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ORIGINAL ARTICLE

Effects of temperature and diet on the growth and longevity of phlebotomine sand flies (Diptera: Psychodidae)

Hilda Guzmán, Robert B. Tesh

Department of Pathology, Center for Tropical Diseases, University of Texas Medical Branch, Galveston, Texas, U.S.A.

A series of experiments were done to study the effects of different ambient temperatures (28 to 10 °C) and diets on the growth rate, size and longevity of phlebotomine sand flies. Four different laboratory colonies of these insects were used: *Phlebotomus papatasi*, *P. perniciosus*, *Lutzomyia longipalpis* (Brazil) and *L. longipalpis* (Colombia). The developmental times and survival of the insects were highly variable, depending on the temperature as well as species. At 18 °C and below, most of the *L. longipalpis* larvae and pupae died. At 15 °C, the developmental time of *P. papatasi* and *P. perniciosus* was markedly prolonged (150 to 412 days) and many of the immature forms died. Within a range of 28 to 15 °C, the longevity of adult *P. papatasi* and *L. longipalpis* increased as the ambient temperature decreased; to 15 °C; the mean survival times of *P. papatasi* and *L. longipalpis* females were 63.2 and 37.1 days, respectively. The effect of two different diets (animal feces/liver powder and decomposing leaves) on the growth rate and size (pupal weight) of *L. longipalpis* was also determined. Insects maintained on the diet of animal feces/liver powder developed faster, were more synchronous and were larger than those fed on decomposing leaves. Results of this study provide new information on the effects of environmental factors on the growth and longevity of phlebotomine sand flies under laboratory conditions.

Key words: sand flies, Psychodidae, vector biology, insect colonization.

Efectos de la temperatura y la dieta sobre el crecimiento y la longevidad de flebotomíneos (Diptera: Psychodidae)

Se realizó una serie de experimentos para estudiar los efectos de diferentes temperaturas ambientales (28 a 10 °C) y tipos de dieta sobre la tasa de crecimiento, el tamaño y la longevidad de los flebotomíneos. Se utilizaron cuatro colonias de laboratorio diferentes: *Phlebotomus papatasi*, *P. perniciosus*, *Lutzomyia longipalpis* (Brasil) y *L. longipalpis* (Colombia). Los períodos de desarrollo y supervivencia de los insectos fueron muy variables según la temperatura y la especie. Por debajo de 18 °C, la mayoría de las larvas y pupas de *L. longipalpis* murieron. A 15 °C, el tiempo de desarrollo de *P. papatasi* y *P. perniciosus* se prolongó considerablemente (150 a 412 días) y muchas de las formas inmaduras murieron. En el rango de 28 a 15 °C, la longevidad de los adultos de *P. papatasi* y *L. longipalpis* aumentó a medida que la temperatura ambiente disminuía; a 15 °C los tiempos promedio de supervivencia de las hembras de *P. papatasi* y *L. longipalpis* fueron de 63,2 y 37,1 días, respectivamente. El efecto de dos tipos de dieta diferentes (heces de animal/polvo de hígado y hojas en descomposición) sobre la tasa de crecimiento y el tamaño (peso de la pupa) de *L. longipalpis* también se determinó, estableciéndose que los insectos alimentados con heces de animal/polvo de hígado se desarrollaron más rápido y fueron más sincronizados y más grandes que aquellos alimentados con hojas en descompisición. Los resultados de este estudio entregan nueva información sobre los efectos de factores medioambientales en el crecimiento y la longevidad de flebotomíneos bajo condiciones de laboratorio.

Palabras clave: flebotomíneos, Psychodidae, biología de vectores, colonización de insectos.

The effects of ambient temperature and diet on the growth rate, size and longevity of mosquitoes under laboratory conditions have been the subject of numerous investigations (1-3). In contrast, little is known about the effects of these external factors on the development and longevity of phlebotomine sand flies. The paucity of such basic information about this important group of insect vectors is due in large part to the difficulty in rearing phlebotomines in the laboratory. Consequently, developmental studies with sand flies usually have been done with laboratory colonies maintained at a single optimal temperature (usually 23-30 °C) on nutrient-rich diets based on animal feces, blood, yeast and/or liver powder (4-27). To learn more about the effects of varying environmental conditions on sand fly growth and survival, a series of laboratory experiments were carried out with three phlebotomine species to determine the effects of different ambient temperatures and diets on the insects' development and longevity. This paper reports the results of those studies.

