. 2) An additional column "t_x" was inserted between l_x (number surviving at the beginning of the age interval) and $d_x f$ (factors responsible for death). The time spent in age interval x is recorded in this column. This should not be confused with "T_x" of Southwood (1978), which represents the total number of animal x age units beyond age x. This addition should provide a more comprhensive picture of the life history and dynamics of a colony population.

In Table 3-7, the l_x for eggs is based strictly on the number of eggs laid, and does not account for irreplaceable loss due to egg retention by females or potential eggs lost due to nonfeeding or unmated females. The l_x values for larvae and pupae represent the number that survived the previous stage.

As the life table shows, the greatest loss (d_x) occurs in the egg stage (64%) due to infertility, excessive moisture, dessication and mold. This is rather high when compared with the corresponding figure of 4.4% for a colony of Lu. longipalpis (Killick-Kendrick et al., 1977). The 2nd most important loss is 1st-instar larvae, the most delicate instar. About 5% of the larvae will die as 1st intars, five-fold more than in all other instars combined. Most deaths of 1st instars probably result from entrapment in moisture (usually in condensation droplets on the sides of the vial), entrapment in mold, or from dessication. The later instars are less susceptable to entrapment in moisture and mold, although some still occurs in the 2nd instar. The extent to which cannibalism and fungal infections contribute to mortality in 2nd through 4th instars is unknown. In

some cultures containing a known number of larvae, individuals would mysteriously disappear, possibly from cannibalism. On one occasion a 4th-instar larva was observed eating another 4th instar. The victim was dead at the time, and it is not known if it was killed by the other larva, or if it died from other causes. Killick-Kendrick et al. (1977) listed cannibalism as one of the factors contributing to mortality in later instars of Lu. longipalpis, especially in cultures that were underfed or contained large numbers of larvae. The pupa is the most robust of all the immature stages, and losses have always been very low. At present, about 99% give rise to adults, comparing favorably with the 96% or greater reported in Lu. longipalpis colonies (Killick-Kendrick et al., 1977).

In Lu. diabolica reared under current colony conditions of 27 ± 1°C, 70 ± 10% RH, 16:8 LD photoperiod and a diet of "gator grits," 30% survival to the adult stage can be expected (70% mortality). Accordingly, if we start with 35 gravid females depositing an average of 28.9 eggs apiece, for a total of 1,000, approximately 300 adult progeny will be produced, 55% males and 45% females. The rate of increase per generation is calculated by dividing the number of female progeny by the number of female parents, and in this case it is 137/35 = 3.9. x per generation.

Reliability of life tables is only as good as that of the basic data used to construct them (Morris and Miller, 1954). If these data are valid and rearing conditions are relatively constant, the table may be used to predict the number of individuals that will be available at a given point in the life cycle, or during a given

generation. This will greatly enhance planning of experimental work requiring large numbers of individuals in a given life stage.

CHAPTER 4
EXPERIMENTAL TRANSMISSION OF *Leishmania mexicana* BY BITES OF *Lutzomyia diabolica* (HALL) AND *Lutzomyia shannoni* (DYAR) WITH NOTES ON THE EXTRINSIC DEVELOPMENT OF THE PARASITE

Introduction

From a medical perspective, the most important aspect of the biology of a sand fly is whether or not it can transmit leishmaniasis (Killick-Kendrick, 1978). Making such a determination is time consuming and usually requires field and laboratory investigations of the insect's biology, followed by labor-intensive experimentation in vector-parasite-host relationships. Consequently, experimental transmissions by bites of sand flies are infrequently demonstrated. A historical review of the incrimination of the major vectors of leishmaniasis is presented in Chapter 1.

The WHO Scientific Working Group on the Leishmaniases (1977) gave long-term, high priority to the establishment of complete life cycles of *Leishmania* in the laboratory by using natural vertebrate and invertebrate hosts. This is necessary to determine the dynamics and mechanics of transmission and to study the developmental morphology and physiology of the parasites in these hosts.

At present, five species of *Leishmania*, *L. donovani*, *L. chagasi*, *L. infantum*, *L. tropica*, and *L. mexicana* ssp. have been experimentally transmitted to human volunteers or laboratory animals by bites of about a dozen sand fly species (Killick-Kendrick, 1981a).

Competence for transmission of L. mexicana ssp. has been demonstrated for several New World sand flies, only one of which, Lu. anthophora (Addis), is indigenous to the USA (Killick-Kendrick, 1979; Endris, pers. comm., 1984).

The specific objectives of this study were to:

1. investigate, under controlled laboratory conditions, the vector capacity of Lutzomyia diabolica (Hall), as compared with Lu. shannoni (Dyar), for leishmaniasis; and

2. examine by means of the electron microscope the morphology of L. mexicana parasites found in the sand fly vector.

Materials and Methods

Infection of Sand Flies and Parasite Development

Leishmania mexicana (strain WR-411), isolated in 1980 from a single-lesion infection of an 11-year-old white male from Uvalde, Texas, was the principal pathogen used. It was provided by Walter Reed Army Institute of Research (WRAIR), Washington, D.C., and has been maintained by serial passage in Syrian hamsters for the past three years. Other strains used included L. m. amazonensis (strain untyped) provided by Dr. K. P. Chang (Department of Microbiology UHS/Chicago Medical School, North Chicago, Illinois) who obtained it in 1979 from original stock isolated by Dr. P. Marsden from a patient in Tres Bracos, Brazil; L. braziliensis guyanensis (strain MHOM/SR/80/CUMC 1), provided by Dr. Jan Keithly, Cornell University Medical Center, New York City; and L. donovani infantum (untyped) from a naturally infected dog that was presented to the University of

Florida School of Veterinary medicine. These strains have also been maintained by serial passage in Syrian hamsters and Balb/c mice. Infected hamsters were kept in individual isolation cages inside a Bioclean® portable laminar air-flow enclosure (Hazleton Systems Inc., Aberdeen, MD) at 23°C in a secure animal room. Laboratory-reared Lu. diabolica and Lu. shannoni from established colonies were used in these experiments. The former originated from wild-stock collected at Garner State Park, Uvalde County, Texas (see Chapters 2 and 3) and the latter from wild-stock collected in Alachua and Levy Counties, Florida (Perkins, 1982).

Hamsters with large histiocytomas on the ear, nose or feet were used to infect sand flies (Fig. 4-1). They were anesthetized with 0.18 to 0.20 cc ketamine chloride given intramuscularly (IM) in the hind thigh. Two to three-day-old laboratory-reared Lu. diabolica and Lu. shannoni were placed in individual or group feeding vials with large-mesh screen lids. The surface of the screen was then placed directly against a histiocytoma on an infected hamster. These areas, rich in parasites, were fed upon readily by the sand flies. The procedure was carried out in a three-sleeved isolation chamber (Fig. 4-2), within the confines of a class II laboratory facility.

The size of each blood meal was graded according to the following scale: probe = no blood visible; #1 = trace of blood in the thorax; #2 = abdomen about one quarter to one half full; #3 = abdomen about two thirds to three quarters full; #4 = abdomen full of blood and distended. Females that fed on infected hamsters were maintained individually in 7-dram oviposition/rearing vials in a Hotpack environmental chamber at 27 $\pm$ 1°C and near 100% RH. Dissections of

Figure 4-1. Histiocytoma on the right ear of a hamster infected with Leishmania mexicana.

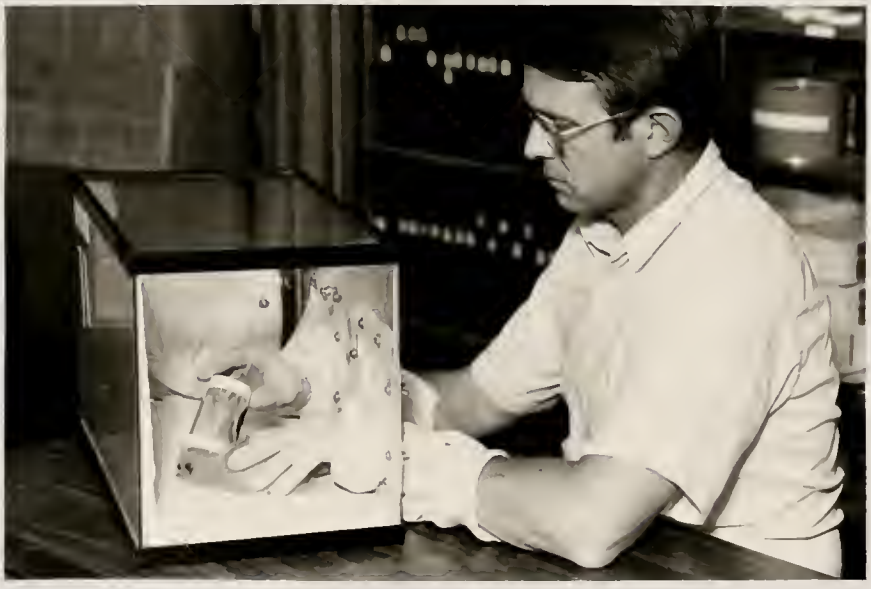


Figure 4-2. Infecting sand flies in a three-sleeved isolation chamber by feeding them on a leishmanial histiocytoma on a hamster's ear.

potentially infected flies were performed according to the technique of Johnson et al. (1963). Since the initial aim was to demonstrate transmission of parasites, most dissections occurred post mortem, or when the fly was obviously too weak to refeed. Data recorded for each specimen included eclosion date, feeding information (to include size of blood meal and host), oviposition date and number of eggs deposited, longevity, and pararasite information.

To establish the complete life cycle of L. mexicana in Lu. diabolica, "healthy" infected flies were immobilized by refrigeration for ten minutes at 0°C, and dissected at 12, 18, and 24 hrs, to determine when promastigotes first appeared, then at 24-hr intervals for the next seven days. Ten flies were dissected at each time interval. A brief description of the infection, location, morphology and behavior of the parasites was recorded for each dissection. Phase-contrast photomicrographs of live and Giemsa-stained parasites and measurements of the various forms were taken through a Zeiss Photo III microscope.

Ultrastructure Studies

The purpose of these studies was to examine the ultrastructure of amastigotes in hamsters infected by bite of Lu. diabolica, and promastigotes present in the gut of infected flies. To obtain the amastigotes in macrophage cells, aspirates were drawn from histiocytomas of infected hamsters and centrifuged at 1,000 gravities for one minute to concentrate the cells into a small pellet. To maintain the integrity of the cell pellets during fixation and embedding, they were first fixed in 1% osmium tetroxide for 1 hr, then

suspended in 3% bacto-agar (Akin, pers. comm., 1984). They were postfixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) for 1-1/2 hrs. Sand flies that had fed on an infected hamster five and six days previously were the source of promastigotes. The flies were immobilized by refrigeration at -1°C for ten minutes and washed in insect Ringer's solution (pH 7.2; Cavanaugh, 1956) to remove body hairs. Whole flies or dissected guts were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer for 1-1/2 hrs before postfixation in 1% osmium tetroxide for 1 hr. The fixed specimens were dehydrated in graded concentrations of ethanol and cleared in absolute acetone. They were infiltrated with graded concentrations of 30, 70 and 100% Spurr's resin (Spurr, 1969) for 1, 1, and 12 hrs, respectively, followed by imbedding in 100% Spurr's resin at 60°C for 48 hrs.

Ultrathin sections were cut using an LKB Ultratome III, model 8800, and were poststained with uranyl acetate followed by lead citrate. Specimens were subsequently examined using a Hitachi HU 11-E or Philips EM 301 electron microscope.

Transmission Trials

At first, potentially infected females were offered daily blood meals on anesthetized, uninfected hamsters. It was found, however, that they would not refeed prior to oviposition, so in all later trials, second blood meals were offered only after the commencement of oviposition. Potentially infected hamsters were checked at least weekly for six months for signs of infection. Infections due to Leishmania were confirmed by microscopic examination of Giemsa-stained aspirates drawn from cutaneous lesions that subsequently developed.

Results

Infection of Sand Flies and Parasite Development

Leishmania mexicana (strain WR-411, Uvalde, Texas). Four hundred sixty (87.9%) of 523 *Lu. diabolica* and 145 (94.8%) of 153 *Lu. shannoni* became infected when fed on histiocytomas of hamsters infected with *L. mexicana* (Table 4-1). Thirty-one (5.9%) of the *Lu. diabolica* and 18 (11.8%) of the *Lu. shannoni* females took visible second blood meals from uninfected hamsters. First feedings by *Lu. diabolica* on histiocytomas of the ear averaged 11 min, 22 sec (range = 6 min, 1 sec to 14 min, 30 sec; n = 20), while second feedings on ears of uninfected hamsters averaged only 6 min, 24 sec (range = 2 min, 30 sec to 10 min 58 sec; n = 18). Infected females of both species were less prone to feed to repletion at the second blood meal than uninfected flies. Nonetheless, many took full blood meals. A few had difficulty inserting their mouthparts and probed many times until they either succeeded or gave up. The average time between first and second blood meals was 7.0 and 7.5 days for *Lu. diabolica* and *Lu. shannoni*, respectively, and ranged from 3 to 14 and 5 to 13 days. Of the *Lu. diabolica* females, 40 (7.6%) died within one to three days after the first blood meal from a ruptured peritrophic sac (Endris, 1982) and ruptured midgut epithelium. The corresponding figure for *Lu. shannoni* was 6 (3.9%). Such deaths were invariably accompanied by heavy bacterial infections and were characterized by perfusion of the blood meal throughout all parts of the body, including thorax, legs, and antennae. Feedings on bacterially contaminated lesions seemed to produce a higher mortality due to peritrophic sac rupture.

Table 4-1. Infection rates in laboratory-reared Lutzomyia diabolica and Lu. shannoni sand flies fed on hamsters with histiocytomas due to Leishmania mexicana (strain WR-411).

Sand Fly Species	Initial Feeding		Refeeding			
	Number Fed	Number (%) Infected	Number (%) Probed Only	Number (%) Taking #1 to #4 Blood Meal	Interval Between Feeds $\bar{x}$±1SD(range)	Number (%) Peritrophic Sac Rupture
Lu. diabolica	523	460 (87.9)	10 (1.9)	31 (5.9%)	7.0±2.6(3-14)	40 (7.6)
Lu. shannoni	153	145 (94.8)	11 (7.2)	18 (11.8%)	7.5±3.0(5-13)	6 (3.9)

The development of L. mexicana in both Lutzomyia species was virtually identical, with most parasites concentrated in the abdominal midgut and cardia and, as the infection progressed, extending forward into the pharynx and mouthparts, and rearward into the hindgut (Fig. 4-3). Tables 4-2 and 3 record the locations of flagellates in the alimentary tract of laboratory-reared Lu. diabolica and Lu. shannoni, respectively, upon post mortem dissection, one to 14 days after the infective feed. Table 4-4 describes infections in live Lu. diabolica dissected between 12 hrs and 8 days after the infective feed.

