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METHODS FOR THE COLONIZATION OF THE PHLEBOTOMID SANDFLY LUTZOMYIA LONGIPALPIS[1]

by

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1. INTRODUCTION

For studies on the life-cycles of Leishmania spp in phlebotomid sandflies (Killick-Kendrick et al., 1974; Molyneux et al., 1975) and the transmission of leishmaniasis by the bite of experimentally infected flies, a closed laboratory colony of Lutzomyia longipalpis (Lutz & Neiva, 1912) from Brazil has been established in England (Killick-Kendrick et al., 1973). Workers in Latin America had already shown that it was possible to breed several generations of this fly in the laboratory (e.g. Sherlock & Sherlock, 1959; Christensen, 1972).

It was essential for our work to have a species of sandfly which would readily take blood from hamsters experimentally infected with leishmaniasis. L. longipalpis was known to meet this requirement (Coelho et al., 1967). Up to now 21 consecutive generations of this fly have been bred, and females from the colony will take blood from hamsters, guinea-pigs, mice and man.

There are only few reports of colonies of Neotropical sandflies bred for more than 10 generations without replenishment from the field. Since the present work is in a country where sandflies are not indigenous, a prime consideration has been the difficulty of supplementing the colony in this way. From the initiation of the colony, therefore, careful calculations were made for each generation and methods of rearing have been modified to ensure the survival of the colony and increase production.

2. ESTABLISHMENT OF COLONY

Collection site. The colony was established with eggs from engorged females collected during September 1972 in one of the Lapinha caves, Lagoa Santa, 60 km north-west of Belo Horizonte, Minas Gerais, Brazil (longitude 43°57', latitude 19°03') (Espinola & da Silva, 1965).

Collection of eggs. On the first day of collection few flies were in the cave, and only two engorged females were seen. At the suggestion of Mr Alberto Falcão a caged cockerel was

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left in the cave and two days later numerous fed flies were found resting on the roof of the cave. From 1500 to 1730 hours, 75 engorged females were caught and placed in individual plastic tubes (6 x 2 cm) lined on one side with damp filter paper. During the six days of transit from Belo Horizonte to the Imperial College Field Station in Ascot, England, they were kept in a polystyrene box lined with damp paper. Sucrose solution was provided on swabs of cotton wool placed on gauze covering the mouths of the tubes (Ward & Killick-Kendrick, 1974).

By the 18th day after collection, 53 flies had laid a total of 1726 eggs with which the colony was established. The females were dissected and identified, and unlaid eggs were counted. All flies were L. longipalpis, except for one specimen of L. renei.

3. METHODS OF COLONIZATION

The combination of methods of colonization at present in use is based largely on the work of Adler & Theodor (1935), Hertig & Johnson (1961), Harwood (1965) and Gemetchu (1971).

3.1 Immature stages

Five to six days after the eggs are laid, they are counted and moved with a camel-hair brush onto small pieces of wet filter paper shaped differently to identify each batch. Eggs of a similar age are grouped in totals of 80-120 and placed to hatch in Hilton pots (Killick-Kendrick et al., 1973) made from the plastic tubes of WHO insecticide testing kits. Each tube, when cut in half, makes two open-ended screw-cap pots, 6 cm in length and 4.5 cm in diameter. The inside of the pots is roughened with acetone and lined with plaster of Paris. Once the lining has set, the bottoms of the pots are filled to a depth of 2 cm with more plaster. The coarse mesh of the lids is replaced with fine gauze stuck on with glue.

Moisture in the pots is maintained by standing them on filter paper wetted with distilled water. The best way to control water and humidity is to keep the pots in snap-top polyethylene food containers which also prevent mite infestations. Particular care is taken to ensure that eggs and first instar larvae do not become dry. Later instars, notably the fourth, seem to prefer somewhat drier conditions than the early instars. The closed containers are kept at 25°C in the dark. If opened daily, they dry progressively at a rate that is suitable for the development of the immature stages.

The larvae are fed on desiccated liver powder[1] (Gemetchu, 1971), small amounts of which are added daily according to the size and number of larvae. Overfeeding encourages the growth of fungi which can usually be controlled by scraping uneaten food and faeces off the plaster. After tending a pot, instruments are flamed to avoid transferring harmful fungi to fresh pots. Some fungi are eaten by the larvae, notably by the third and fourth instars, but excessive growth is a danger to younger larvae.

The optimum number of larvae reared in each pot is not critical. The percentages of first instar larvae successfully reared to adult flies in 25 pots of the fifth generation which each contained 26-110 larvae were compared. Within this range, the success was unrelated to density.

3.2 Adult flies

When flies emerge from pupae, they are released into a gauze-covered cage $(18\ cm)^3$, and caught in small tubes of clear plastic. The flies are then counted, sexed and set free in another similar cage; the ratio of males to females is 1.2:1.0. Not more than 120 flies are kept in any one cage. The yield of three days or less is pooled to give a series of cages with flies of similar age. A 30% sucrose solution (mass/volume) is freely provided on cotton wool in a dish, and is changed thrice weekly. A high humidity (100% RH) is obtained by sealing each cage in a clear plastic bag containing a swab of wet cotton wool. The cages are kept in the dark at 25°C.