Materials and methods

Sand flies: four old established laboratory colonies were used: *Phlebotomus papatasi* Scopoli, initiated from specimens collected in Aurangabad, Maharashtra State, India (19); *Phlebotomus perniciosus* Newstead, started from insects collected in Toscana, Italy (21); *Lutzomyia longipalpis* Lutz & Neiva, started from females captured in Lapinha Cave in Minas Gerais State, Brazil (14), and hereafter referred to as the Brazil strain; and *Lutzomyia longipalpis*, initiated from insects collected in El Callejón, Cundinamarca Department, Colombia (28), and hereafter referred to as the Colombia strain. Recent studies (29-32) suggest that *L. longipalpis* is a species complex and that the Brazil and Colombia strains actually represent two distinct species in this complex.

Rearing methods

All sand flies used in the experiments were produced from colonies maintained at the Yale Arbovirus Research Unit, Yale University School of Medicine. The basic technique for rearing the insects has been described before (19). Unless noted otherwise, larvae were fed on our standard larval diet which consisted of an aged mixture of rabbit feces, rabbit chow and beef liver powder (19). Insects in the normal laboratory colonies (adults and immature forms) were maintained at 25 °C with a 12:12 (light:dark, L:D) photoperiod. In experiments comparing the effect of various temperatures on larval development, several different L:D regimes were used, as we had only a limited number of shared constant temperature chambers. Insects at 10 °C were housed in a constant temperature refrigerator (Ambi-Hi-Lo Chamber, Lab Line, Melrose Park, IL) without illumination. Insects maintained at 15, 18, 20 and 23 °C were held in programmed illuminated incubators (Model #818, Precision Scientific, Chicago, IL) with a 14:10 (L:D) photoperiod; insects maintained at 25 and 28 °C were kept in the same type of incubators but with a 12:12 photoperiod.

Effect of temperature on larval and pupal development

Newly emerged adult female sand flies were released into cloth holding cages (19) with males for approximately 7 days to allow mating. These insects were maintained on a 30% sucrose solution at 25 °C and a 12:12 (L:D) photoperiod. After 1 week, the flies were allowed to feed on an anesthetized hamster. Fully engorged females were subsequently removed and held under the same conditions for an additional 5 days to permit blood digestion and egg maturation. On the fifth day, 25 gravid females each were confined in six 125 ml polymethylpentene jars (Cat. No. 2117-0500, Nalge Co., Rochester, NY) with a thin layer of moist plaster of Paris on the bottom (19). One oviposition jar was placed in each of six programmed incubators maintained at the following temperatures: 28, 25, 23, 20, 18 and 15 °C. In preliminary studies, it was observed that the number of eggs laid, and the percentage hatching, at 15 °C or lower were markedly reduced for all species. Thus, in later experiments done at 15 and 10 °C, the gravid females were initially confined at 25 °C until oviposition, and their eggs were held

Correspondence:
Robert B. Tesh
Tel: (409) 747-2431; fax: (409) 747-2429
e-mail: rtesh@utmb.edu

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at this same temperature until hatching began. Then the larvae were transferred to 10 or 15 °C.

When gravid sand flies were confined in the oviposition containers at 25 °C, they usually laid their eggs within 2 to 7 days after confinement. But since the eggs were laid singly and did not all appear on the same day, it was not possible to determine precisely on which day oviposition occurred. Thus, the date of maternal confinement (day 0) was recorded, as was the date when eggs were first noted on the moist plaster surface of the oviposition container. Approximately 3 to 7 days after maternal confinement, or when most of the gravid females had oviposited, the dead and surviving sand flies were removed from the jars, the total number of eggs in each container was counted, and a small quantity of the standard larval diet was added. The eggs and developing larvae were subsequently examined under a stereo zoom microscope at regular intervals (every 2 days at higher temperatures and once weekly at lower temperatures). At each examination, the larval food was gently excavated, so that a sample of the developing insects could be clearly observed. Because sand flies are not very synchronous in their development (especially at lower temperatures) and because the larvae burrow into the food, it was not feasible to count all of the immature insects at every examination. Instead, the number of eggs in each jar was initially counted, as described above; and the total number of adult flies emerging from the containers was also recorded. At each other visual examination during the insects' development, we simply noted the various life stages present and any obvious mortality. Additional food and humidity (drops of water) were also added to the jars, as needed, at the time of the visual examinations.