No parasites were seen in dissections of live flies made less than 18 hrs after the infecting blood meal. The first indication of development of ingested amastigotes appeared between 18 and 24 hrs when light to moderate infections of ovoid, slightly motile promastigotes with short flagella were observed in the abdominal midgut, swimming through the blood meal, within the peritrophic sac (Fig. 4-4a). About 50% of these were dividing by binary fission (Fig. 4-4b). As they became older they elongated and many formed rosettes consisting of a dozen or more individuals arranged radially about a common point, with their flagella directed toward the center (Fig. 4-4c). Without the flagellum they measured about 4 µm long and 2-3 µm wide. The flagellum was shorter than the body, measuring about 3 µm or less.

By day two, the parasite population had increased many-fold, but was still confined primarily to the abdominal midgut with the blood meal. Division continued in about 50% of the ovoid, slightly motile promastigotes. Many, however, had transformed into long-slender, highly motile forms (Fig. 4-4d). In a small percentage of two-day-old

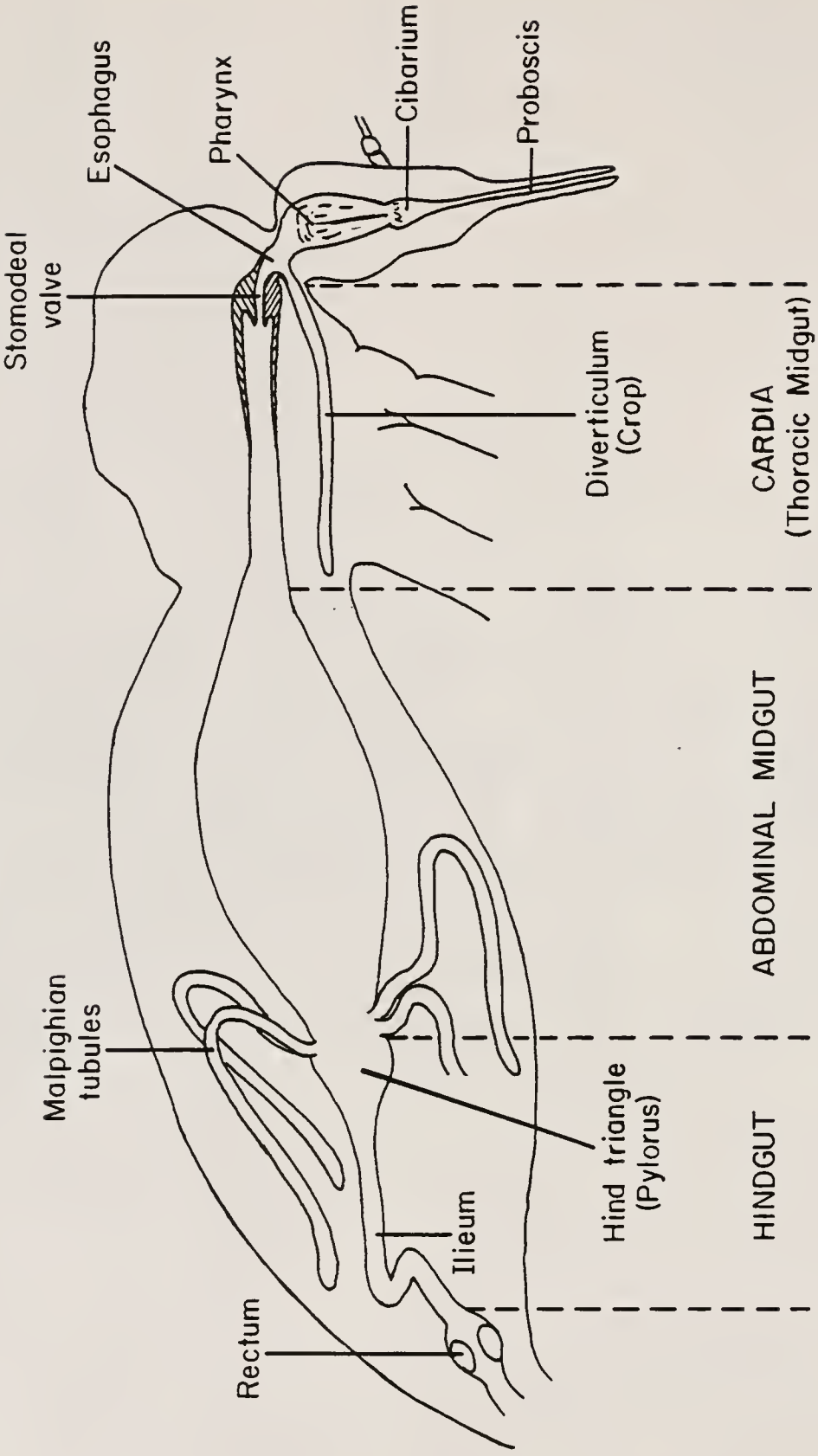


Figure 4-3. Diagram of a sand fly alimentary tract.

Table 4-2. Distribution of Leishmania mexicana (strain WR-411) in the alimentary tract of Lutzomyia diabolica dissected post mortem after feeding on infected hamsters. Infected flies were held at 27 ± 1°C and near 100% RH until death; only flies in which the entire alimentary tract was visible were included in the table.

Days After Infecting Feed	Sand Flies No. (%)	Free in Hindgut No. (%)	Midgut No. (%)	Cardia No. (%)	Pharynx No. (%)	Mouthparts No. (%)
1	2(0.5)	- -	2(100.0)	- -	- -	- -
2	19(4.9)	3(15.8)	18(94.7)	8(42.1)	- -	- -
3	29(7.4)	9(31.0)	28(96.6)	20(69.0)	2(6.9)	1(3.4)
4	62(15.9)	6(9.7)	59(95.2)	52(83.9)	14(22.6)	9(14.5)
5	81(20.8)	18(22.2)	81(100.0)	68(84.0)	24(29.6)	11(13.6)
6	100(25.6)	20(20.0)	96(96.0)	90(90.0)	31(31.0)	19(19.0)
7	42(10.8)	3(7.1)	40(95.2)	39(92.9)	10(23.8)	7(16.7)
8	20(5.1)	6(30.0)	20(100.0)	20(100.0)	8(13.8)	5(25.0)
9	17(4.4)	1(5.9)	17(100.0)	15(88.2)	5(29.4)	2(11.8)
10	11(2.8)	1(9.1)	11(100.0)	10(90.9)	2(18.2)	2(18.2)
11	3(0.8)	- -	3(100.0)	3(100.0)	- -	- -
12	2(0.5)	- -	2(100.0)	2(100.0	1(50.0)	- -
13	1(0.3)	- -	1(100.0)	1(100.0)	- -	- -
14	1(0.3)	- -	1(100.0)	- -	- -	- -

Total Flies = 390

Table 4-3. Distribution of *Leishmania mexicana* (strain WR-411) in the alimentary tract of *Lutzomyia shannoni* dissected *post portem* after feeding on infected hamsters. Infected flies were held at 27 ± 1°C and near 100% RH until death; only flies in which the entire elimentary tract was visible were included in the table.

Days After Infecting Feed	Sand Flies No. (%)	Free in Hindgut No. (%)	Midgut No. (%)	Cardia No. (%)	Pharynx No. (%)	Mouthparts No. (%)
1	7(4.7)	- -	7(100.0)	3(42.9)	- -	- -
2	10(6.7)	- -	10(100.0)	7(70.0)	- -	- -
3	7(4.7)	1(14.3)	7(100.0)	6(85.7)	- -	1(14.3)
4	14(9.4)	- -	14(100.0)	14(100.0)	- -	- -
5	21(14.1)	3(14.3)	21(100.0)	20(95.2)	4(19.0)	4(19.0)
6	44(29.5)	7(15.9)	41(93.2)	41(93.2)	8(18.2)	11(25.0)
7	16(10.7)	3(18.8)	16(100.0)	15(93.8)	6(37.5)	7(43.8)
8	12(8.1)	2(16.7)	12(100.0)	12(100.0)	5(41.7)	5(41.7)
9	11(7.4)	4(36.4)	11(100.0)	10(90.1)	3(27.3)	4(36.4)
10	4(2.7)	1(25.0)	4(100.0)	4(100.0)	1(25.0)	3(75.0)
11	1(0.7)	- -	1(100.0)	1(100.0)	- -	- -
12	1(0.7)	- -	- -	- -	- -	- -
13	0(0.0)	- -	- -	- -	- -	- -
14	1(0.7)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)

Total Flies = 149

Table 4-4. Description and location of *Leishmania mexicana* in the alimentary tract of laboratory-reared *Lutzomyia diabolica* dissected 12 hrs to 8+ days after an infecting blood meal. Flies were held at 27 ± 1°C and near 100% RH.

Time After Infecting Blood Meal	Number of Infected Flies	Observations
<24 hrs	13	No parasites were observed in dissections prior to 18 hrs postfeeding. In three-18 hr dissections short-ovoid, slightly motile, dividing promastigotes were seen in the blood meal within the abdominal midgut. Heavy midgut infections with short-ovoid, dividing promastigotes were seen in 5 dissections. Many were formed into rosettes. A few long-slender, highly motile forms were observed. All parasites were in the midgut, within the peritrophic sac.
2 days	10	Heavy infections of short-ovoid, slightly motile, dividing forms were seen in abdominal midguts of all dissections. Many were transforming to long-slender, highly motile promastigotes. Rosettes of short-ovoid, as well as long-slender forms were seen. The infections were confined within and around the perimeter of the blood meal.
3 days	10	Heavy infections of short-ovoid, and long-slender forms were seen in the abdominal midgut, with some short forms dividing. Rosettes were common. Long-slender forms predominated and had established a heavy infection in the cardia. Many broad forms were attached to the anterior cardia and stomodeal valve; some were dividing. A few parasites were swimming freely in the hind triangle in four dissections.
4 days	10	Broad-dividing forms were seen packed behind the stomodeal valve in nine dissections. Long-slender forms predominated in the abdominal midgut; broad-dividing forms predominated in the cardia and at the stomodeal valve. In seven dissections, long-slender promastigotes were seen swimming freely in the hind triangle. Several dead promastigotes were seen in the hindgut. Many short-slender, highly active promastigotes were seen in the pharynxes of eight flies and in the mouthparts of one.

Table 4-4. continued

Time After Infecting Blood Meal	Number of Infected Flies	Observations
5 days	10	Massive infections were observed in the anterior portion of the abdominal midgut and cardia, with the predominant form in the abdominal midgut being long-slender promastigotes. Broad-dividing forms predominated in the cardia and at the stomodeal valve. Long-slender forms were seen swimming freely in the hindgut of all dissections. Short-slender, highly active forms were present in the pharynxes of eight flies. Small-slender, highly active forms were seen moving rapidly in all directions in the mouthparts of five flies; some were seen throughout the alimentary tract. Parasites in the cardia and attached to the stomodeal valve were joined by a gel-like matrix. A long-slender, trinucleated form and an extremely long mononucleated form were seen in the midgut of one fly.
6 days	10	Massive infections were observed in the anterior portion of the abdominal midgut and cardia, with long-slender forms predominating in the abdominal midgut and broad-dividing forms and short-slender, highly active forms in about equal numbers in the cardia and at the stomodeal valve; the short-slender forms did not attach to the valve. Nests of short-slender, highly active forms were present in the cardia and pharynxes of eight specimens. Three had mouthpart infections. Short-slender, highly active forms were present throughout the alimentary tract. Parasites in the cardia were joined by a gel-like matrix.
7 days	10	Massive infections of broad forms and short-slender, highly active forms were seen in the cardia. No division was observed in the cardia, but broad-dividing forms were observed in the abdominal midgut. Midgut infections were heavy but not as heavy as in the cardia. Free-swimming promastigotes were observed in the hind triangle. Pharyngeal infections of short-slender, highly active forms were seen in five flies; four of these had mouthpart infections. Promastigotes in the cardia were joined by a gel-like matrix.

Table 4-4. continued

Time After Infecting Blood Meal	Number of Infected Flies	Observations
8+ days	7	Parasites were seen in the same forms and locations as in 5 to 7-day infections, but numbers were much less. Mouthpart infections were common. One 10-day infection had parasites only in the abdominal midgut. One 10-day infection showed massive numbers of short-slender, highly active forms throughout the alimentary tract, including the mouthparts, pharynx, and rectum.

infections, these long-slender promastigotes were seen swimming in the anterior portion of the abdominal midgut, cardia and hind triangle (Fig. 4-5). They measured about 12 and 2 μm in length and width, respectively, with a flagellum as long as or longer than the body. Intermediate forms between the ovoid and long-slender promastigotes were also observed.

Three-day-old infections were characterized by tremendous numbers of long-slender, highly motile promastigotes packed in the abdominal midgut, with anterior migration to the cardia, where shorter and broader dividing forms were seen attached to the stomodeal valve (Fig. 4-4e). These broad dividing forms measured 5-6 and 2-3 μm in length and width, respectively, with the flagellum equal in length to the body. The smaller dividing forms seen on days one and two were present in lesser numbers in the abdominal midgut on day three. In dissections where the alimentary tract was still intact, the parasites were packed in so tightly that the gut was noticably swollen and movement of parasites was restricted to stationary flagellar undulations. When the gut wall was ruptured, parasites spewed into the dissecting medium as if under pressure (Fig. 4-6). Masses of them appeared to be joined together by a gel-like matrix. In only one fly each, of three-day infected Lu. diabolica and Lu. shannoni the parasites had advanced beyond the stomodeal valve and into the esophagus, gaining entry into the pharynx and mouthparts (Tables 4-2 and 4-3). In addition, small numbers of long-slender promastigotes were seen swimming freely in the hind triangle and throughout the hindgut to the rectal ampullae (Fig. 4-5).

By the fourth day a massive infection was established in the anterior cardia at the stomodeal valve (Fig. 4-7). The commonest morphological form in this region was the broad dividing promastigote (Fig. 4-4e). They were packed behind the stomodeal valve and were attached to its surface by their flagella. When the anterior aspect of the cardia was severed, they blossomed out into the saline, most of them remaining attached to the cuticular surface of the valve (Fig. 4-8). Concomitantly, a heavy infection of long-slender, highly motile promastigotes continued to remain in the abdominal midgut, with small numbers appearing in the hindgut. In one four-day infection, promastigotes were observed swimming in the diverticulum (crop).

