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REPRODUCTIVE ISOLATION BETWEEN DIFFERENT FORMS OF *LUTZOMYIA LONGIPALPIS* (LUTZ & NEIVA), (DIPTERA: PSYCHODIDAE), THE VECTOR OF *LEISHMANIA DONOVANI CHAGASI* CUNHA & CHAGAS AND ITS SIGNIFICANCE TO KALA-AZAR DISTRIBUTION IN SOUTH AMERICA

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The males of the sandfly Lutzomyia longipalpis *occur in two forms, one which bears a single pair of pale spots on tergite 4 and another in which an additional pair of spots characterizes tergite 3. In crosses between laboratory reared stocks of the two forms originating from allopatric and sympatric sites in Brazil nearly all males of one form fail to inseminate females of the other. In addition, insemination failure between some allopatric populations of* Lu. longipalpis *with similar tergal spot patterns is recorded, indicating the existence of additional forms in an apparent species complex. The possibility that* Lu. longipalpis sensu latu *represents more than a single taxon is discussed and the relevance of these findings to future epidemiological studies on kala-azar is considered.*

The male of *Lu. longipalpis* occurs in two forms; one in which the 4th abdominal tergite bears a pair of dorsolateral pale spots and the other in which a second pair of spots is found on the 3rd segment. These differences were first recorded by Mangabeira (1969) in a posthumous publication on the sandflies of Ceará State, north-eastern Brazil. He noted that male *Lu. longipalpis* captured in Pará State, N. Brazil bore a single pair of spots whilst those from Ceará had two pairs. Mangabeira observed that the two forms lived under completely contrasting ecological conditions and felt that they might represent two species or varieties. With considerable foresight he wisely commented, however, that

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until the two forms had been compared in colonies they should continue to be treated as a single species.

In 1972 Killick-Kendrick, Leaney & Ready (1973) established a colony of *Lu. longipalpis* from the Lapinha cave (LC) near Belo Horizonte, Minas Gerais (Fig. 1).

Fig. 1 – Origins of *Lutzomyia longipalpis* colonies used in crossing experiments.

Later in 1973, one of us (RDW) established another colony of the same species with flies collected by Dr. R. Lainson from under stacked roof tiles in gardens at Morada Nova (MN), Ceará (Fig. 1). The contrasting origins of the two colonies and minor behavioural differences led in 1974 to attempts at cross-mating them. Despite poor hatch rates from the crosses, it was concluded that they were identical (White & Killick-Kendrick, 1975).

In 1981 during morphological studies of *Lu. longipalpis* we found that males of the Morada Nova colony were uniformly of the type described by Mangabeira with two pairs of pale spots, whilst those from Lapinha cave bore only a single pair, similar to those described from Pará (Fig. 2). The crossing data from 1974 were re-examined and new