Effect of diet on larval development

Two different diets were used in these experiments. One was the standard larval diet which consisted of an aged mixture of rabbit feces, rabbit chow and liver powder (19). The other diet consisted of a mixture of decomposing maple (*Acer*) and oak (*Quercus*) leaves taken from a compost pile in the authors' garden. The latter mixture was frozen at -30 °C for several days prior to use in order to kill any potential nematode or arthropod predators in the compost. In two experiments utilizing the Brazil and Colombia strains of *L. longipalpis*, one group of larvae was maintained exclusively on the standard larval diet at 25 °C, while the second group was maintained solely on the leaf compost mixture under the same temperature and light conditions. Observations on larval and pupal development were made every 2 or 3 days as noted before. In addition, the weights of a sample of pupae and the number of adult insects emerging from each experimental group were recorded. In order to determine weights, early pupae (those with pink eyes) were removed from the rearing jars, and the larval skin was carefully removed from its posterior attachment to the pupal case, revealing the developing imago's terminal genitalia and allowing its sex to be determined. Weights of individual male and female pupae were measured on a Mettler balance (Model AE240, Mettler Instrument Corp., Highstown, NJ). During removal of the larval skin and prior to weighing, the pupae were held in Petri dishes on moist filter paper to prevent desiccation.

Effect of ambient temperature on adult longevity

Two experiments were done, using *L. longipalpis* (Colombia) and *P. papatasi*, to determine the longevity of adult sand flies at different ambient temperatures. Newly emerged adult sand flies (<24 h old) from the same generation were divided into four groups, which were maintained in separate programmed illuminated incubators at 28, 25, 20 and 15 °C. The insects were held in cloth cages (19), which were enclosed within 61 x 75 cm plastic bags containing a moist sponge. The flies were provided with cotton pads soaked in 30% sucrose solution as a source of nourishment and liquid. Cages were examined daily. Any dead flies were removed, and their sex was determined and recorded.

Results

Effect of temperature on larval and pupal development

Phlebotomus papatasi: figure 1 shows the effect of various ambient temperatures (15, 18, 23, 25

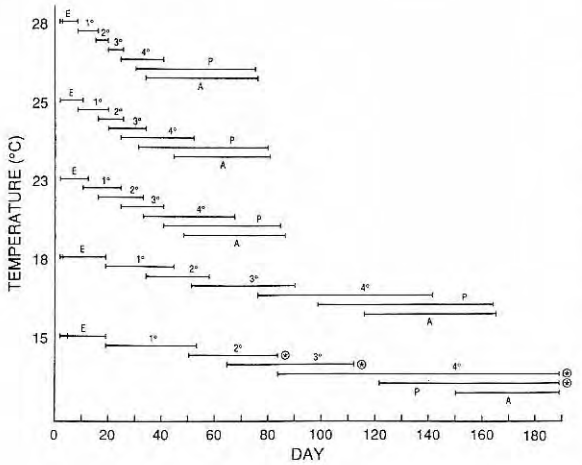

Figure 1. Developmental time of *Phlebotomus papatasi* at various ambient temperatures. Symbols: E: egg; 1°: 1st larval instar; 2°: 2nd larval instar; 3°: 3rd larval instar; 4°: larval instar; P: pupa; A: adult. Each horizontal line indicates the duration time after confinement of the gravid parent females that each life stage was observed in the cultures.
* significant mortality observed

and 28 °C) on the growth rate of *P. papatasi.* Because the total number of immatures was not determined at each examination, it was not possible to calculate mean development time for the various life stages at different temperatures. Instead, results were recorded as the range of days after confinement that a particular life stage was observed in the culture. For example, at 28 °C the eggs were laid 2 to 3 days after maternal confinement, and eclosion began about 7 days later; the first stage larvae were present from day 9 to day 16 after confinement; second stage larvae were observed from days 15 to 20, etc.