At five days, many parasites began to migrate beyond the stomodeal valve, into the head, invading the esophagus, pharynx and mouthparts. This anterior migration was accompanied by a reduction in size from the larger broad forms to short-slender, highly active promastigotes. These measured about 4-5 μm in length and 1-2 μm in width. The flagellum was longer than the body, measuring about 10 μm in length. Although these tiny flagellates were observed as early as three days in the mouthparts of a very few specimens, they were usually not observed until the infection was at least five days old or older (Fig. 4-4f). Their movement was incredibly rapid and appeared to be random. In a few six to ten-day infections they were observed throughout the alimentary tract from the mouthparts to the ileum. In some five to eight-day infections, nests of short-slender, highly active promastigotes could be seen in the region of the stomodeal valve in striking contrast to masses of larger, broader, sessile promastigotes inhabiting the same area. In one five-day infection a long-slender

nondividing, binucleated form and an extremely long mononucleated form were seen in the abdominal midgut (Fig. 4-9 and 4-10).

The highest degree of infection anterior to the stomodeal valve occurred from day four through eight in Lu. diabolica and day five through day eight in Lu. shannoni. Once inoculated with leishmanial parasites, sand flies of both species usually maintained the infection for life, but the degree of infection tended to diminish beyond eight days.

Leishmania mexicana amazonensis (strain untyped). Twenty-four (96%) of 26 Lu. diabolica became infected when fed on histiocytomas of the foot of a Syrian hamster infected with L. mexicana amazonensis. Six (25%) took second blood meals on uninfected hamsters, five to seven days after the infecting blood meal. For the first three days of the infection, the parasite growth pattern paralleled that of L. mexicana (strain WR-411), with the heaviest infections in the abdominal midgut and anterior migration to the cardia (Table 4-5). Massive infections were not observed, nor was attachment to the stomodeal valve. The infections seemed to be more localized, with the predominant form after three days being a rounded promastigote rather than the long-slender form seen previously in infections of L. mexicana (strain WR-411).

Leishmania braziliensis guyanensis (strain MHOM/SR/80/CUMC 1). Thirteen (46.4%) of 28 Lu. diabolica and 3 (42.9%) of 7 Lu. shannoni became infected when fed on a histiocytoma at the base of the tail of a white mouse infected with L. b. guyanensis. Initial development and establishment of the infection was in the hind triangle where short, rounded promastigotes attached in bunches to the hindgut wall

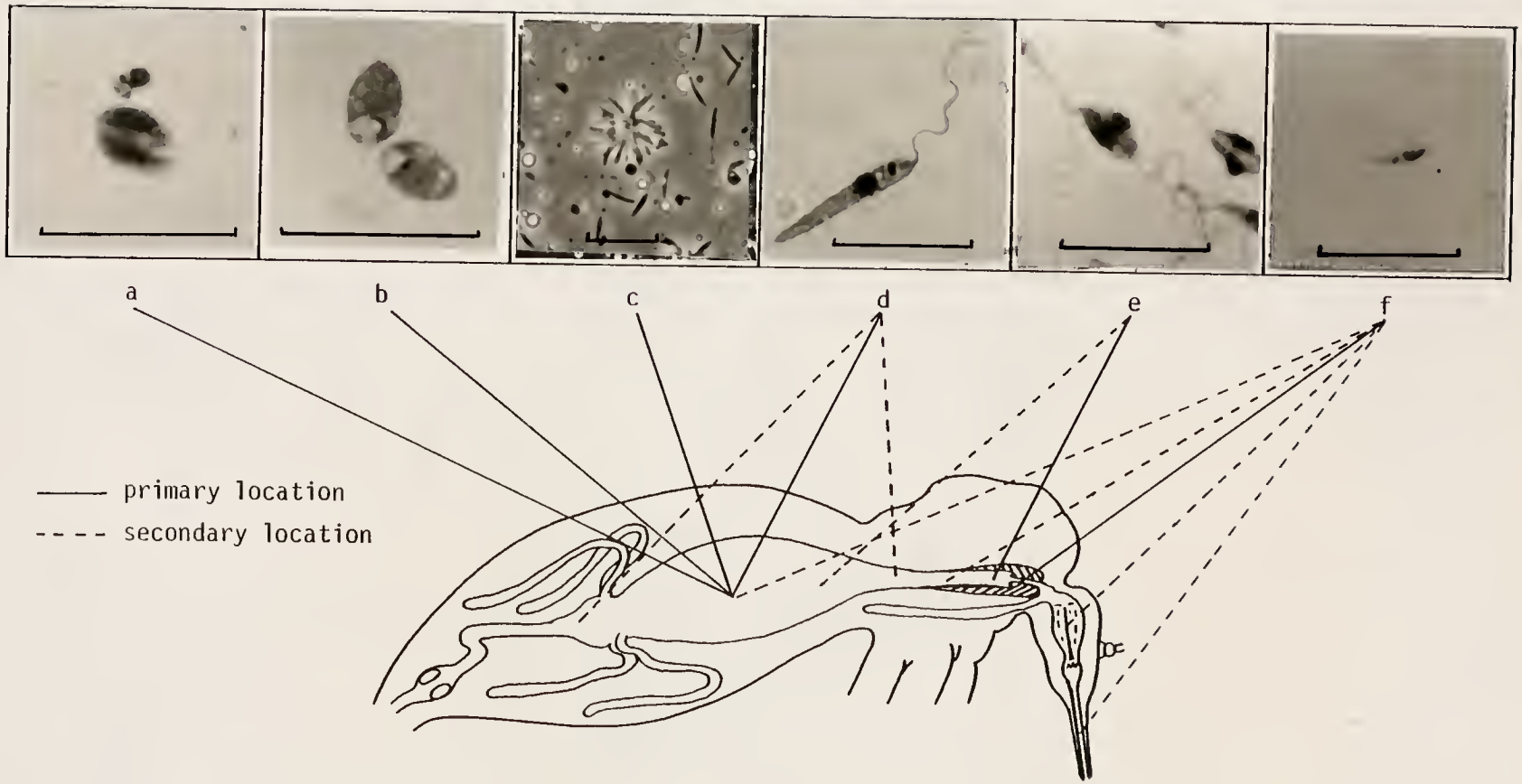


Figure 4-4. Development of Leishmania mexicana in Lutzomyia diabolica: (a) short-ovoid, dividing form, 18-48 hrs postinfective feed; (b) short-ovoid, slightly motile form, 18-48 hrs postinfective feed; (c) rosette of elongated promastigotes, 24-72 hrs postinfective feed; (d) long-slender, highly motile promastigote ("nectomonad"), 48 hrs - death; (e) short-broad, dividing promastigote ("haptomonad"), 3 days - death; (f) short-slender, highly active form ("infective stage"), 5 days - death. See Figure 4-3 for site designations. Bars represent 10 μm.

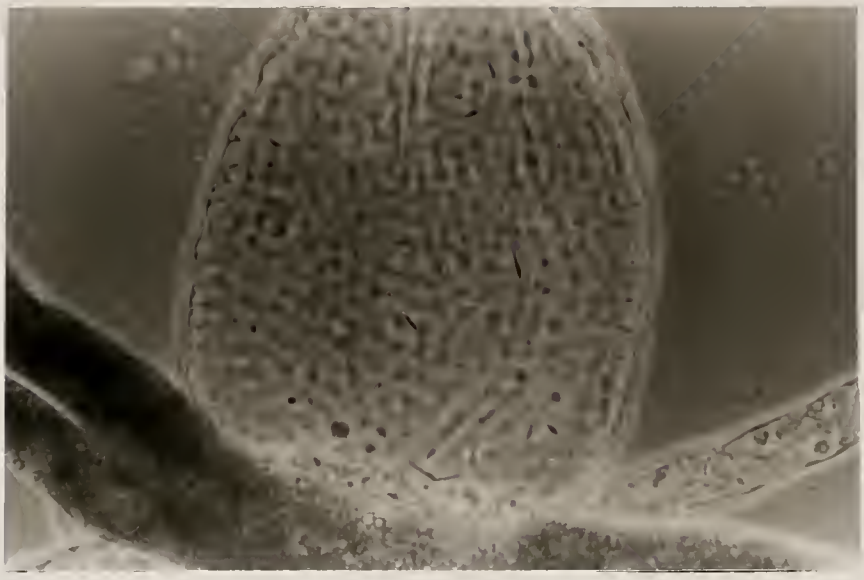


Figure 4-5. Long-slender promastigotes of Leishmania mexicana swimming freely in the hind triangle of an infected sand fly, Lutzomyia diabolica (2-day infection; approx. magn. x 1050).

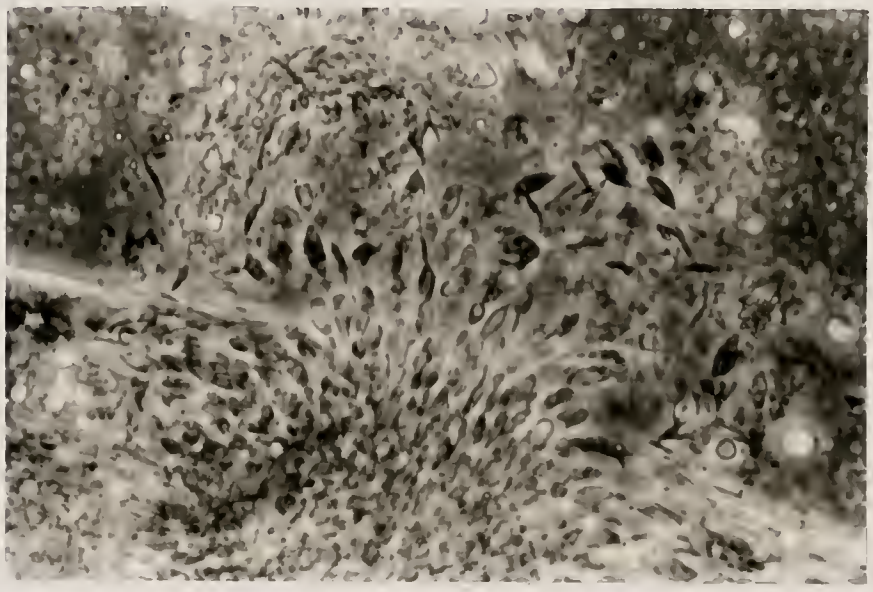


Figure 4-6. Short-broad promastigotes ("haptomonads") of Leishmania mexicana spewing into the dissecting medium at the site of a rupture in the gut wall of a sand fly, Lutzomyia diabolica (3-day infection; approx. magn. 2640).

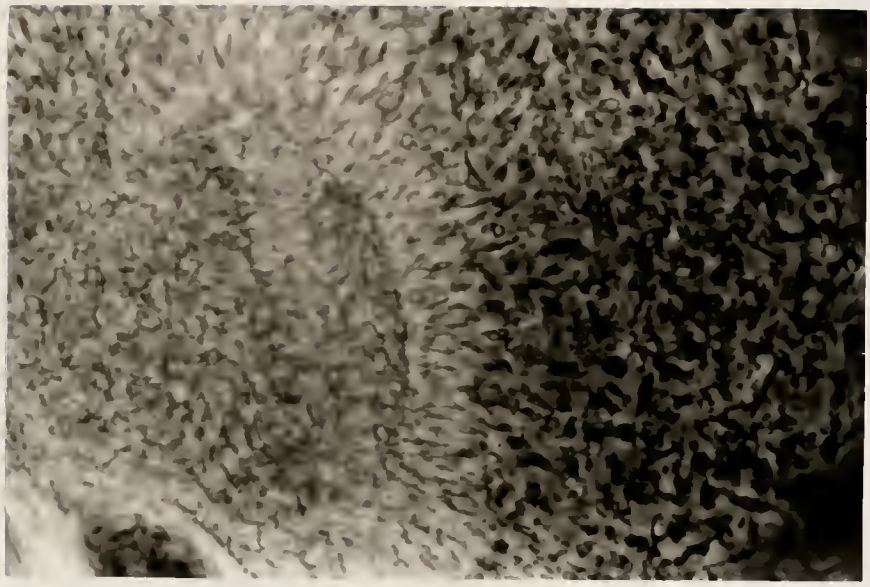


Figure 4-7. Short-broad, dividing promastigotes ("haptomonads") massed at the stomodeal valve in the gut of a female sand fly, Lutzomyia diabolica, infected with Leishmania mexicana (4-day infection; approx. magn. x 2500).

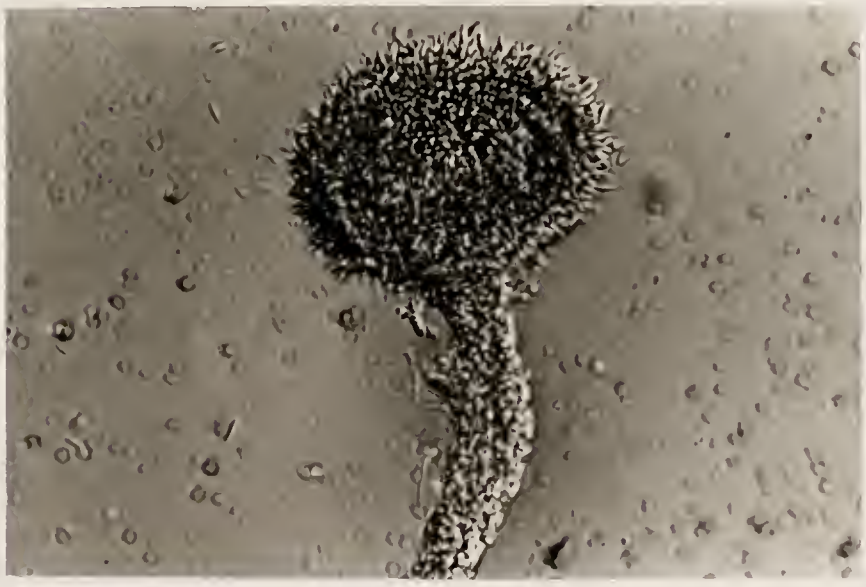


Figure 4-8. Severed anterior aspect of the cardia in a female sand fly, Lutzomyia diabolica, showing massive numbers of Leishmania mexicana promastigotes ("haptomonads") attached to the stomodeal valve (4-day infection; differential interference contrast; approx. magn. x 1050).