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[1] Armour Pharmaceutical Company Ltd., Eastbourne, Sussex, England.

The flies mate freely in the cages, and pairs may be seen in copula at any time from 24 hours after emergence onwards. Mating takes place before, during or after the females take a bloodmeal.

Female flies are offered a bloodmeal 3-5 days after emergence. Younger flies feed with reluctance or not at all, and older flies have a less than optimum rate of survival to oviposition. From the time the culture was started, the main line has been maintained on hamsters which are anaesthetized with sodium pentobarbitone given intraperitoneally (0.4 mg/10 g body weight, increasing as tolerance develops). Hair on the abdomen is trimmed with scissors, and sticky tape is attached to each leg and pinned to a cork board with the animal on its back. The board bearing the hamster is placed on the floor of the cage. A line fed on man is offered blood from a volunteer's hand and wrist inserted in the cage. Flies will feed at any time of the day, both in the dark and in normal light. The proportion of females of optimum age which takes blood varies from 30%-90% in apparently identical conditions. Differences in time of day, light, ambient temperature, relative humidity, or the host animal, do not seem to explain these variations.

3.3 Oviposition

Engorged flies are maintained in the dark at 25°C until oviposition.[1] Two different methods are used.

Method 1. Each female is collected in a small glass tube (4 x 2 cm) lined on one side with a triangle of filter paper, the tip of which covers the base of the tube. A 30% sucrose solution is provided ad libitum on swabs of cotton wool placed on gauze covering the mouths of the tubes. For the first three days after the bloodmeal the filter paper is left dry and humidity is controlled at 95% RH by standing the tubes over dilute sulfuric acid of specific gravity 1.1 (Buxton & Mellanby, 1934; Adler & Theodor, 1935; Solomon, 1951) either on a platform in a desiccator or on a perspex rack in a closed plastic box. From the fourth day onwards the paper is kept damp with distilled water applied through the gauze with a syringe and hypodermic needle. The flies then begin to lay eggs after which they seldom survive more than 24 hours; most flies die while ovipositing. The yield of eggs from batches of flies treated this way is normally more than 40% greater than when the filter paper is wetted as they are tubed, and the mean pre-oviposition period is reduced from 7 (4-12) to 6 (5-9) days.

As flies oviposit and die, the dead females are removed and 0.5 ml of distilled water is added to the tubes; the eggs are then kept in the humidity chambers for 5-6 days before being transferred to Hilton pots. To monitor productivity, the females are dissected and retained eggs are counted.

Method 2. Groups of three females are narcotized with carbon dioxide from a hand dispenser,[2] and then transferred to damp Hilton pots for oviposition. A temporary cover of gauze is held over the mouths of the pots with an elastic band, and sugar solution is given in the usual way. The pots are kept at 25°C on damp filter paper in a snap-top plastic box. After the females have laid eggs and died they are removed and the faeces are scraped from the plaster. The gauze is then replaced with a screw cap and the eggs are left to hatch. The larvae are then reared as described above.

This method has the advantage of giving less work because the eggs do not have to be transferred and water does not have to be added daily. Disadvantages are: (i) the lack of information on the performance of individual flies; (ii) the comparatively early growth of fungi introduced into the pots with the engorged females; and (iii) the difficulty of feeding newly hatched larvae if any female has a prolonged pre-oviposition period.

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[1] Recently, however, it was found that egg production was improved by keeping the flies in a 12-hour dark-light regime.

[2] "Sparklets Corkmaster", British Oxygen Company Ltd., London, England.

4. DEVELOPMENTAL TIMES OF LABORATORY-BRED L. LONGIPALPIS

Generation time. At 25°C in environmental cabinets with the water and humidity controlled, the time of development from engorgement to the first emergence of adults of the next generation is 40 days.

Pre-oviposition period. The time from bloodmeal to oviposition is shortest when flies are tubed individually and kept at 95% RH with the filter papers in the tubes left dry until the fourth day after the bloodmeal. The mean time of the pre-oviposition period in these conditions is just under six days (range 5-9).

Hatching time. At 25°C, the mean hatching time of eggs in 86 pots of the fifth to eighth generations was a little less than seven days (range 5-9). Diapause of eggs as reported for some Neotropical sandflies (Lindquist, 1936; Johnson & Hertig, 1961; Ward & Killick-Kendrick, 1974) was not observed.

Larval development. Ranges of minimum developmental times of larvae were obtained by observing the first appearance of each stage in the 86 pots of the fifth to eighth generations. They were: 1st instar, 3-6 days; 2nd instar, 2-4 days; 3rd instar, 1-5 days; and 4th instar, 3-9 days. In general, the normal pattern of larval growth is 1st instar larvae for the first week, 2nd and 3rd for the second and 4th for the third. Diapause of 4th instar larvae as recorded for Holarctic sandflies (e.g. Theodor, 1934; Chaniotis, 1967) and one Neotropical species (Barretto, 1943) did not occur.