The data in figure 1 demonstrate two characteristics of sand fly development: 1) that sand flies are rather asynchronous in their development, particularly at lower temperatures, and 2) that the insects' developmental rate is inversely related to the ambient temperature. For example, the development time from initial confinement until emergence of the F_1 adults of *P. papatasi* reared at 28 °C varied from 34 to 76 days; in contrast, at 15 °C the F_1 adults appeared 150 and 189 days after confinement. The % emergence (total adults emerging/total eggs x 100) of *P. papatasi* also decreased as the ambient temperature was lowered, suggesting increased mortality among the immature forms at lower temperatures (table 1). At 15 °C, there was considerable mortality among the larvae and pupae, and only 6 adults (0.4%) emerged from 1,424 eggs originally placed at this temperature.

Two other experiments were done (not shown) in which *P. papatasi* eggs were first allowed to hatch at 25 °C, and then the larvae were placed in incubators maintained at 15 and 10 °C, respectively. In the first experiment, newly hatched first instar larvae were placed at 15 °C; considerable mortality occurred in this group in all of the immature stages. From 1,806 eggs hatched at 25 °C and then the first instar larvae placed at 15 °C, only 12 adults emerged (0.7% emergence). These 12 adults emerged from 258 to 360 days after confinement. In a second experiment, eggs were hatched at 25 °C; then first, second and third stage larvae reared at this temperature were transferred to 10 °C. At this lower temperature, all of the larvae eventually died and

Table 1. Productivity of sand fly laboratory colonies maintained at various ambient temperatures.

Ambient temperature (°C)	*Phlebotomus papatasi*		*Phlebotomus perniciosus*		*Lutzomyia longipalpis* (Colombia)	
	Number of eggs	Percent emergence *	Number of eggs	Percent emergence	Number of eggs	Percent emergence
28	1,283	57.8	686	58.9	478	47.9
25	1,285	44.4	709	24.7	1,053	37.1
23	1,678	33.5	738	43.8	206	17.0
20	NT	NT	547	44.8	526	29.5
18	1,511	6.2	645	17.8	283	1.1
15	1,424	0.4	212	2.4	133	0.0 **

* Percent emergence: total adults emerging/total eggs x 100
** None of the eggs held at this temperature (15 °C) hatched

none were able to molt to the next larval stage. For example, second instar *P. papatasi* larvae placed at 10 °C survived for up to 166 days, but none molted to the third instar. Third instar larvae transferred to 10 °C survived up to 346 days, but none reached the fourth instar. *P. papatasi* larvae held at 10 °C moved on stimulation but were not observed feeding.

Phlebotomus perniciosus: figures 2 and 3 summarize the results obtained with *P. perniciosus* maintained at ambient temperatures from 28 to 10 °C. The development time of *P. perniciosus* from confinement to the F_1 adult ranged from 32 to 45 days at 28 °C; in contrast, it was 192 to 412 days at 15 °C. As with *P. papatasi*, considerable mortality occurred among the third and fourth instar larvae and pupae of *P. perniciosus* at lower temperatures (table 1). At 18 and 15 °C, adult emergence was 17.8% and 2.4%, respectively. When *P. perniciosus* larvae were allowed to hatch at 25° and then were placed at 15 °C, the results were similar (figure 3). Of 602 *P. perniciosus* eggs hatched at 25 °C and then transferred to 15 °C, most of the developing larvae and pupae died, and only 44 adults emerged (7.3% emergence). When newly hatched *P. perniciosus* larvae were transferred to 10 °C, development was very slow and all larvae died by the third instar.