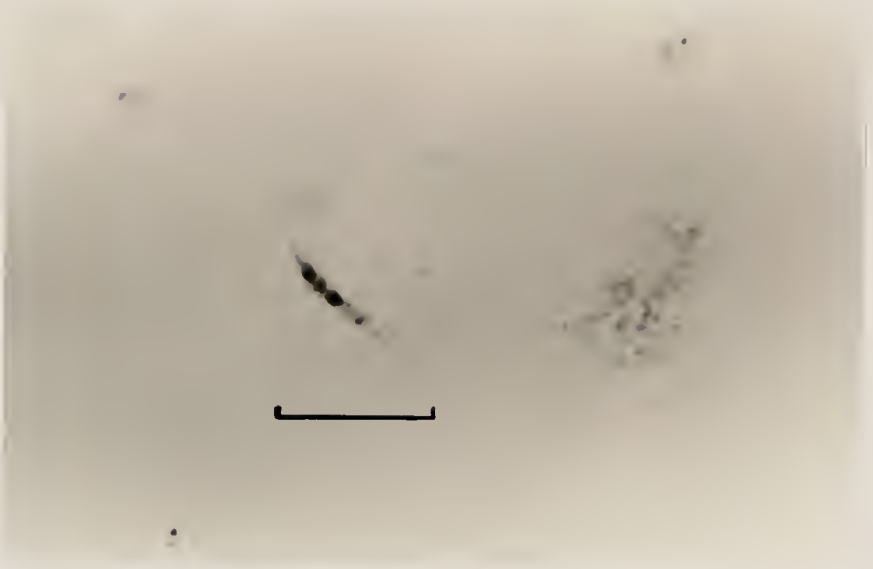


Figure 4-9. Long-slender, nondividing, binucleated promastigote seen in a 5-day infection of Leishmania mexicana in Lutzomyia diabolica (bar represents 10 μm).

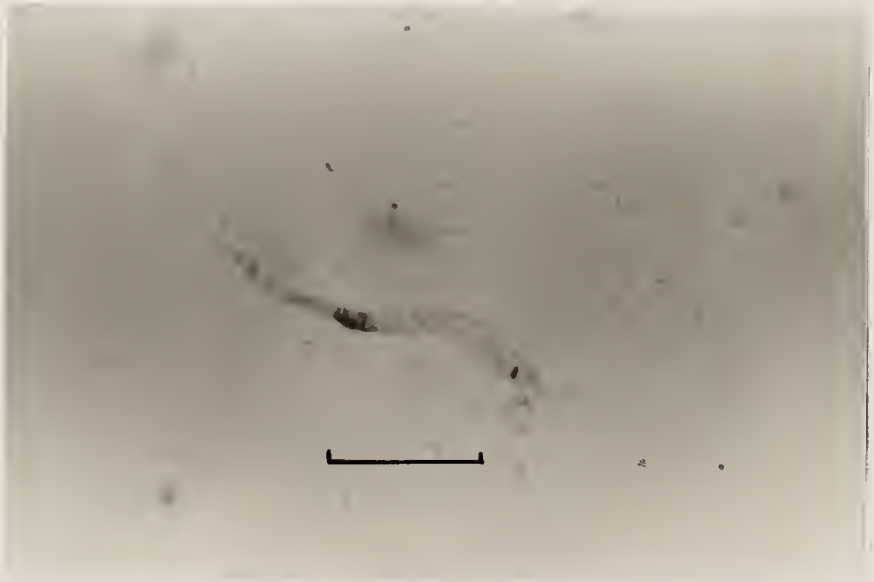


Figure 4-10. Extremely long mononucleated form seen in a 5-day infection of Leishmania mexicana in Lutzomyia diabolica. (bar represents 10 μm).

(Table 4-6). After about three days, long-slender and short-broad promastigotes appeared in the abdominal midgut. In one four-day infection, short- broad dividing forms were packed in the anterior cardia and attached by their flagella to the stomodeal valve, as observed in L. mexicana infections. Parasites were not observed anterior to the stomodeal valve and short-slender, highly active forms were not seen.

Leishmania donovani infantum (strain untyped). Fifty-one (89.5%) of 57 Lu. diabolica became infected when fed on the ear and abdomen of a dog naturally infected with visceral leishmaniasis, apparently due to L. d. infantum. The dog had lived in Sicily for three years. Fourteen (24.6%) of the flies refed on uninfected hamsters. Twelve of those that refed were found to be infected upon dissection. The growth pattern of L. d. infantum in Lu. diabolica paralleled almost exactly that of L. mexicana. These infections and transmission trials with visceral leishmaniasis will comprise the subject of a later paper.

Concurrent infections. Large, peanut-shaped flagellates with large eccentric nuclei were also observed in about 50% of Leishmania-infected sand flies and in some uninfected flies (Fig. 4-11a). Their movement was rather sluggish and they appeared to be more ameboid than the smaller leishmanial forms, frequently changing their shape. They did not occur in large numbers, with rarely more than a dozen seen in a single fly. Without the flagellum they measured about 15 μm in length and about 4 μm in width. The flagellum was about half as long as the body, measuring 8 to 9 μm. Upon fixation in 100% methanol they rounded up, losing their characteristic peanut shape (Fig. 4-11b). Their presence did not appear to be deleterious to either leishmanial

Table 4-5. Distribution of Leishmania mexicana amazonensis (strain untyped) in the alimentary tract of Lutzomyia diabolica dissected post mortem, after feeding on histiocytomas of infected hamsters. Flies were held at 27 ± 1°C and near 100% RH; only flies in which the entire alimentary trace was visible were included in the table.

Days After Infecting Feed	Sand Flies No. (%)	Free in Hindgut No. (%)	Midgut No. (%)	Cardia No. (%)	Pharynx No. (%)	Mouthparts No. (%)
3	4(16.6)	1(25.0)	4(100.0)	3(75.0)	- -	- -
5	2(8.3)	1(50.0)	2(100.0)	1(50.0)	- -	- -
6	10(41.7)	2(20.0)	9(90.0)	4(40.0)	- -	- -
7	2(8.3)	-	2(100.0)	2(100.0)	- -	- -
8	3(12.5)	-	3(100.0)	2(66.7)	- -	- -
9	2(8.3)	-	2(100.0)	2(100.0)	1(50.0)	1(50.0)
10	1(4.2)	-	1(100.0)	1(100.0)	- -	- -

Total Flies = 24

Table 4-6. Distribution of *Leishmania braziliensis guyanensis* (strain MHOM/SR/80/CUMC 1) in the alimentary tract of *Lutzomyia diabolica* dissected *post mortem* after feeding on a histiocytoma on the tail of a white mouse. Flies were held at 27 ± 1°C and near 100% RH; only flies in which the entire alimentary tract was visible were included in the table.

Days After Infecting Feed	Sand Flies No. (%)	Attached to Hindgut No. (%)	Midgut No. (%)	Cardia No. (%)	Pharynx No. (%)	Mouthparts No. (%)
3	8(61.5)	5(62.5)	5(62.5)	- -	- -	- -
4	1(7.7)	1(100.0)	1(100.0)	1(100.0)	- -	- -
5	4(30.8)	3(75.0)	4(100.0)	- -	- -	- -

Total Flies = 13

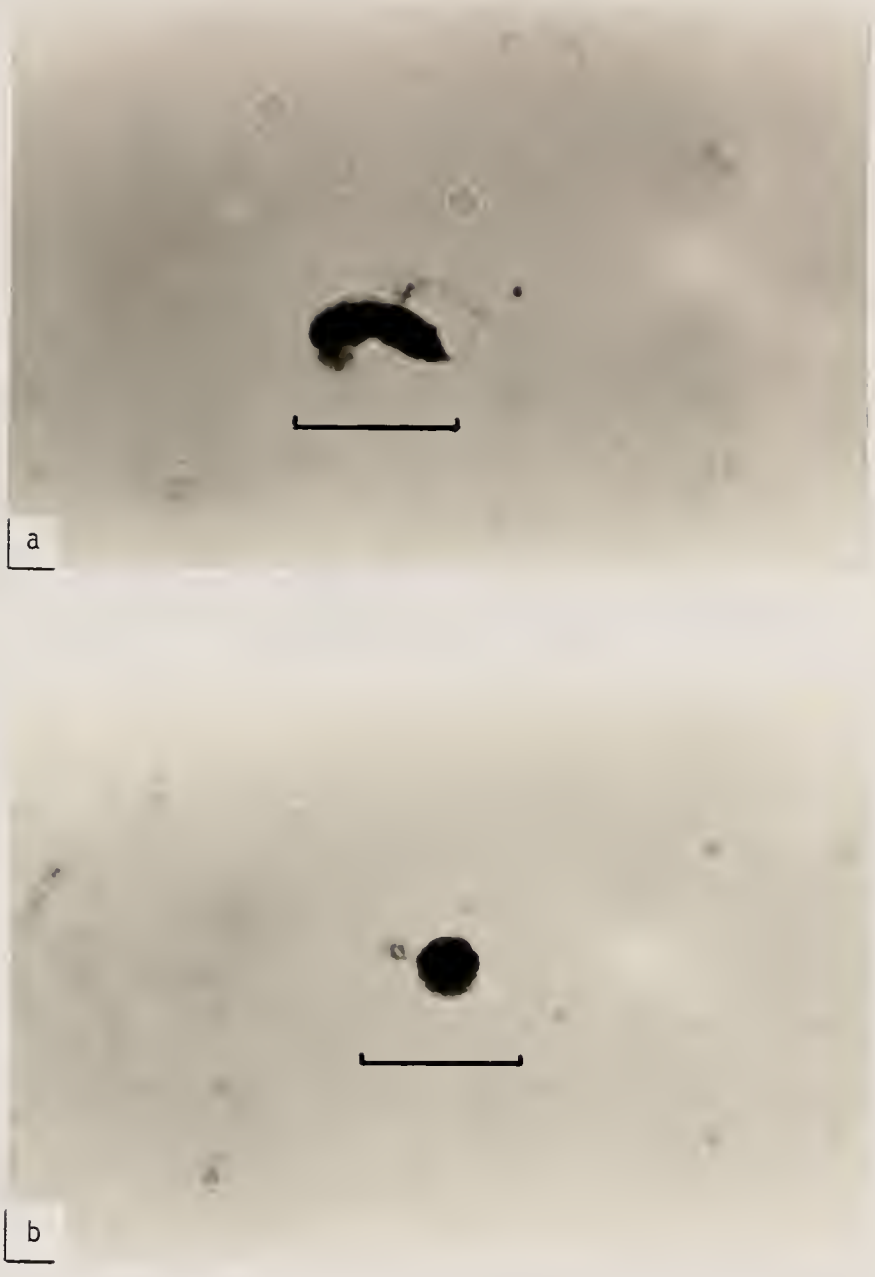


Figure 4-11. Large peanut-shaped flagelate observed in infected and uninfected laboratory-reared Lutzomyia diabolica females: a. normal; b. after fixation in 100% methanol (bars represent 10 μm).

parasites or to the sand fly. On the other hand, concurrent infections with bacteria resulted in rapid degeneration of the leishmanial infection and premature death of the host fly, due to rupturing of the peritrophic sac. The large, peanut-shaped flagellates did not seem to be affected by the presence of the bacteria.

Ultrastructure Studies

Leishmania mexicana (strain WR-411) amastigotes from hamsters infected by the bite of Lu. diabolica were examined with an electron microscope. Up to 17 parasites were observed in cross-sections of a single macrophage (Fig. 4-12). Their shape was elliptical and size was fairly uniform, measuring about 3.5 µm long by 1.5 µm wide. The parasites (Fig. 4-13) were surrounded by a pellicle (P) below which subpellicular microtubules (SM) ran spirally along the longitudinal axis. The number of microtubules counted in transverse sections of amastigotes averaged 89 and ranged from 81 to 97 (n = 43). Other conspicuous organelles included a short flagellum (F), with a typical nine-plus-two microtubule configuration, emerging eccentrically from a flagellar reservoir (FR), a kinetoplast (K), and an electron-dense nucleus (N) (see Gardener, 1974).

Promastigotes of L. mexicana were examined in the midgut and cardia of female Lu. diabolica that had been fixed six days after the infecting blood meal. As expected, a much greater variation in size and shape was observed in promastigotes than in amastigotes. Figure 4-14 shows several promastigotes in transverse section. The pellicle (P) and subpellicular microtubules (SM) surrounding the parasites are

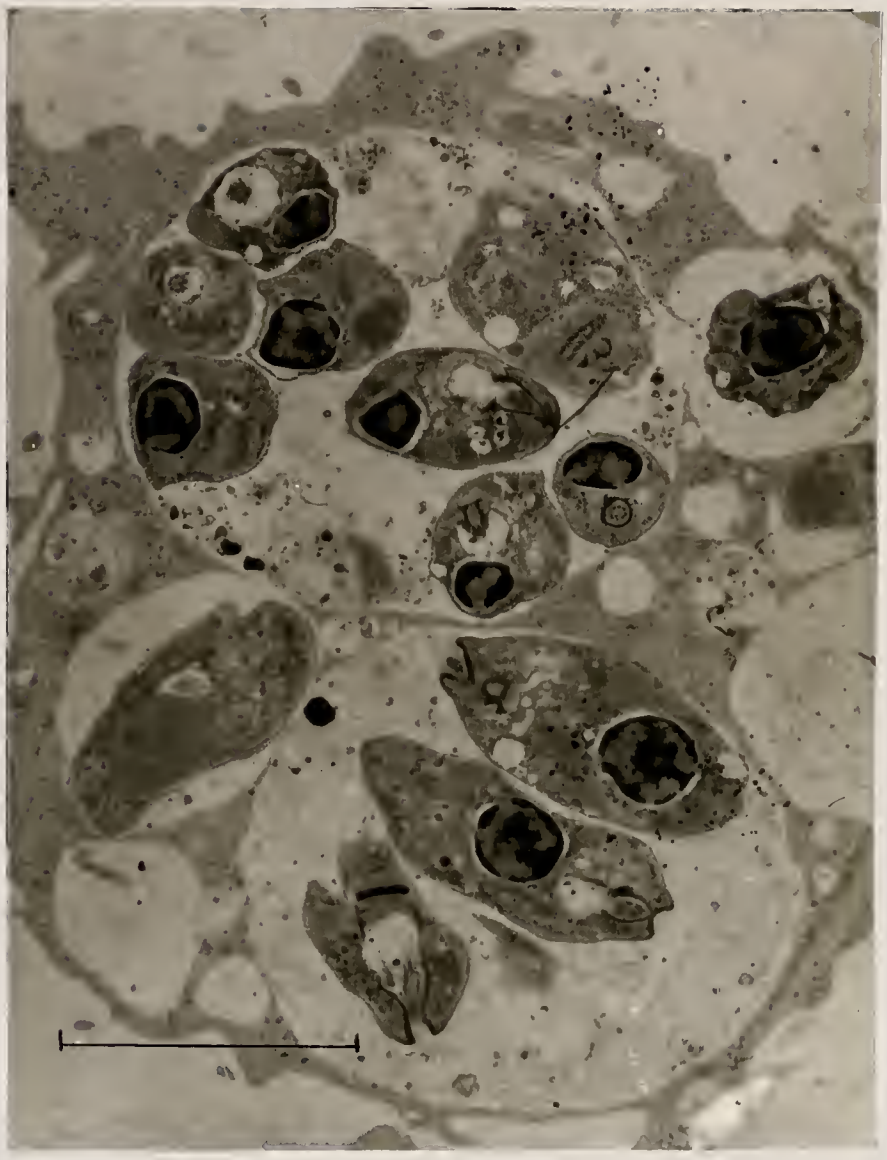


Figure 4-12. Electron micrograph of Leishmania mexicana in large vesicles within a macrophage cell of a hamster infected by the bite of a female Lutzomyia diabolica (approx. magn. x 12,500; bar represents 3 μm).