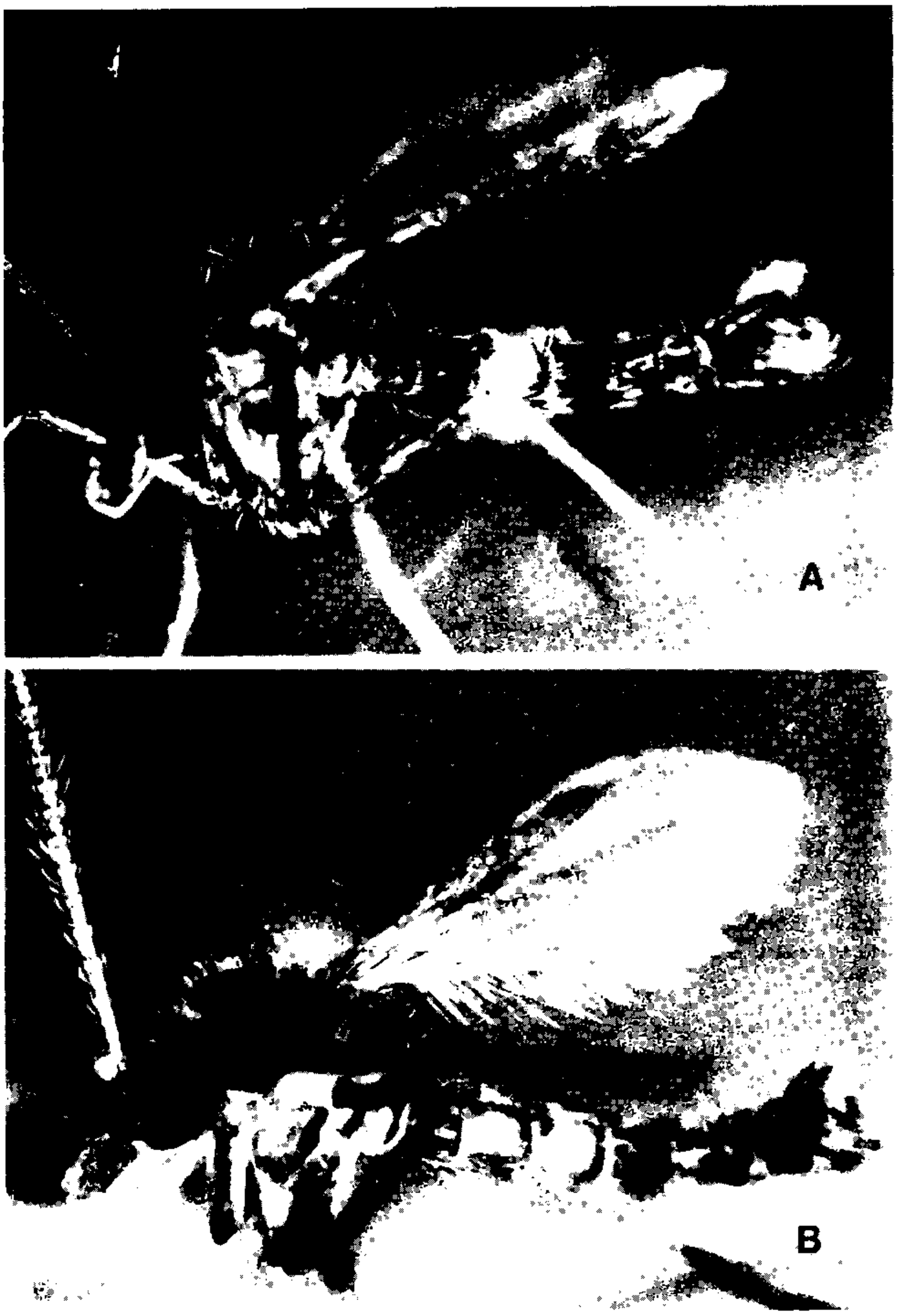

Fig. 2 – (A) The male of *Lu. longipalpis* from Lapinha cave, Belo Horizonte, Minas Gerais, Brazil showing the pale spot on tergite 4. (B) The male of *Lu. longipalpis* from Morada Nova, Ceará, Brazil showing the pale spots on tergites 3 and 4.

crosses carried out in Liverpool in 1980-81 between the two original colonies. Following poor hatch rates in reciprocal crosses between the two forms further experiments and spermathecal dissections showed a high failure rate in the males' ability to inseminate females.

In the present paper we re-examine the crossing results between the Lapinha cave and Morada Nova colonies made in 1974 and describe those carried out in 1980-81.

We were aware, however, that as the colonies had been maintained in laboratories for more than 8 years they might have undergone some artificially induced genetic changes. Therefore we also describe the results of new field collections made in Brazil during April-May 1982 and subsequent crosses between new colonies of the two forms established from flies collected together from a cow shed in Sobral, Ceará (Fig. 1).