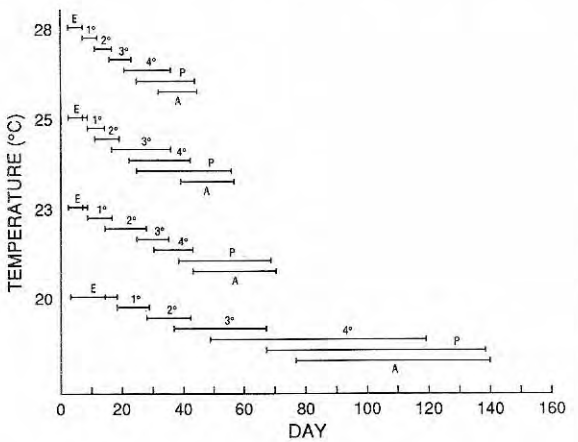

Figure 2. Developmental time of *Phlebotomus perniciosus* at various ambient temperatures. Symbols are described in figure 1.

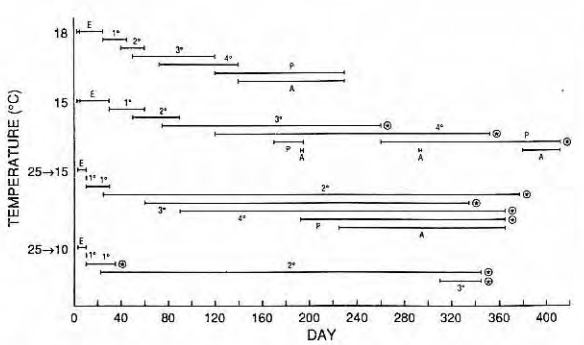

Figure 3. Developmental time of *Phlebotomus perniciosus* at various ambient temperatures. See figure 1 for description of symbols. 25 → 15 and 25 → 10 indicate that eggs were held at 25 °C until hatching, then the larvae were transferred to 10 or 15 °C.

Lutzomyia longipalpis: figures 4 and 5 and table 1 summarize the results obtained with *L. longipalpis* (Colombia) maintained at constant ambient temperatures from 28 to 15 °C. The development time of *L. longipalpis* from confinement to F_1 adult ranged from 37 to 53 days at 28 °C; at 18 °C, it ranged from 193 to 216 days. This tropical species was less tolerant of lower temperatures than *P. papatasi* or *P. perniciosus* and considerable larval and pupal mortality occurred at 18 °C (table 1). Only 1.1% adult emergence occurred at this temperature; and none of the *L. longipalpis* eggs laid at 15 °C hatched. A total of 542 *L. longipalpis* eggs were hatched at

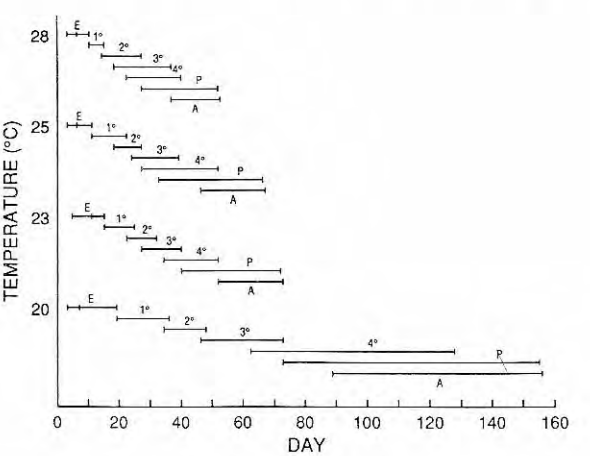

Figure 4. Developmental time of *Lutzomyia longipalpis* (Colombia) at various ambient temperatures. Symbols are described in figure 1.

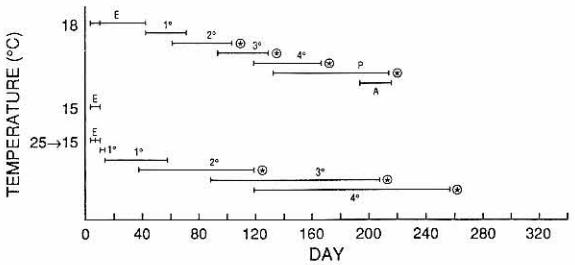

Figure 5. Developmental time of *Lutzomyia longipalpis* (Colombia) at various ambient temperatures. Symbols are the same as given in figure 3.

25 °C and the newborn larvae then were transferred to 15 °C; all of these larvae died during the second, third or fourth instars (figure 5). This species was not tested at 10 °C.