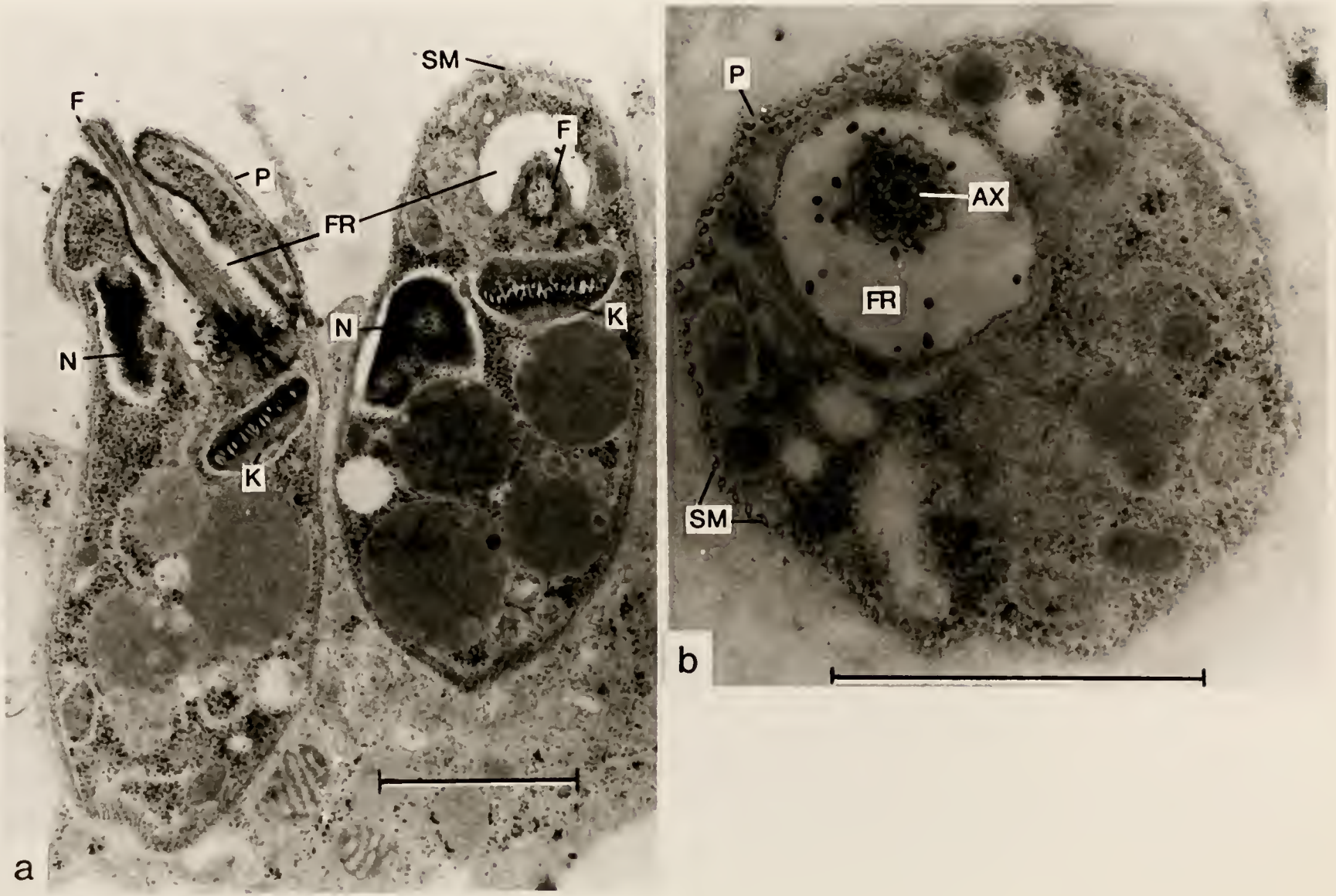
clearly visible. The number of microtubules averaged 86.5 and ranged from 49 to 151 (n = 62), increasing in number with the diameter of the parasite. Conspicuous organelles included the flagellum (F) emerging eccentrically from the flagellar reservoir (FR), kinetoplast (K), mitochondrion (M), golgi apparatus (G), nucleus (N), and dense inclusion vesicles (DV) (Fig. 4-14 and 4-15; see Molyneux et al., 1975).

The flagellum of the promastigote is surrounded by a somewhat corrugated flagellar sheath (FS) (Fig. 4-15). Associated with the flagellar reservoir are four microtubules (RT) which run parallel to its axis (Fig. 4-14). They have the same diameter as the subpellicular microtubules. The axoneme (AX) of the flagellum has a typical nine-plus-two configuration (Fig. 4-14). The flagella of promastigotes in the abdominal midgut were commonly associated with the microvilli (MV) lining the gut wall, although no points of attachment were seen. In the region of the stomodeal valve, where there are no microvilli, many broad promastigotes were attached to the cuticular lining (CT) of the valve by foot-like enlargements, or hemidesmosomes (HD), at the tips of their flagella (Fig. 4-15; see Killick-Kendrick et al., 1974).

Transmission Trials

Five (24%) of 21 hamsters were infected by bites of 25 potentially infected Lu. diabolica (Table 4-7). Of the successful trials, four were single feedings and one was a double feeding. The four flies involved in single feeding transmissions took partial blood meals and the two involved in the double feeding fed to repletion.

Figure 4-13. Electronmicrograph of saggital (a) and transverse (b) sections of Leishmania mexicana amastigotes in macrophage cells of an infected hamster; P = pellicle; SM = subpellicular microtubules; F = flagellum; FR = flagellar reservoir; K = kinetoplast; N = nucleus; AX = axoneme [approx. magn. x (a) 33,440 and (b) 63, 889; bar represents 1 μm].


SM
F
F
P
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N
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AX
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SM
b

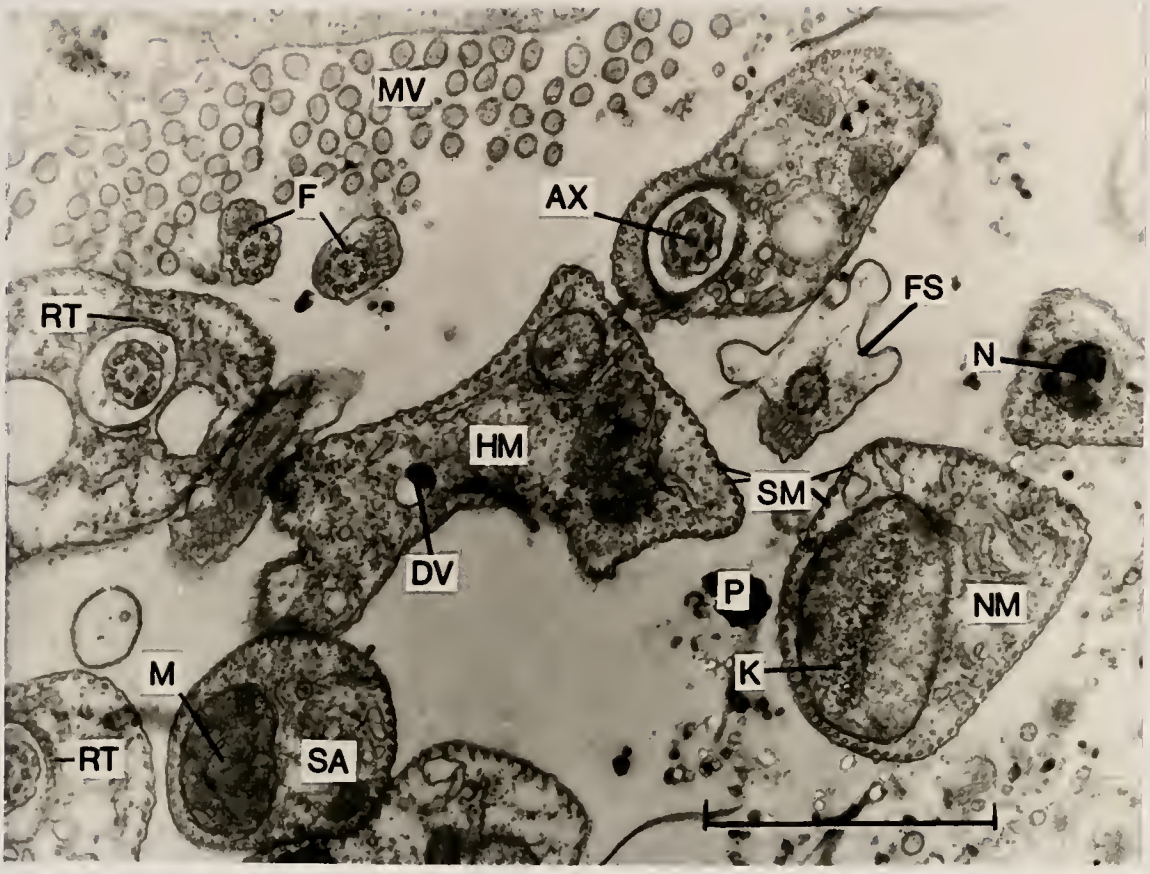
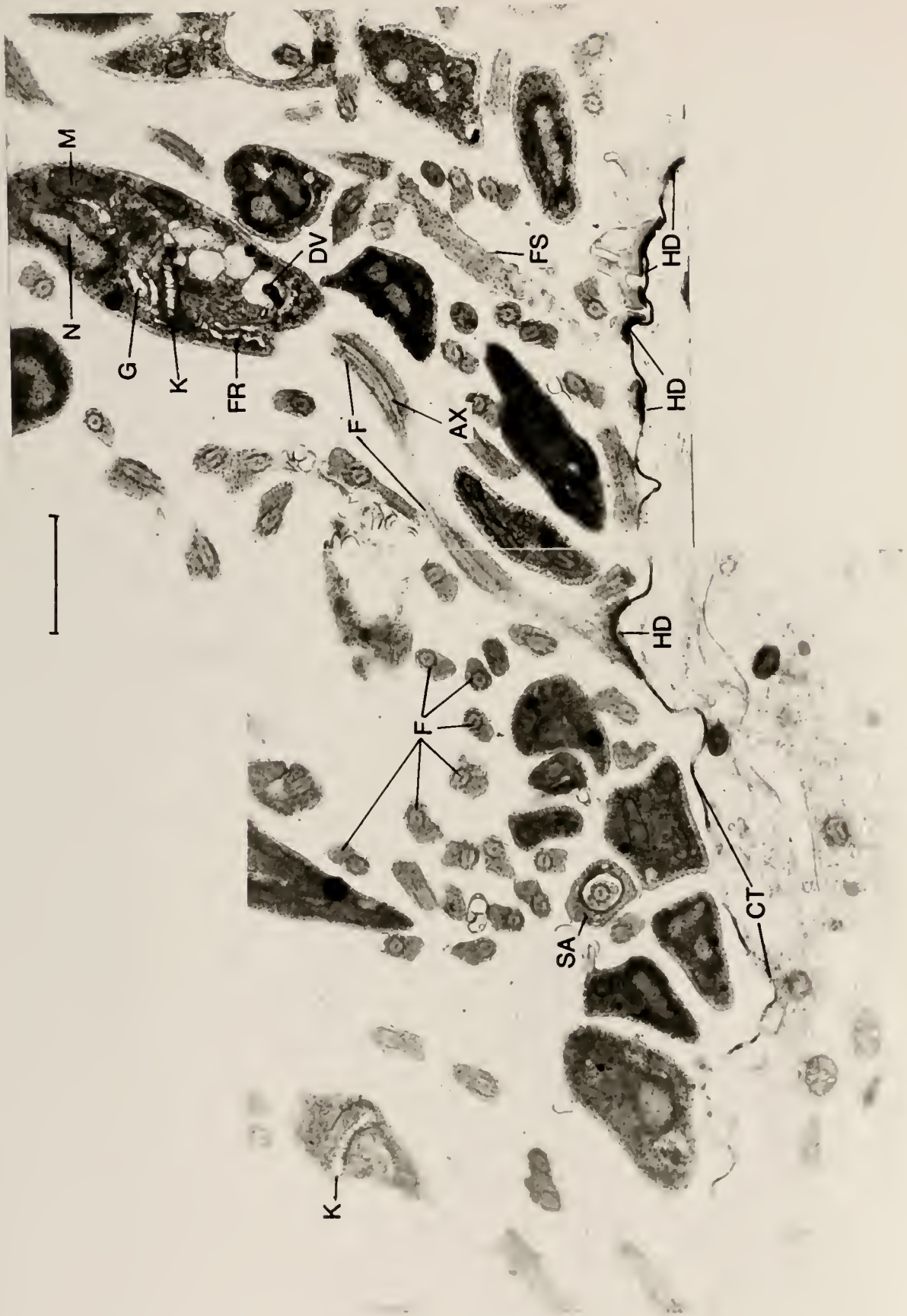


Figure 4-14. Electronmicrograph of Leishmania mexicana promastigotes in the abdominal midgut of an infected Lutzomyia diabolica female; P = pellicle; SM = subpellicular microtubules; F = flagellum; FR = flagellar reservoir; K = kinetoplast; M = mitochondrion; N = nucleus; DV = dense inclusion vesicle; FS = flagellar sheath; RT = reservoir tubules; AX = axoneme; MV = microvilli of the midgut; NM = nectomonad; HM = haptomonad; SA = short-slender, highly active form (approx. magn. x 38,000; bar represents 1 μm).