Whilst visiting Brazil in 1982 collections of *Lu. longipalpis* were also made at Lapinha cave to establish a 3rd new colony. In addition we were kindly given eggs of a recently established colony from Camara, Marajó island (MI), Pará (Lainson et al, 1983) and have established a 4th new colony in Liverpool. The results of crosses between these new colonies (LC and MI) are also presented (both bear a single pair of pale tergal spots).

MATERIALS AND METHODS

The colonies of *Lu. longipalpis* were maintained in the laboratory as described previously (Ward, 1977). Individual pupae were isolated in vials before adults were released for crossing experiments. Equal numbers of males and females were released into 12x12x12" cages and left for 3-4 days before feeding on golden hamsters. Twenty-four hours later fed females were isolated for oviposition in vials lined with filter paper. The numbers of eggs laid, hatched, and the tergal colouration of the reared males from each petri dish were recorded.

To test if females were inseminated during copulation large numbers of males of one type were placed in experimental cages and single virgin females were released at intervals into the cage. If no copulation was observed after 20 minutes, the female was removed from the cage and another substituted. Female flies of the two forms were released alternately into the cage of males and those that copulated were observed until they separated from the male, when they were removed from the cage for spermathecal dissection.

Spermathecal dissections were carried out using very fine entomological pins mounted in wooden cocktail sticks. The two terminal abdominal segments of each female are removed in a drop of saline and teasing of the chitinized furca from within the tissues in these segments usually results also in the removal of the spermathecae. The latter are then examined under a x 100 oil immersion objective whilst slowly draining the saline from beneath the cover slip with a small piece of filter paper. In some individuals sperm can be observed undulating along the length of the spermathecae until removal of the saline creates downward pressure from the cover slip and deters further movement. If saline is added quickly to the edge of the cover slip following cessation of sperm movement, the sperm become active again. In other spermathecae little movement is detectable, though the opaque globule containing semen in such individuals contrasts with the translucent appearance of uninseminated spermathecae (Fig. 3). Most female *Lu. longipalpis* were dissected before oviposition as there is a very high mortality rate during egg laying. Others were allowed to lay eggs and the hatch rate was used to indicate insemination.

The recently estabished colonies of *Lu. longipalpis* from Sobral, Ceará were the progeny of more than 300 blood fed females collected on 1/5/82 from a cow stable