Effect of diet on larval development

Experiments also were done comparing the effects of two different larval diets on the developmental time and size (pupal weight) of the Brazil and Colombia strains of *L. longipalpis* maintained at a constant temperature of 25 °C. The two diets tested were our standard larval diet (animal feces base) and decomposing leaves. The results are shown in figure 6 and table 2. With both *L. longipalpis*

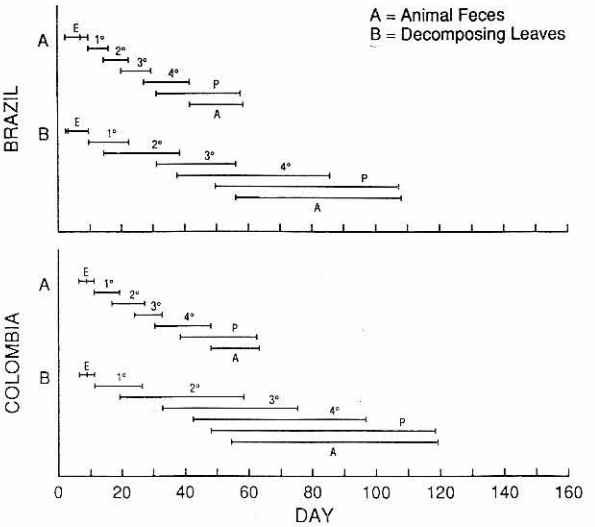

Figure 6. Effect of two different larval diets (animal feces base and leaf compost) on the development of the Brazil and Colombia strains of *L. longipalpis* maintained at 25 °C.

strains, the insects maintained on the standard larval diet were more synchronous and developed faster than their counterparts maintaned on decomposing leaves. Despite their longer development time, the two groups of larvae maintained on decomposing leaves were able to

Table 2. Developmental time and size (pupal weight) of *L. longipalpis* (Brazil and Colombia strains) reared on animal feces or decomposing leaves at 25 °C.

	Brazil		Colombia	
	Animal feces*	**Decomposing leaves**	**Animal feces***	**Decomposing leaves**
Initial number of eggs	450	450	669	835
Adult development time (days)[a]	41-58	56-108	48-63	54-119
Adults emerging	139	130	228	213
Sex ratio	66:73	62:68	104:124	104:109
% emergence[b]	30.9	28.9	34.1	25.5
n	50	22	51	24
Mean (SD)	0.42 (0.03)	0.31 (0.03)	0.39(0.03)	0.30 (0.03)
Range	0.36-0.48	0.24-0.36	0.30-0.46	0.24-0.38
Pupal weight (mg) - Females				
n	50	29	52	24
Mean (SD)	0.53 (0.04)	0.38 (0.06)	0.41(0.04)	0.35 (0.04)
Range	0.47-0.67	0.27-0.49	0.35-0.54	0.26-0.42

* Animal feces: standard larval diet

[a] Adult development time: range of days after maternal confinement between the emergence of the first and the last F_1 adult flies in the group.

[b] % emergence: total adults emerging/total eggs x 100

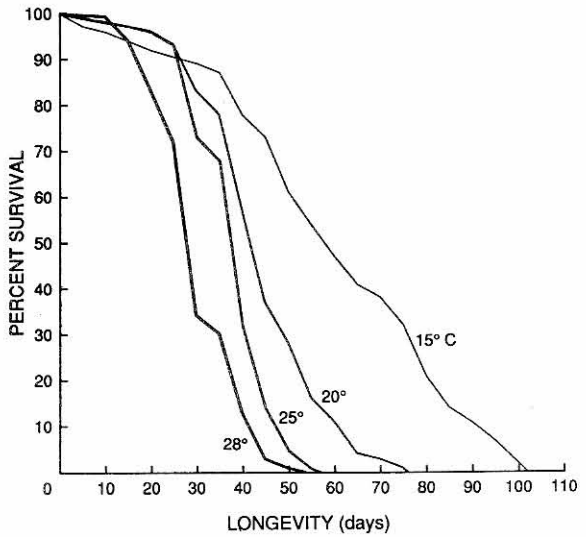

Figure 7. Longevity (percent survival) of *Phlebotomus papatasi* adults (both sexes) maintained at four different constant ambient temperatures.

complete their life cycle and the percent adult emergence among the four groups was not notably different. However, the mean pupal weights of insects maintained on the standard larval diet were significantly heavier than those of insects reared on decomposing leaves at the same temperature (table 2). This was true for both sexes.