Figure 4-15. Electronmicrograph of Leishmania mexicana promastigotes in the cardia (thoracic midgut) of an infected Lutzomyia diabolica female; F = flagellum; FR = flagellar reservoir; K = kinetoplast; M = mitochondrion; G = golgi apparatus; N = nucleus; DV = dense inclusion vesicle; FS = flagellar sheath (corrugated); AX = axoneme; CT = cuticular linlining of the stomodeal valve; HD = hemidesmosome (approx. magn. x 20,100; bar represents 1 μm).


M
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SA
CT
K

Table 4-7. Results of transmission trials of *Leishmania mexicana* (strain WR-411) by bites of laboratory-reared *Lutzomyia diabolica*.

Hamster Number	Number of Flies that Fed	Location of Bite(s)	Size of* Blood Meal	Days Between 1st and 2nd Blood Meals	Transmission Status		
					+ or -	Days Til 1° Lesion	Site of Lesion(s)
1	1	outside right ear inside right ear	1x probe #2 BM	7	+	25	outside right ear
2	1	inside right ear inside left ear	#2 BM 1x probe	4	+	52	inside right ear
3	1	inside right ear	5x probe, #2 BM	7	+	56	inside right ear
4	2	insight right ear inside left ear	1x probe, #4 BM 1x probe, #4 BM	6	+	148	left hind foot
5	2	inside right ear	2x probe, #4 BM	9	-	-	-
6	1	inside right ear	#4 BM	13	-	-	-
7	1	inside right ear	#3 BM	9	-	-	-
8	2	inside left ear inside right ear	#4 BM #4 BM	9	-	-	-
9	1	inside right ear	#2 BM	5	+	41	inside right ear
10	1	inside right ear	#3 BM	7	-	-	-
11	1	inside right ear	#4 BM	9	-	-	-
12	1	inside right ear	#3 BM	6	-	-	-
13	1	inside right ear	#4 BM	3	-	-	-
14	1	inside right ear	#4 BM	5	-	-	-
15	1	inside right ear	#4 BM	5	-	-	-
16	1	inside right ear	#4 BM	5	-	-	-

Table 4-7. Continued.

Hamster Number	Number of Flies that Fed	Location of Bite(s)	Size of* Blood Meal	Days Between 1st and 2nd Blood Meals	Transmission Status		
					+ or -	Days Til 1° Lesion	Site of Lesion(s)
17	1	inside right ear	#4 BM	7	-	-	-
18	1	inside right ear	#4 BM	5	-	-	-
19	1	inside right ear	#3 BM	10	-	-	-
20	1	inside right ear	>10x probe, #3 BM	5	-	-	-
21	2	inside left ear inside right ear	#4 BM #4 BM	6	-	-	-

* Size of blood meal: probe = no blood visible; #1 = trace of blood in thorax; #2 = abdomen 1/4 to 1/2 full; #3 = abdomen 2/3 to 3/4 full; #4 = fully engorged, abdomen distended.

Coincidentally, the first female Lu. diabolica infected with L. mexicana transmitted it to an uninfected hamster by a 45-sec bite on the outside of the right ear, seven days after the infecting blood meal. A lesion appeared at the site within 25 days (Fig. 4-16). The second, third, fourth, and ninth trials were also successful, with lesions appearing between 52 and 148 days after the second blood meal. In the fourth trial, two infected females were allowed to begin feeding on the right ear, were interrupted after a short time, then allowed to feed to repletion on the opposite ear. No lesions appeared on either ear, but one appeared on the left hind foot in 148 days after the transmitting bite(s). The mean time between blood meals of Lu. diabolica in successful transmissions was 5.8 days and ranged from four to seven days. Table 4-8 gives the results of postfeeding dissections of female Lu. diabolica involved in transmission of L. mexicana (strain WR-411) to hamsters. Of the 19 flies that did not transmit Leishmania, three were negative for parasites, nine had heavy bacterial infections and only remnants of leishmanial infections were seen. Four of the bacterial infections resulted in ruptured peritrophic sacs. The remaining six had light to heavy infections of Leishmania, one of which showed a rather heavy mouthpart infection.

In all, five (100%) of five hamsters were infected with L. mexicana (strain WR-411) by bites of Lu. shannoni (Table 4-9). In the first trial, 12 potentially infected sand flies fed between six and eight days postinfective feed on the ears of an uninfected hamster. Only four of the 12 took visible blood meals. The others probed several times but blood was not seen in their guts. A cutaneous lesion appeared 14 days after the first infected fly fed on the back

Figure 4-16. Lesion on the ear of a hamster approximately two weeks after it was bitten by a female Lutzomyia diabolica that was experimentally infected with Leishmania mexicana.

Table 4-8. Results of postfeeding dissections of laboratory-reared Lutzomyia diabolica sand flies involved in experimental transmissions of Leishmania mexicana (strain WR-411) to Syrian hamsters.

Sand Fly Number	Age of Infection	Infected + or -	Location of Parasites				
			Hindgut	Midgut	Cardia/ S. Valve[1]	Pharynx	Mouthparts
			Hamster #1				
ILD-1	10	+	-	+	+	-	-
			Hamster #2				
ILD-99	4	+	-	+	+	-	-
			Hamster #3				
ILD-119[2]	6	-	-	+	+	-	-
			Hamster #4				
ILD-134	6	+	-	+	+	-	-
ILD-135	6	+	-	+	+	-	-
			Hamster #5				
ILD-227	5	+	-	+	+	-	+

1. S. Valve = Stomodeal valve.

2. Heavy bacterial infection at time of dissection, very few Leishmania seen.

Table 4-9. Results of transmission trials of Leishmania mexicana (strain WR-411) by bites of laboratory-reared Lutzomyia shannoni.

Hamster Number	Number of Flies that Fed	Location of Bite(s)	Size of Blood Meal[1]	Days After Infecting Blood Meal	Transmission Status		
					+ or -	Days Til 1° Lesion	Site of Lesion(s)
1	12	abdomen	6x probe,[2] 1x #1 BM	5 at 6 days 4 at 7 days 2 at 8 days	+	14	1 on left ear 4 on right ear
		left ear	2x probe[2]				
		right ear	2x probe,[2] 1x #1 BM, 1x #2 BM, 1x #3 BM				
2	15	left ear	6x probe,[2] 1x #3 BM, 1X #2 BM	1 at 6 days 9 at 7 days 5 at 8 days	+	>30	4 inside left ear 4 inside right ear
		right ear	4x probe, 2x #2 BM, 1x #3 BM				
3	1	inside right ear	#2 BM	8 days	+	<36	1 inside right ear
4	1	scrotum	#1 BM	5 days	+	<43	1 on scrotum
5	1	inside right ear	#1 BM	5 days	+	22	1 inside right ear

1. Size of blood meal: probe = no blood visible; #1 = trace of blood in thorax; #2 = abdomen 1/4 to 1/2 full; #3 = abdomen 2/3 to 3/4 full; #4 = fully engorged, abdomen distended.
2. Some flies that took visible blood meals also probed at other locations.

of a hamster's right ear. Four other lesions appeared shortly thereafter, corresponding to the sites of the other infective bites.

Similarly, in the second transmission trial, 15 potentially infected flies probed or fed on the ears of an uninfected hamster six to eight days after the infecting blood meal. This trial resulted in eight lesions, four on each ear, appearing within 30 days. Seven of the flies probed only, eight took full or partial blood meals, and several that took blood also probed first at different locations before inserting their mouthparts.

The remaining three trials with Lu. shannoni were individual feedings, where only one potentially infected female was allowed to feed, five to eight days after the infecting meal, each on an uninfected hamster. All three took partial blood meals and all bites resulted in transmission of Leishmania, with lesions appearing between 22 and 43 days (Table 4-9). Two of these latter transmissions were from bites that drew only a trace of blood. The mean interval between first and second blood meals of Lu. shannoni involved in these transmissions was 6.9 days, and ranged from five to eight days. Table 4-10 shows the results of postfeeding dissections of female Lu. shannoni involved in the transmissions.

No transmissions were obtained in limited refeeding of flies infected with L. m. amazonensis or L. b. guyanensis. Results of trials with L. d. infantum will be forthcoming due to the long incubation period of the parasite in hamsters.

Table 4-10. Results of postfeeding dissections of laboratory-reared Lutzomyia shannoni sand flies infolved in experimental transmissions of Leishmania mexicana (strain WR-411) to Syrian hamsters.

Sand Fly Number	Age of Infection	Infected + or -	Location of Parasites				
			Hindgut	Midgut	Cardia/ S. Valve[1]	Pharynx	Mouthparts
			Hamster #1				
ILS-38	5	+	+	+	+	-	-
ILS-39	8	+	+	+	+	-	+
ILS-43	5	+	-	+	+	-	-
ILS-32	7	+	-	+	+	+	+
ILS-10	8	+	-	+	+	+	-
ILS-52[2]	7	+	-	+	+	-	-
ILS-53[2]	7	-	-	-	-	-	-
ILS-59	6	+	-	+	+	-	-
ILS-61	7	+	-	+	+	-	+
ILS-119[2]	7	+	+	+	+	+	+
ILS-132	6	+	+	+	+	+	+
ILS-136[2]	7	+	-	+	+	+	-

Table 4-10. continued

Sand Fly Number	Age of Infection	Infected + or -	Location of Parasites				
			Hindgut	Midgut	Cardia/ S. Valve[1]	Pharynx	Mouthparts
			Hamster #2				
ILS-52[2]	7	+	-	+	+	-	-
ILS-53[2]	7	-	-	-	-	-	-
ILS-65	6	+	-	+	+	-	-
ILS-93	10	-	-	-	-	-	-
ILS-99	13	+	+	+	+	+	+
ILS-119[2]	7	+	+	+	+	+	+
ILS-123	8	+	+	+	+	+	+
ILS-125	7	+	+	+	+	+	+
ILS-130	7	+	-	+	-	-	-
ILS-136[2]	7	+	-	+	+	+	-
ILS-150	8	+	-	+	+	-	+
ILS-151	7	+	-	+	+	+	+
ILS-154	7	+	-	+	+	-	+

Table 4-10. continued

Sand Fly Number	Age of Infection	Infected + or -	Location of Parasites				
			Hindgut	Midgut	Cardia/ S. Valve[1]	Pharynx	Mouthparts
ILS-155	7	+	-	+	+	-	+
ILS-159	8	+	+	+	+	-	+
			Hamster #3				
ILS-162	8	+	-	-	-	+	+
			Hamster #4				
ILS-165	5	+	-	+	+	+	+
			Hamster #5				
ILS-167	5	+	-	+	+	+	-

1. S. Valve = Stomodeal valve.

2. Fed on both hamsters 1 and 2.

Discussion and Conclusions

Infection of Sand Flies and Parasite Development

General observations. A key factor in successful experimental transmission studies is obtaining a high leishmanial infection rate to produce large numbers of heavily infected sand flies, thus increasing the probability of transmission. Several methods of infecting sand flies from histiocytomas on hamsters have been used with varying degrees of success. Johnson and Hertig (1970) restrained sand flies in small cages made of bolting-cloth stretched over a wire frame. The cages were fitted over the head or leg of the infected hamster and fastened with a drawstring, thus confining the flies to the general area of the lesion. They obtained infection rates of 22 to 66%, with four leishmanial strains in Lu. sanguinaria (Fairchild and Hertig) and Lu. gomezi (Nitz.), respectively. Lainson et al. (1977) obtained only a 10% infection rate in Lu. longipalpis (Lutz and Neiva) following blood meals on L. chagasi-infected hamsters. Because of this low infection rate they resorted to a less natural method of feeding the flies on amastigote suspensions through chick-skin membranes, and obtained a 100% infection rate. Tesh and Modi (1984) fed amastigote suspensions of six species of Leishmania to Phlebotomus papatasi Scopoli and Lu. longipalpis through a chick-skin membrane and obtained high rates of infecton (73-100%) with all but two of the Leishmania species. Christensen and Herrer (1980) obtained 88.9% and 82.6% infection rates in Lu. sanguinaria and Lu. gomezi, respectively, by feeding the flies on anesthetized hamsters with histiocytomas of the nose, feet and ears. Perkins (1982) found that when an anesthetized

hamster with histiocytomas due to L. mexicana was placed inside a colony chamber, only 10.4% of the females that fed became infected. When the flies were restrained in 120 ml screen-lidded feeding containers, and the screen pressed directly against a histiocytoma, 96.3% of those that fed became infected. This latter method was used in the present study to obtain L. mexicana infection rates of 87.9% and 94.8% in Lu. diabolica and Lu. shannoni, respectively. This insured that a large number of infected flies were available for transmission studies.

The time required by female sand flies to complete the infecting blood meals on leishmanial histiocytomas averaged five minutes longer than that observed for first, noninfective feedings on uninfected hamsters. This may be due to restricted blood flow within the histiocytoma. Infected flies feeding on ears of uninfected hamsters fed to repletion in about the same time as uninfected flies.

The death of a small percentage of infected flies from ruptured peritrophic sacs was expected. This was also observed in uninfected, laboratory-reared Lu. diabolica. Endris (1982) reported mortalities of 6.6 and 9.9% due to peritrophic sac rupture in three laboratory generations of Lu. anthophora. He stated that in these flies, death followed feeding within one to four days, with 75% dead within 24 hrs. He hypothesized that death occurred as a result of changes in the hemolymph osmoticum and release of digestive enzymes. Adler and Theodor (1957) stated that the alimentary tract of fed and unfed sand flies is bacteriologically sterile and that chance bacterial contamination interferes with digestion of the blood meal and is fatal to the insect. Lu. diabolica were probably contaminated with bacteria

during feeding, either from the surface of the host's skin, or from secondary infections in bacterially contaminated leishmanial lesions. Disinfecting the feeding surface with alcohol or administering antibiotics to the hamster may be useful precautions to reduce bacterial contamination.

Leishmania mexicana (strain WR-411) and Leishmania mexicana amazonensis (strain untyped). Brief descriptions of the development of several L. mexicana strains (to include L. m. amazonensis) in Central and South American sand flies, were provided by Strangways-Dixon and Lainson (1966), Ward et al. (1977), Lainson and Shaw (1977), and Christensen and Herrer (1980). These are consistent with the development of L. mexicana (strain WR-411) and L. m. amazonensis (strain) in Lu. diabolica, and are typical of the suprapylarian leishmanias. According to Lainson and Shaw (1979), leishmanias in this taxonomic section have lost the primitive hindgut development in the sand fly host. Flagellates are restricted to the abdominal midgut and cardia, with later anterior migration to the pharynx and mouthparts. Transmission is inoculative by the bite of the sand fly.