Pupal development. At 25°C most adults emerge from pupae on day 10, although a few emerge as early as day 7. Males begin to emerge before females. As a guide to possible deleterious effects of inbreeding, randomly selected pupae of several generations were weighed 1-2 days before emergence. The weights varied but never fell to less than at the first generation.

Longevity of adults. L. longipalpis is relatively robust. When left in an uncovered cage on the laboratory bench for 24 hours, flies of both sexes survive well and, when handling flies, no control of humidity or ambient temperature is necessary.

Sucrose solution is always available to adults of the colony. At 25°C in the dark, males and the females which have not taken a bloodmeal normally live for at least two weeks and many for more than a month. The length of life of females which have taken blood is determined by the time at which they lay eggs. Few survive oviposition more than 24 hours, but gravid females denied a damp surface do not oviposit, and most live for more than a month.

5. PRODUCTIVITY OF THE COLONY

From the initiation of the colony in September, 1972, until the nineteenth generation in May, 1975, 19 392 flies of the hamster line were reared from 82 808 eggs laid by 2725 fed females. For each generation about 120 fed flies are set up in individual tubes to provide eggs from which 800-1000 flies are bred. The somewhat less reliable procedure of permitting engorged flies to digest their meal and oviposit directly in Hilton pots is employed if more flies are needed. Losses have been analysed by calculating the irreplaceable mortality (see Southwood, 1966) of each stage when reared in the best conditions. For this purpose, the figures for potential adults from 100 females were based on a mean number of 80 eggs developed per female; eggs lost by females, refusing a bloodmeal or dying prematurely after absorbing blood, were taken into account.

Losses of eggs. Since the proportion of females taking blood has increased in later generations, the number of eggs lost by females refusing a meal has steadily fallen to an acceptable level now represented by an irreplaceable mortality (IM) of less than 3%. Similarly, improvements in the maintenance of engorged females have resulted in a low IM of 0.6% for eggs lost by engorged flies dying before oviposition.

By far the highest IM of nearly 13% is that of eggs retained by ovipositing females. This represents nearly 40% of the eggs produced. The mean number of eggs produced per fed female is 80, with a maximum of 146, and that of eggs laid is 50, with a maximum of 136. The IM represented by eggs not hatching is 4.4%.

Losses of larvae. The second most important loss is of 1st instar larvae. This is the most delicate instar, and the IM of 7% is more than double that of the other instars combined. Most deaths of 1st instar larvae probably result from the difficulty of standardizing the precise water requirements of this stage (Parrot, 1931; Teixeira, 1947). In later instars, losses are largely attributed to the complete failure of a few individual pots due to the growth of uncontrollable fungi and to cannibalism in pots with large numbers of larvae.

Losses of pupae. The pupa is the most robust stage, and losses have always been low. Currently, the IM is 0.8%. In the first generation, the proportion of pupae giving rise to adults was 88%; in later generations it has never fallen below 96%.

Results of controlling water and humidity. There were improvements in productivity following the adoption of methods to control water and humidity in the maintenance of engorged females. The output rose by 185%, i.e. the production of adults from the potential eggs of females offered a bloodmeal rose from 3.7% to 10.4%. In recent generations, 18-25% of the eggs actually laid were successfully reared to adult flies.

RÉSUMÉ

Pour étudier le cycle biologique et la transmission de Leishmania spp, il faut disposer d'un vecteur qui se nourrisse facilement du sang de hamsters infectés à titre expérimental. Lutzomyia longipalpis répond à cette condition et, dans un laboratoire d'Angleterre, on a établi une colonie fermée de ce phlébotome du Brésil. Etant donné que les phlébotomes ne sont pas indigènes en Angleterre, l'une des principales difficultés a été d'obvier au renouvellement de la colonie, opération sans laquelle les colonies de phlébotomes néotropicaux dépassent rarement la dizième génération. La combinaison de méthodes utilisées a donc été décrite pour chaque stade allant de la ponte et de l'éclosion des oeufs, aux stades larvaire et pupaire et jusqu'à l'apparition de l'insecte adulte et la période de pré-ponte.

Depuis le début de la colonie, en septembre 1972, jusqu'à la 19ème génération en mai 1975, 19 392 insectes de la lignée hamster ont été élevés à partir de 82 808 oeufs pondus par 2725 femelles gorgées. L'adoption de méthodes de régulation portant sur l'eau et l'humidité utilisées pour l'entretien des femelles gorgées de sang s'est accompagné d'une amélioration particulièrement sensible de la productivité. Le rendement s'est élévé de 185%, c'est-a-dire que la production d'insectes adultes à partir des oeufs potentiels provenant des femelles ayant pris un repas de sang est passée de 3,7% à 10,4%. Dans les générations récentes, 18 à 25% des oeufs effectivement pondus ont été élevés jusqu'au stade de l'insecte adulte.

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