Effect of temperature on adult longevity

Figures 7 and 8 and table 3 show the survival of *P. papatasi* and *L. longipalpis* (Colombia) adults maintained at 15, 20, 25 and 28 °C. In general, the mean survival of female sand flies was longer than that of their male counterparts (table 3). Within the temperature range tested, the mean survival of both species increased as the ambient temperature was lowered. Although 15 °C was lethal to the immature stages of both *P. papatasi* and *L. longipalpis* (table 1), this temperature did not seem to be harmful to adults, as their longevity was longest at this lower temperature (figures 7 and 8). The longevity of *P. papatasi* was longer than that of *L. longipalpis* at all temperatures tested.

Discussion

Results of this study demonstrate that the duration of the life cycle of phlebotomine sand flies can be

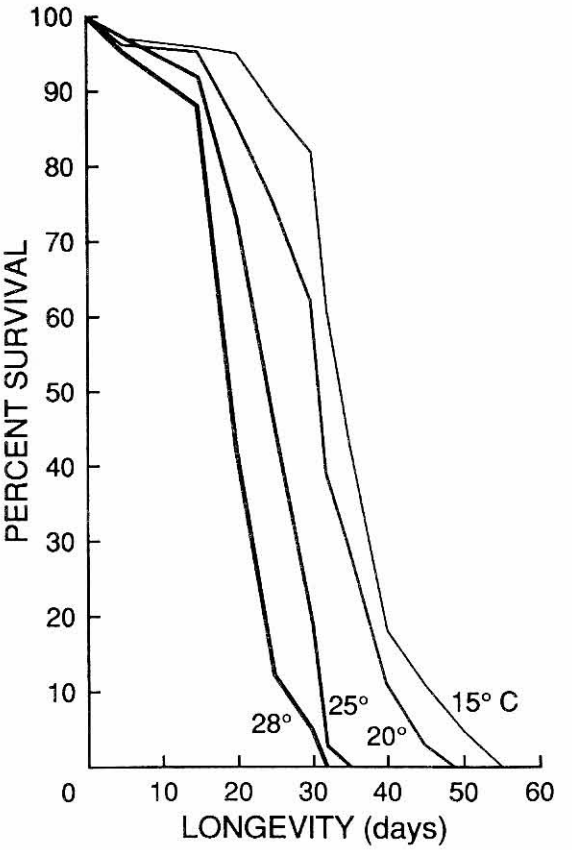

Figure 8. Longevity (percent survival) of *Lutzomyia longipalpis* adults (both sexes) maintained at four different constant ambient temperatures.

highly variable and that ambient temperature, larval diet and species are all important factors in determining the developmental time of these insects. Likewise, adult longevity varies with ambient temperature and species. Although the effect of humidity was not determined in our experiments, Theodor (33) previously showed that the survival time of *P. papatasi* shortens as the relative humidity decreases. The results of our studies are not surprising; but until now, most information on the effect of diet and temperature on sand fly development has been anecdotal. Most previously published studies of sand fly development (4,6-11,15-23,25,26,33,34) have been done under optimal laboratory conditions, using a single nutrient-rich diet and constant temperature (usually in the range of 23-30 °C), consequently it is often assumed that the duration of the life cycle

Table 3. Longevity of adult *Phlebotomus papatasi* and *Lutzomyia longipalpis* (Colombia) maintained at various ambient temperatures.