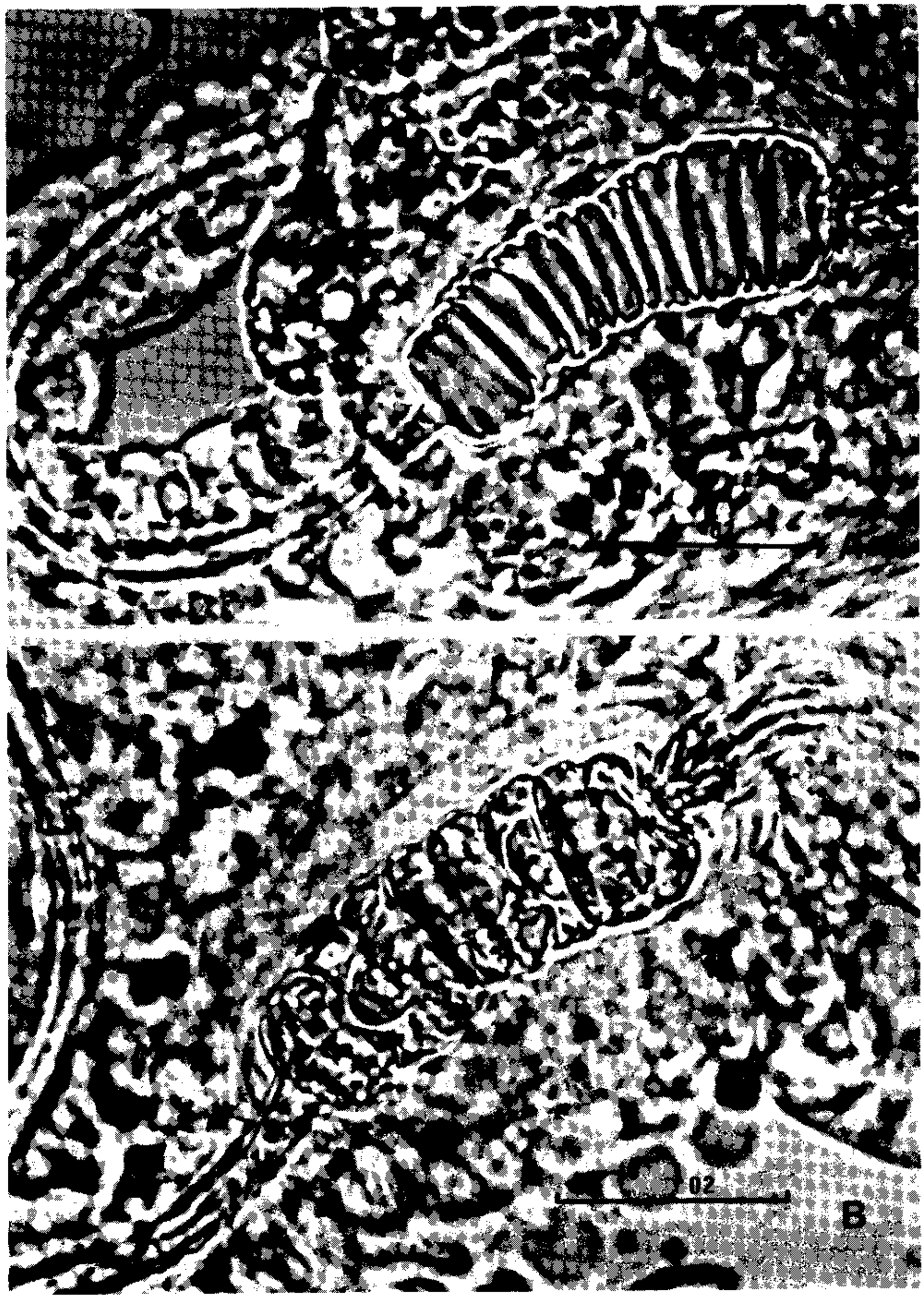

Fig. 3 – (A) Uninseminated spermatheca of *Lu. longipalpis* from Morada Nova. (B) Inseminated spermatheca of *Lu. longipalpis* from Lapinha cave (X1,600).

adjacent to a house at Boqueirão de Renato Parente, about 10 kms north of Sobral, Ceará. The females were aspirated from the stable wall and released into a cage from which individual females were isolated in plastic vials lined with damp filter paper. From the fed

flies, 49 individual egg batches were reared separately in plastic sterile petri dishes lined with filter paper. Larvae were fed on finely ground *Daphnia* until pupation. The male adults from each of the 49 progeny series were scored separately for tergal spotting and with their associated females separated into two daughter colonies (1 spot and 2 spot Sobral). In a later laboratory generation the males of the 1 spot and 2 spot Sobral colonies were all examined to determine if each had remained monomorphic. The progeny from 250 wild-caught females were released together into a cage, giving a mixed population of the one and two spot type males and their associated females. From these flies individual egg batches from 57 females were subsequently reared separately and the morphology of the reared males was recorded.

In the following descriptions of the crosses carried out between the colonies females are designated before males.

RESULTS

(a) Morada Nova (MN), (2 spot) x Lapinha cave (LC), (1 spot) 1974 and 1980-81.

The crosses made in 1974 and 1980-1 between the LC and MN colonies are summarized in Table I. Reciprocal crosses between LC and MN colonies produced a small number of hybrid progeny. These hybrids were crossed as summarized in Table II, and low hatch rates of 8.3 and 9.0% were recorded. The results suggest either hybrid breakdown or, more possibly, insemination failures similar to those observed in crosses between the parental types. Two male and 8 female F_1 hybrids (MN/LC) were backcrossed with LC adults and the results of this experiment are summarized in Table II. The low hatch rates of 8.3 and 9.0% seen in hybrid crosses are not as marked in F_1 hybrid backcrosses where rates of 25.5 and 47.6% were seen, though the amount of data is limited probably due to poor insemination rates.