The failure of L. m. amazonensis promastigotes to attach to the stomodeal valve, and lack of massive midgut and cardia infections in Lu. diabolica, may indicate that this sand fly is a less suitable host than Lu. flaviscutellata (Mangabeira), the natural vector of the parasite (Ward et al., 1977). The small number of infection trials (26) is insufficient to formulate conclusions as to the ability of Lu. diabolica to harbor this subspecies.

Strangways-Dixon and Lainson (1966), working in Belize, studied the development of two strains of L. mexicana in local Lutzomyia

species. They noted that although cutaneous lesions on which sand flies fed contained very large numbers of amastigotes, surprisingly few leishmaniae were ingested, and a long search was needed to find them in the sand fly host. This may account for the failure to see amastigotes in dissections made within 18 hrs after the infecting feed in Lu. diabolica.

Strangways-Dixon and Lainson (1966) said that the first indication of amastigote development in the insect host was between 12 and 24 hrs when a few large, highly vacuolated, aflagellate forms were observed. They also suggested that this early growth phase was followed by a period of binary fission of the amastigote before development of the flagellum. No clear evidence of this early amastigote development was observed in L. mexicana-infected Lu. diabolica, although dividing forms with extremely short flagella were seen. The above authors observed transformation into promastigotes between 24 and 36 hrs after the infecting feed in flies maintained at 25 C and 100% RH. The earlier appearance of promastigotes between 18 and 24 hrs after the infecting feed in Lu. diabolica may be due to a higher holding temperature of 27°C, or it may reflect genetic differences between strains. The early promastigotes figured by Strangways-Dixon and Lainson (1966) are similar in size and appearance to those observed in the present study.

Killick-Kendrick (1979) maintained that leishmanias have basically three morphological forms in the fly, amastigotes, promastigotes, and paramastigotes. The dominant form is the promastigote, of which he observed two types in L. m. amazonensis.

One type, for which he resurrected the term "nectomonad" corresponds in size and shape to the long-slender, highly motile forms observed in the midguts of L. mexicana-infected Lu. diabolica and Lu. shannoni two to three days postinfective feed. The second type appeared as the promastigotes migrated forward to colonize the thoracic midgut (cardia). This type corresponds to the short-broad, dividing forms seen after three days, packed behind and attached to the stomodeal valve. For these, Killick-Kendrick (1979) revived the name "haptomonad." According to him, the change from nectomonad to haptomonad is a modification associated with establishment of infection in a different part of the gut, and represents a change in habitat. It appears to be associated with the indispensable step of forming hemidesmosomes which attach to the cuticular parts of the stomodeal valve (Fig. 4-15).

The significance of the nondividing, binucleated promastigote and the extremely long promastigote (Fig. 4-9 and 4-10) observed in a five-day infection of L. mexicana in Lu. diabolica remains obscure. Molyneux (1983) reported that "giant multinucleate forms" and other bizarre forms have been observed in other trypanosomatids (Trypanosoma brucei and T. rangeli) and suggested that they could possibly have something to do with genetic exchange in these parasites.

Killick-Kendrick (1979) observed that L. m. amazonensis divides in the abdominal midgut of Lu. longipalpis but never in the cardia. In this respect, L. mexicana (strain WR-411) in Lu. diabolica behaved differently, since division occurred in forms in the abdominal midgut, cardia, and attached to the stomodeal valve. Lainson et al. (1977)

also found dividing promastigotes of L. chagasi (= L. d. chagasi) in the thorax as well as in the abdominal midgut of Lu. longipalpis.

Massive infections of the cardia and stomodeal valve were described by Killick-Kendrick (1979), which appear identical to those seen in three-day and older infections of L. mexicana in Lu. diabolica, to include the gel-like matrix that joins masses of promastigotes together. He suggested that this matrix might be of parasitic origin. Its significance remains obscure.

Killick-Kendrick (1979) stated that the commonest morphological form in the foregut and hindgut of the sand fly is the "paramastigote", a round or oval parasite with the kinetoplast level with or posterior to the nucleus, and a free flagellum. Although forms that roughly fit this description were seen in L. mexicana infections of Lu. diabolica, they were so far outnumbered by "haptomonads" and "nectomonads" as to be inconspicuous.

Although parasites were seen on one occasion in the diverticulum, infections in this site are generally assumed to be aberrant and not a normal part of the life cycle (Killick-Kendrick, 1979). The diverticulum acts as a receptacle for sugar taken by the sand fly and may play a role in osmoregulation, but does not participate directly in digestion of the blood meal. According to Killick-Kendrick (1979, infections of the diverticulum may arise in two ways: During the act of feeding, infected blood may leak into it, or parasites in the esophagus may be swept into the diverticulum as the fly takes solutions of sugar.

Other workers have described short-slender, highly active promastigotes with long flagella, the appearance of which coincides

with the invasion of the pharynx and proboscis, in several combinations of parasite and fly (Killick-Kendrick, 1979). Nonetheless, there is no universally accepted opinion on the form of Leishmania deposited in the skin by the infected fly. Adler and Theodor (1931) described forms in the proboscis in late infections of L. d. infantum in P. perniciosus Newstead as very short flagellates with flagella longer than the body. They considered these the end point of the life cycle in the sand fly and felt that under natural conditions they were the most likely to enter the vertebrate host. Adler and Ber (1941) measured flagellates (L. tropica), presumed to be proboscis forms, found in a smear of fluid seen emerging from the mouthpart puncture immediately after a female P. papatasi had fed on a human volunteer. They measured 6.5 µm to 13 µm in length without the flagellum; the flagella were 13 to 24 µm long. These measurements are somewhat longer than the dimensions of proboscis forms of L. mexicana found in Lu. diabolica and Lu. shannoni. Killick-Kendrick (1979) concluded that since no other morphological form has been seen in the proboscis in Leishmania-infected sand flies, the description of Adler and Theodor (1931) must apply to all mammalian leishmanias.

It is uncertain which parasites are the precursors of the short-slender, highly active proboscis forms. Killick-Kendrick (1979) said that they originate from paramastigotes attached to the cuticular lining of the pharynx. He likened the pharynx to the "base camp" and the proboscis to the "summit." He then suggested a sequence of parasite development as follows: multiplication in the midgut or hindgut (depending on the species); migration forward and attachment of promastigotes to the stomodeal valve; cessation of division and

migration to the pharynx accompanied by a morphological change to sessile paramastigotes; metamorphosis of a few paramastigotes to short-slender, highly active proboscis forms; and a final anterior migration to the mouthparts.

Since few paramastigotes of L. mexicana were observed in the pharynx of Lu. diabolica, and were not obvious in the cardia and stomodeal valve, a "paramastigote" precursor is open to question, at least in the parasite-host combination used in this study. It is suggested that the broad promastigotes attached to the stomodeal valve (haptomonads) may be the precursors of the proboscis forms, putting the "base camp" at the valve rather than in the pharynx. This agrees with a report by Adler and Theodor (1931) that proboscis forms of L. d. infantum in P. pernisciosus first appeared in the thoracic midgut (cardia). Furthermore, it appears that more than just a "few" of these precursors metamorphose to short-slender, highly active forms, since in some dissections they numbered in the thousands, and instead of a directed movement from "base camp" (stomodeal valve) to "summit" (proboscis), they moved randomly in all directions. This is substantiated by their presence in all parts of the alimentary tract in some old infections and suggests that the term "proboscis form" is a misnomer.

Leishmania braziliensis guyanensis (strain MHOM/SR/80/CUMC 1). The small sample of flies infected with L. b. guyanensis (strain MHOM/SR/80/CUMC 1) was too small for any conclusions to be drawn about the ability of Lu. diabolica to harbor the parasite. The high percentage of hindgut infections (Table 4-6) is consistent with findings of Johnson and Hertig (1970) that all Panamanian strains of

L. braziliensis, whether isolated from man, other mammals, or wild sand flies, produced infections in Lu. sanguinaria and Lu. gomezi in the hind triangle (Fig. 4-3). This behavior is typical of the peripylarian leishmanias. Members of this group maintain an obligate hindgut development in their sand fly hosts, but also migrate anteriorly as in the suprapylaria (Lainson and Shaw, 1979). The development of a massive infection at the stomodeal valve four days postinfection in Lu. diabolica indicates a potential for maintaining the complete life cycle of the parasite. This species of Leishmania does not occur naturally in the USA.

Leishmania donovani infantum (strain untyped). The high infection rate and apparently normal suprapylarian development of L. d. infantum in Lu. diabolica is remarkable, since this sand fly is not a natural invertebrate host for the parasite. The results of the present studies with this parasite in Lu. diabolica will clearly be of interest in view of reports by Anderson et al. (1980) of an endemic focus of canine leishmaniasis in north central Oklahoma. Ultrastructural studies of amastigotes isolated from these infections indicate that the parasite is morphologically similar to L. donovani (Kocan et al., 1983). In addition, radiorespirometry tests indicated that the Oklahoma isolates are similar to L. d. infantum (Kocan et al., 1983). These findings underscore the need for further study of Lu. diabolica's competence for vectoring visceral leishmaniasis.

Concurrent infections. Young and Lewis (1977) reviewed the pathogens of Psychodidae, some of which are rarely present and of little consequence. Others may be found in a high proportion of a sand fly population and could be important factors affecting the

development and subsequent transmission of Leishmania in nature, or in laboratory-reared flies (Killick-Kendrick, 1979). The large, peanut-shaped flagellates with large eccentric nuclei (Fig. 4-11), observed in infected and uninfected Lu. diabolica and Lu. shannoni, do not seem to inferfere with Leishmania development in the sand fly and are apparently harmless monoxenous commensals. Their identity remains obscure and further study is needed to determine their complete life cycle.

Ultrastructure Studies

Gardener (1974) and Veress et al. (1980) indicated that ultrastructural studies of amastigotes have shown that cell size and number of subpellicular microtubules may be useful morphological parameters for differentiating between visceral and cutaneous leishmanial forms. Kocan et al. (1984), working with isolates of visceral leishmaniasis from dogs naturally infected in Oklahoma, compared subpellicular microtubule counts for Oklahoma dog (OKD) isolates with information published on two visceral human strains, VL strain (Veress et al., 1980) and LV23 strain (Gardener et al., 1977). Mean microtubule counts for the OKD, VL, and LV23 isolates were 67, 91, and 81, respectively, with ranges of 51 to 82 (OKD), 68 to 116 (LV), and 58 to 118 (LV23). Based on these numbers, and the mean maximum diameters, Kocan et al. (1983) concluded that the OKD strain was morphologically similar to L. donovani.

In the present study, the mean microtubule number (89) compares well with that of the VL isolate described above (mean = 91), but the range, 81 to 97, is much narrower. Other authors (Adler, 1964) have

reported microtubule numbers in L. mexicana of 130 to 200, about twice the number found in this study, indicating that the reliability of microtubule counts as a means of differentiating between visceral and cutaneous leishmanial forms is doubtful.

Molyneux et al. (1975) examined the ultrastructure of L. m. amazonensis promastigotes in the midgut and pharynx of Lu. longipalpis. They said that the distance between subpellicular microtubules is constant and therefore the microtubule count is not a specific characteristic of Leishmania but is a function of the diameter of the parasite. It follows that, in forms in the sand fly, the microtubule number is not a reliable character for separating species, but may be useful in separating developmental forms, providing all counts are made at the same level within the body of the parasite, e.g. at the level of the nucleus or kinetoplast.

Molyneux et al. (1975) and Killick-Kendrick (1979) reported a range of microtubule numbers in nectomonads (long-slender forms) of 76 to 96 and in haptomonads (shorter and broader forms) of 115 to 138. They did not give microtubule counts for the short-slender, highly active pharyngeal and proboscis forms. Electronmicrographs of L. mexicana (strain WR-411) in the cardia of Lu. diabolica reveal a wide variety of sizes and shapes. The range of microtubule numbers (49 to 151) is broader than the combined ranges of nectomonads and haptomonads (76 to 138) reported by Molyneux et al. (1975) and Killick-Kendrick (1979). The wider range reflects the three morphological forms commonly seen in the cardia of Lu. diabolica in 6-day infections of L. mexicana, with the short-slender, highly active

forms having microtubule counts falling at the lower end of the range, between about 49 and 65.

Based on descriptions and microtubule number provided by the previously mentioned authors, the parasite in the lower-right hand corner of Figure 4-14, with the large kinetoplast, represents a nectomonad (NM) (78 microtubules). The larger irregular shaped parasite in the center of the electronmicrograph has 125 microtubules and probably represents a haptomonad (HM). The smaller parasite, round in cross section, in the lower-left hand corner of the figure has only 61 microtubules and may represent one of the short-slender, highly active forms (SA). The parasite in the lower-left portion of Figure 4-15 may also be a short-slender, highly active form (SA).

The descriptions provided by Molyneux et al. (1975) and Killick-Kendrick (1979) of ultrastructural features within the bodies of the leishmanial parasite are consistent with those seen in L. mexicana (strain WR-411). Corrugations in the flagellar sheath were also seen by these authors and tentatively attributed to the method of fixation. The four microtubules associated with the flagellar reservoir (RT) (Fig. 4-14) apparently extend posteriorly through the cytoplasm, past the kinetoplast towards the nucleus. Their relationship to the nucleus is obscure (Molyneux et al., 1975).

Foot-like modifications of the flagellum associated with attachment of Leishmania to the gut wall and stomodeal valve, called hemidesmosomes (HD) (Fig. 4-15), were described by Killick-Kendrick et al. (1974). Flagellar adhesion by hemidesmosomes seems to be most efficient when it is to cuticular surfaces. According to Brooker (1971), it enables the parasite to maintain a series of physiologically

favorable positions where they may undergo their proper cyclical development. Killick-Kendrick et al. (1974) suggested that the food ingested by infected flies may have an affect on the anterior spread of promastigotes by enhancing or interfering with attachment and subsequent colonization of the anterior part of the fly.

More extensive investigations of the ultrastructure of Leishmania in Lu. diabolica are needed to characterize all morphological forms at various times throughout their development in the alimentary tract of the insect. Special emphasis should be directed to describing the short-slender, highly active forms, which may represent a morphologically-distinct infective stage.