	Temperature (°C)							
	15 °		20 °		25 °		28°	
	Males	Females	Males	Females	Males	Females	Males	Females
P. papatasi								
n	62	54	42	60	54	68	46	62
Survival range (days)	3-102	3-102	3-66	19-76	7-43	2-57	7-38	12-54
Mean survival (days)	55.3	63.2	37.8	45.8	32.2	39.6	25.8	31.8
± SD	24.1	23.1	12.8	12.3	7.7	8.1	6.8	9.2
L. longipalpis (Colombia)								
n	81	69	74	90	61	71	50	80
Survival range (days)	3-49	4-55	3-48	3-49	11-35	11-38	3-27	3-32
Mean survival (days)	32.0	37.1	31.3	30.0	23.7	25.3	19.6	20.6
± SD	8.9	8.9	9.0	9.3	5.8	6.1	5.3	5.6

of most sand fly species is in the range of 20-45 days (35-37). Under natural conditions, their developmental time is undoubtedly much more variable. The longevity of adult *P. papatasi* and *L. longipalpis* (Colombia) increased as the ambient temperature was lowered from 28 to 15 °C (table 3). From an epidemiological perspective, this is important, because the duration of a vector's life has a major impact on its vectorial capacity (38). Although estimates of sand fly longevity based on laboratory studies are suspect and obviously need field verification, there is currently little information of this type available. Few estimates of adult sand fly survival have been made under field conditions, largely because of the difficulty in estimating the ovarian age of parous females (39) and because mark-recapture studies rarely encounter marked individuals beyond 10 or 11 days after their release (40). In attempting to calculate adult longevity or larval developmental rates in nature, it is important to study the exact conditions of the insects' environment (41). But since the precise larval breeding sites of most phlebotomine species are unknown, it is difficult to measure or to duplicate the exact conditions of their environment. It is presumed that sand flies spend most of their lives hidden in protected, dark, damp and relatively warm places such as caves, deep rock crevices, tree holes, animal burrows, basements or other enclosed structures which protect them from climatic extremes (35,42-44). Only during periods of nocturnal adult activity (i.e. host seeking) are they exposed to less favorable conditions. However, even in very hot and dry climates, the temperature is usually lower and the humidity is higher during the night when the adults are out of their normal resting places. Most sand fly species that occur in temperate regions enter diapause with the onset of cold weather and survive the winter months in the egg stage or as fourth instar larvae (5,8,16,35,40,45,46). During larval diapause, development is temporarily arrested and the insects become very sluggish and feed only occasionally. Although little is known about winter diapause in sand flies under natural conditions, presumedly its onset and duration vary at different latitudes and among species. With the onset of warmer weather (and possibly longer day length), the diapausing larvae again become active and development resumes. This same pattern can be observed with *P. perniciosus* in the laboratory; if the larvae are exposed to temperatures of 10 to 15 °C for a few weeks or months development essentially ceases; but when the ambient temperature is again raised to 23-28 °C, normal development resumes again (46).

In the current series of experiments, larvae were exposed to constant temperatures. Consequently, the larval and pupal mortality observed among *P. papatasi* and *P. perniciosus* held continuously at 10 and 15 °C probably would have been less if they had been returned to warmer temperatures after a few months, as normally might occur under natural conditions (5,14,16). In the case of *L. longipalpis* (Colombia), a tropical species, considerable mortality occurred in larvae and pupae held at 18 °C, and all of the immature forms died at 15 °C (table 1). In contrast, *P. papatasi* and *P. perniciosus*, species that are found in more

temperate zones (46), survived better at these lower temperatures (table 1). These data are in general agreement with the findings of others. Theodor (33) observed that *P. papatasi* larvae from Jerusalem died within 4.5 days when placed at 0 °C and within 2 to 3 days when held at -2 °C. Ready and Croset (16) found that *P. perniciosus* from southern France became completely immobile but survived exposure to temperatures of 2 to 10 °C, although their longevity at these temperatures was not determined. Thus, it seems likely that both *P. papatasi* and *P. perniciosus* larvae can survive for short periods of time at temperatures between 0 and 10 °C. However, because of the protected nature of their normal breeding sites, they probably are not long exposed to such low temperatures, even during winter.

In the current experiments, the longevity of adult sand flies was not determined at temperatures below 15 °C. However, Theodor (5) previously reported that adult *P. papatasi* developed cold paralysis at 10 °C and that all insects placed at this temperature died within 19 days. At 6 °C, their life span was considerably shorter. On the basis of his studies, he concluded that temperatures under 10 °C were unfavorable for adult *P. papatasi*.

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