TABLE I

Crosses between laboratory reared *Lu. longipalpis* from Lapinha Cave and Morada Nova (1974 and 1980-81)

Cross	*Total no. of eggs laid*	*No. of eggs hatched*	*% hatch*	*Phenotype of reared* ♂♂	
♀♀ ♂♂				1 spot	2 spot
MN x MN	2590	1209	46.7	–	–
LC x LC	354	156	44.0	–	–
LC x MN	369	76*	20.6	–	–
LC x MN	323	33	10.2	0	17
MN x LC	933	3	0.3	0	1
MN x LC	714	38	5.3	–	–

*The 76 larvae hatched from the eggs of 2 ♀♀; eggs of a further 6 ♀♀ failed to hatch.

Further crosses and spermathecal dissections were carried out to determine the cause of poor egg hatches in crosses between adults of the two forms. The results of these observations are shown in Table III and Fig. 4, where it is evident that in crosses between different forms most males were unable to inseminate females despite observed periods of

TABLE II

Hybrid crosses and backcrosses using *Lu. longipalpis* from the Morada Nova and Lapinha Cave colonies (1980-81)

Cross	*Total no. of eggs laid*	*No. of eggs hatched*	*% hatch*	*Phenotype of reared ♂♂*	
♀♀ ♂♂				1 spot	2 spot
MN/LC x MN/LC	240	20	8.3	–	–
MN/LC x MN/LC	177	16	9.0	1	2
MN/LC x LC	231	59	25.5	8	1
LC x MN/LC	63	30	47.6	1	0

copulation lasting up to 10 minutes. In crosses between different forms more than 70% of the males flapped their wings for part or most of the time during copulation and females often attempted to resist either by lowering their abdomen and/or by moving away from the male, thus dragging him around the cage. In crosses between flies from Morada Nova and Lapinha Cave 26-42% of females attempted to repulse males though none of the Lapinha Cave females rejected their own males. A comparison of copulation durations for the two experiments – LC x MN and LC x LC shows them to be significantly different ($P < 0.02$) (Bailey, 1981-Wilcoxon's rank sum test for two samples). It is interesting however, that MN x LC crosses lasted longer than the others. Unfortunately the MN x MN control cross was not carried out due to low colony numbers.

TABLE III

Insemination rates following reciprocal crosses between laboratory reared *Lu. longipalpis* from Lapinha Cave and Morada Nova (1980-81)

Cross	*No. of males in cage*	*No. of females released*	*No. of females copulating*	*No. of females inseminated*
♀♀ ♂♂				
LC x LC	52	36	19	17
MN x LC	52	32	19	0
LC x MN	37	19	15	0

(b) Sobral, (1 spot) x Sobral, (2 spot) 1982.

From the 49 individual egg batches reared from *Lu. longipalpis* collected at Sobral, 17 females gave rise to 313 male progeny, all with a single pair of pale spots on tergite 4. A further 23 individual batches gave rise to 287 males which bore pale spots on tergites 3 and 4. Five of the egg batches, however, gave rise to 1 spot and 2 spot males in the following ratios 1:16, 4:2, 4:1, 10:4 and 5:6. These flies were preserved in liquid nitrogen ($-196^{o}C$) for future electrophoretic study. A further 4 egg batches produced only a total of 5 adults and were therefore not classified for spot form. Crosses between adults reared from the apparently monomorphic 1 spot and 2 spot batches are summarized in Table IV.