Transmission of Leishmaniasis

The results of these experiments clearly demonstrate that Lu. diabolica and Lu. shannoni are both competent for transmission of L. mexicana between hamsters by individual bites, six to seven days after the infecting blood meal. The mean intervals between the infecting and transmitting blood meals (5.8 and 6.9 days for Lu. diabolica and Lu. shannoni, respectively) were considerably longer than the three to five days observed by Ward et al. (1977) for L. m. amazonensis in Lu. flaviscutellata, or the just-under four days reported by Strangways-Dixon and Lainson (1966) for L. mexicana in Lu. pessoana (Barretto). The longer time may reflect differences in parasite strain, sand fly species, or holding conditions for infected flies.

Tables 4-7 and 9, indicate that the second blood meal need not be a large one in order for the host to become infected. This agrees with the report of Strangways-Dixon and Lainson (1962) that in the

first transmission of L. braziliensis to a human volunteer by P. paraensis (=Lu. pessoana) the infective bite was merely a 30-sec probe resulting in no blood ingestion.

Transmissions by bite of Lu. diabolica and Lu. shannoni occurred after a critical period of multiplication and development in the sand fly, during which time a massive infection was established in the region of the cardia and the stomodeal valve. This period corresponds to the logarithmic phase of growth described in cultures (Sacks and Perkins, 1984). Once this point in the life cycle of the parasite is reached, division ceases and reduction in size of many promastigotes to short-slender, highly active forms begins. This period corresponds to the stationary phase of growth in cultures (Sacks and Perkins, 1984). The short-slender, highly active forms were first apparent in the region of the cardia and stomodeal valve, and spread forward to the pharynx and mouthparts, as well as rearward to the midgut and hindgut. It is tempting to suggest that they represent a morphologically distinct, highly infective stage.

Sacks and Perkins (1984) stated that although morphological differences between dividing midgut forms and those found anteriorly have been described, there is no evidence that these changes reflect development of promastigotes to an infective stage. They found, however, that the infectivity of L. donovani promastigotes taken from stationary cultures greatly exceeded that of promastigotes from cultures in the logarithmic phase. Further, they found that identical developmental changes occurred during growth of promastigotes in the fly. Leishmania tropica promastigotes, taken from the gut of infect Lu. anthophora three days after fly infection, were essentially

avirulent in Balb/c mice. In contrast, midgut promastigotes obtained seven to ten days after fly infection were highly virulent (Sacks and Perkins, 1984). These authors did not describe the morphologies of the three and ten-day promastigotes obtained from the sand fly midguts. Their findings are consistent with the observations of L. mexicana development in Lu. diabolica in that transmissions to uninfected hamsters occurred only after at least four days of parasite development, corresponding to the point in the life cycle at which division ceased and the short-slender, highly active flagellates first appeared. It is probable that the ten-day inoculum of L. tropica promastigotes from the midgut of Lu. anthophora, used by these workers to infect Balb/c mice, contained short-slender, highly active flagellates, and that the high virulence may have been due to their presence. Because of their rapid, random movement, these parasites, as well as being present in the pharynx and proboscis, are also found throughout the alimentary tract in six to ten-day infections. This is evidence of morphological change to an infective stage which Sacks and Perkins (1984) may have overlooked.

Gross differences in behavior and pathogeneicity between in vivo and culture forms were pointed out by Adler and Theodor (1927). Differences in ultrastructure of Leishmania in the sand fly and in culture were also pointed out by Molyneux et al. (1975), who said that although the ultrastructures of in vivo and in vitro parasites are similar, the changes in the organization or organelles and the configuration of the parasite seen in the different parts of the gut of the vector are not clearly revealed in culture. They concluded that cultural forms may be misleading and should not be considered identical to those in the vector (Molyneux et al., 1975).

In Leishmania transmissions, the presence of parasites in the mouthparts of the infecting sand fly is probably not as critical as massive infections in the region of the stomodeal valve and cardia which give rise to the small "infective" forms. Some Lu. diabolica and Lu. shannoni involved in successful transmissions manifested no parasites in the mouthparts upon dissection but had massive infections in the cardia, with great numbers of short-slender, highly active forms present.

Bray (1974b) stated that "factors governing the numbers of promastigotes in the sand fly capable of being delivered to the skin of the vertebrate, and the mechanism or mechanisms which cause them to be deposited in the skin, remain completely unknown and . . . constitute the most important gap in our knowledge of the disease" (Bray, 1974a, p. 98). Several researchers have suggested that, in the act of biting, parasites from the stomodeal valve, or apparently blocking the pharynx or esophagus, may be forced forward and deposited in the skin (Bray, 1974b; Molyneux, 1977; Lainson et al., 1977). Adler and Theodor (1935) opposed this blockage theory on the grounds that the strong dilator muscles of the cibarium and pharynx would widen the lumen sufficiently for blood to pass without difficulty. Molyneux (1977) accepted Adler and Theodor's explanation but believed the blockage could occur in nonpumping organs such as the esophagus and stomodeal valve. This appears to be true in well developed infections of L. mexicana in Lu. diabolica and Lu. shannoni. In three and four-day-old infections, numbers of attached parasites in the region of and immediately posterior to the stomodeal valve were so great, that the gut became distended, as if under positive

pressure. When the anterior aspect of the cardia was severed, the stomodeal valve usually everted, and the parasites spewed into the dissecting medium, still attached by their flagella. It should be noted that the short-slender, highly active forms were not attached and thus were able to escape the "blockage."

Many workers have noted that infected sand flies have difficulty in engorging, but this does not prevent transmission (Killick-Kendrick et al., 1977). Killick-Kendrick et al. (1977) suggested that the feeding behavior of infected flies may be upset by parasites interfering with the activity of internal sensilla monitoring engorgement. The infected fly would have difficulty engorging and would probe many times, thus increasing the chances of the parasites in the proboscis being deposited in the skin.

The actual process by which the short-slender, highly active promastigotes travel from the stomodeal valve to mononuclear phagocytes in the skin to the mammal is not known. Bray (1981) suggested that sugars in the crop (esophageal diverticulum) are fed into the alimentary canal at the level of the esophagus and exert a chemotactic influence on the promastigotes, thus attracting the promastigotes to the level of the esophagus. This may explain the higher transmission rates with L. donovani in sand flies fed on raisins, reported by Smith et al. (1940). Bray (1981) suggested that sand flies used for transmission studies should be fed 10% or 20% raffinose. Adler and Theodor (1931) maintained that the tendency of flagellates to pass upwards is a real tropism, accounting at least in part, for their migration into the cardia and eventually beyond.

The hamster to hamster transmissions of L. mexicana reported here are the first ever by bites of anthropophilic sand flies indigenous to the USA. Endris et al. (1984) demonstrated transmission of L. mexicana by Lu. anthophora (Addis), a species sympatric with Lu. diabolica in south central Texas. Although Lu. anthophora has bitten humans in the laboratory, this sand fly is not naturally anthropophilic. The successful transmission of trials with Lu. diabolica add substantially to the body of evidence incriminating this species as the major potential vector, if not the only vector, of autochthonous human leishmaniasis in south central Texas.

There are differing opinions as to what constitutes incrimination of a vector. On occasion, a species has been incriminated simply because it is more abundant than others; sometimes it is because the sand fly is a known vector in other foci; or the sole evidence may be that wild-caught specimens were found to be naturally infected (Killick-Kendrick and Ward, 1981). None of these observations alone is enough to incriminate a vector beyond doubt. Killick-Kendrick and Ward (1981) outlined five criteria that must be fulfilled before a suspicion that a sand fly is a vector of human leishmaniasis becomes a reasonable certainty. These are

1. The species must be anthropophilic and present in a place where man becomes infected.

2. The distribution of the suspected vector should accord with the distribution of the disease in man, and the sand fly should be sufficiently abundant to assume that it could maintain the transmission of the parasite in nature.

3. It should be demonstrated that naturally or experimentally infected flies can maintain the infection in the laboratory through the complete life cycle of the parasite.

4. It should be demonstrated experimentally that the sand fly can transmit Leishmania by bite.

5. Leishmania should be isolated from wild-caught sand flies and shown to be indistinguishable from the parasite causing disease in man in the same place.

In the case of Lu. diabolica, the first four criteria have been met as follows:

1. Lutzomyia diabolica were found in man-biting collections in the vicinities of autochthonous human case sites of cutaneous leishmaniasis in south central Texas. In addition, adult females were collected in light traps in the back yard of a patient in D'Hanis, Texas. These adults fed readily on the author's arm immediately after capture (see Chapter 2). Lu. diabolica is the only anthropophilic species of sand fly known from Texas (Young and Perkins, 1984).

2. The known distribution of Lu. diabolica is in accord with that of autochthonous human cases of leishmaniasis in south central Texas (Fig. 2-29, page 73, Chap. 2). Collection records indicate that Lu. diabolica is sufficiently abundant in these areas to maintain the transmission cycle in nature (Table 2-1, page 51, Chap. 2). Semi-weekly New Jersey light trap collections from the case site in D'Hanis, Texas, indicate that the sand fly is present there from early May through early December.

3. It has been demonstrated experimentally that Lu. diabolica can maintain L. mexicana (strain WR-411) infections for life, through the complete extrinsic life cycle of the parasite.

4. The ability of Lu. diabolica to transmit L. mexicana (strain WR-411) by bite has now been confirmed experimentally. The parasite strain used was obtained from a patient living in the type locality of Lu. diabolica.

Additional weight of evidence is provided by observations that Lu. diabolica may be an opportunistic feeder, taking blood meals from a wide range of hosts (page 154, Chap. 3). Moreover, its somewhat peridomestic habits increase the likelihood of possible transmission between natural mammalian reservoirs of leishmaniasis and man and his domesticated animals. No mammmalian reservoir hosts for leishmaniasis are known in south central Texas, but interest in this aspect of the epidemiology of the Texas focus is mounting. Grogl et al. (1984) found immunological evidence of exposure to leishmaniasis in Texas coyotes. In addition, results of serologic tests on four dogs belonging to patients in the Texas focus indicate that these animals may have been exposed to the disease (Gustafson et al., 1984). Wild-caught Lu. diabolica collected at the case site in D'Hanis, Texas, fed readily to repletion on one of these dogs.

One criterion remains to be fulfilled in order to complete the chain of evidence linking Lu. diabolica with the natural transmission of cutaneous leishmaniasis in Texas, namely, the isolation from wild-caught sand flies of Leishmania that are indistinguishable from the parasite causing the disease in man in the same place.

CHAPTER 5
SUMMARY AND RECOMMENDATIONS

During this three-year study, the objectives, outlined in Chapter 1, were achieved as follows:

1. Field surveys were conducted at and near human case sites of leishmaniasis in south central Texas to determine the sand fly fauna and potential vectors of the disease. Five species of Lutzomyia, including one new species, were collected and eight new county records established. Lutzomyia diabolica (Hall), the only anthropophilic sand fly found, was the most common, accounting for 99% of all specimens. Lutzomyia anthophora (Addis), Lu. vexator (Coquillett), and Lu. texana (Dampf) were collected in smaller numbers.

2. The biology of Lu. diabolica was studied under field conditions. Nearly 8,000 eggs were recovered from approximately 1,925 females that were blood fed after capture to stock a laboratory colony. Dissections of wild-caught Lu. diabolica revealed several naturally occurring parasites including Crithidia, other unidentified flagellates, gregarines, microsporidians, and phoretic mites.

3. A productive laboratory colony of Lu. diabolica was established for the first time.

4. Detailed laboratory studies on the biology of Lu. diabolica, both in temperature controlled incubators and under ambient conditions, were conducted through 16 generations. A new larval diet,

developed from a modified horn fly diet, reduced the average time from egg hatch to adult emergence by about 50%. Quiescence and diapause were observed in both the egg and larval stages, with hibernation of up to 270 days in the egg stage of outdoor-reared sand flies.

5. Vector capacities of Lu. diabolica and Lu. shannoni for L. mexicana (strain WR-411) were investigated under controlled laboratory conditions. Eighty-eight percent and 95% infection rates were obtained in Lu. diabolica and Lu. shannoni, respectively, when flies were fed on leishmanial histiocytomas of hamsters. For the first time, transmissions of Leishmania mexicana to hamsters were obtained by bites of infected Lu. diabolica and Lu. shannoni females, six to seven days after the infecting blood meals.

6. Leishmania mexicana amastigotes from hamsters infected by bites of Lu. diabolica, and promastigotes from infected sand flies were examined with an electron microscope. The number of subpellicular microtubules in promastigotes showed a wider range of variation than is reflected in the literature.

To complete the chain of evidence linking Lu. diabolica with transmission of human cutaneous leishmaniasis in south central Texas, it is recommended that field surveys be continued to: 1) more precisely delineate the geographic distribution of Lu. diabolica and to locate adult resting and larval breeding sites; 2) isolate leishmanial parasites from wild-caught sand flies that are indistinguishable from the parasites causing the disease in the same place; and 3) determine the natural mammalian hosts of Lu. diabolica and potential reservoirs of Leishmania in endemic areas.

Future laboratory studies should include development of artificial-membrane feeding systems to facilitate study of the dynamics and mechanisms of Leishmania transmission by sand flies.

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BIOGRAPHICAL SKETCH

Phillip G. Lawyer was born, the third son of goodly parents, in Wenatchee, Washington, 10 May, 1945. Early boyhood years were spent in the communities of Cashmere, Washington, and Anchorage, Alaska. In 1958, his family moved to Utah where he graduated from East High School in Salt Lake City in 1963. He enrolled at the University of Utah in the fall of 1963, but interrupted his studies to accept a mission call from the Church of Jesus Christ of Latter-Day Saints. After serving as a missionary in West Germany for 27 months, he returned to the University of Utah to complete a B.A. in biological sciences in June, 1970. In December, 1969, he accepted a commission in the US Army and was granted an educational delay to complete an M.A. degree in medical entomology, which he received in June, 1971. Phillip commenced active duty in the US Army as a 1LT, medical entomologist, in October, 1971. Following several military assignments, which included tours with the 20th Preventive Medicine Unit, Republic of South Vietnam, the US Army Environmental Hygiene Agency, St. Louis, Missouri, and Fort Meade, Maryland, and the Medical Service Corps Officers Advanced Course, he was assigned to the teaching staff at the US Army Academy of Health Sciences, Fort Sam Houston, Texas.

Phillip entered graduate school at the University of Florida in August, 1981. He has been a member of the Entomological Society of America and on the American Registry of Professional Entomologists since 1973.

Following graduation he will be assigned to Walter Reed Army Institute of Research, Washington, DC, and will continue research on phlebotomine sand flies and leishmaniasis. He will be accompanied by his wife, Joyce, and six children, Natalie, Juliet, Nathan, Joshua, Aaron, and Andrew.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. Jerry F. Butler, Chairman
Professor of Entomology and
Nematology

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Associate Professor of Microbiology
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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