The adult progeny from the 250 Sobral flies that were not individually reared as isolated egg batches were released into a cage and allowed to mate at random. From these

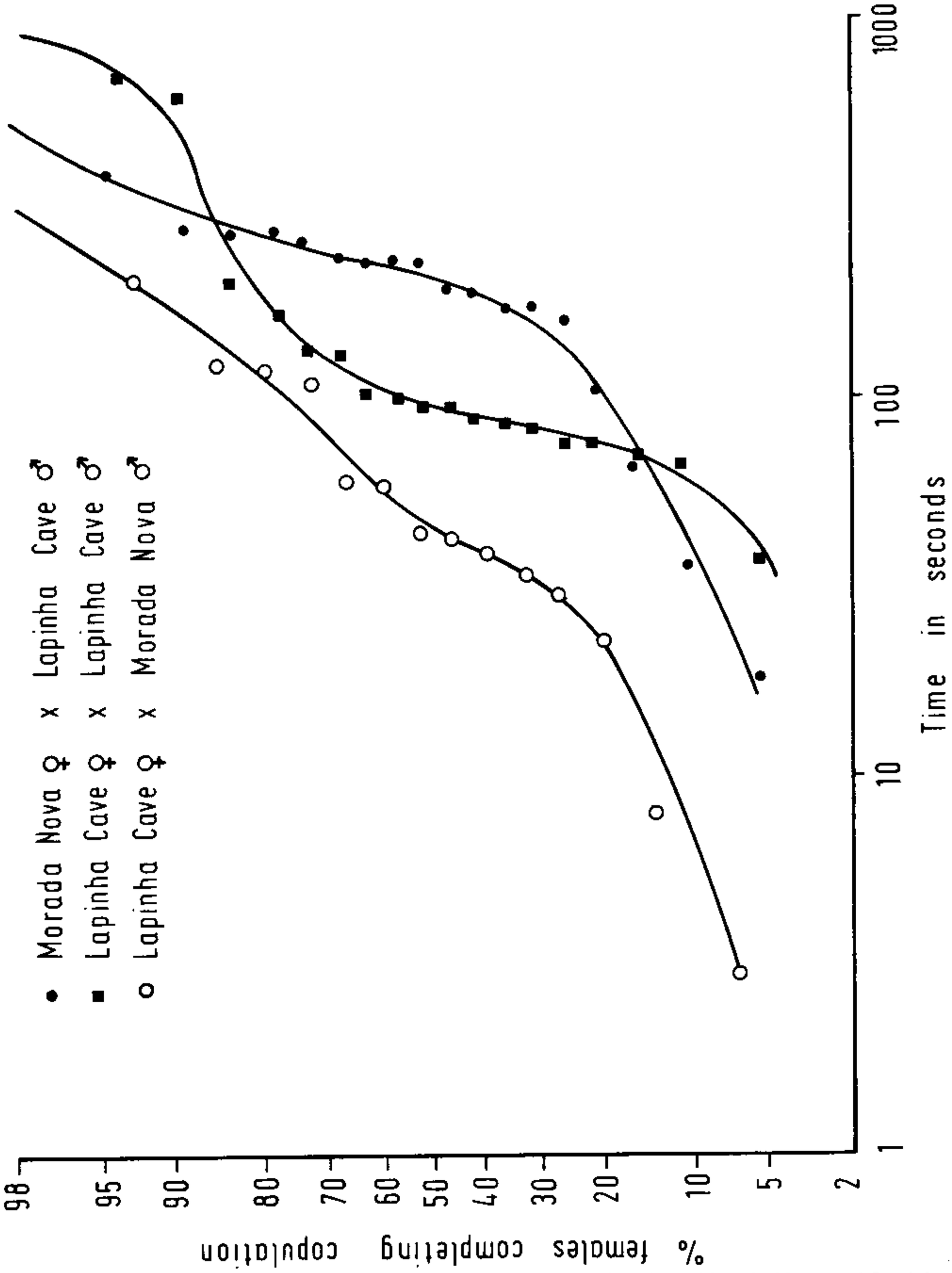

Fig. 4 – A comparison of mating durations between *Lu. longipalpis* from Morada Nova and Lapinha cave (1980-81). Plotted on a probit scale.

flies, 57 individual egg batches were reared separately. Of these, 15 gave rise to only 1 spot males (159 ♂♂), 20 to 2 spot males only (276 ♂♂) and 3 produced males of the 1 and 2 spot form in the following ratios 8:4, 6:1, and 13:3 (35 ♂♂). Nineteen of the individual batches could not be classified as they either gave rise to small numbers of males (less than 4) or only females (20 ♀♀ and 13 ♂♂).

TABLE IV

Crosses between 'one spot' and 'two spot' *Lu. longipalpis* reared from adults collected in a cow shed, Sobral (1982)

Cross	*Inseminated*	*Uninseminated*	*Eggs laid by undissected* ♀♀	*Eggs hatching*	*% hatch*
♀♀ ♂♂					
1 spot x 1 spot	7	1	216	140	64.8
2 spot x 2 spot	12	3**	650	424	65.2
2 spot x 1 spot		34*	99	0	0.0
1 spot x 2 spot		12	152	3	1.9

*5 of these females were caught in copula immediately before dissection.
**Dissected after oviposition and laid eggs which subsequently hatched.

(c) Marajó island (MI) x Lapinha cave (LC), (both 1 spot).

The results of crosses between *Lu. longipalpis* from a colony established from Marajó island, Pará and a new colony from the Lapinha cave, are summarized in Table V. It is of interest that although the males of these two colonies have similar abdominal tergite patterns none of the MI ♂♂ were capable of inseminating LC ♀♀ and only 10.2% of MI ♀♀ laid eggs which hatched after mating with LC ♂♂. The parental crosses, however, gave rise to normal hatch rates of 44-56%.

TABLE V

Crosses between *Lu. longipalpis* from Marajó island and Lapinha cave (both 1 spot).

Cross	*Inseminated*	*Uninseminated*	*Eggs laid by undissected females*	*Eggs hatching*	*% hatch*
♀♀ ♂♂					
MI x MI	10	0	426	240	56.3
LC x LC	5	0	291	128	44.0
LC x MI	0	6	215	0	0.0
MI x LC	2	10	254	26	10.2

DISCUSSION

In the first series of crosses carried out between the allopatric populations of Morada Nova, and the Lapinha cave *Lu. longipalpis*, there were indications that although there was little insemination between them, some fertile hybrids of both sexes were produced. It is not certain however, if in addition to ethological and/or mechanical barriers to mating, there are also post-mating barriers resulting in reduced hatch rates. Despite the production of fertile F_1 progeny of both sexes, hybrid crosses and backcrosses

generally showed lower hatch rates than in parental matings, indicating some reproductive disadvantage to hybrids. The fact that all the hybrid males produced from the cross (LC x MN) in the 1980-81 experiments (Table I) were of the male parental type indicates that hybrid progeny would not be recognised phenotypically. The hybrid crosses and backcrosses (Table II) show also that individual females may produce male progeny of the two phenotypes. With the small number of hybrids produced it will require a much longer series of crosses to establish the formal genetics of pale tergal spot expression.

From observations of mating behaviour it appears that it is the female flies which differentiate between the different forms of males and respond appropriately. The abdominal dipping and dragging of males by females when copulating with males of the wrong type has also been observed in *Drosophila* and termed "decamping behaviour" (Burnett & Connolly, 1974). The response of females to males may be the result of auditory stimuli produced by males which beat their wings when approaching potential mates before copulation. Thus, continual male wing beating was observed throughout crosses between adults of different forms by a large percentage of individuals. This behaviour may represent continued unsuccessful efforts to court females who fail to respond as expected by refusing insemination. Differences in duration of copulation between crosses may be interpreted in different ways. Thus the rapidity of the LC x MN couplings may be due to male loss of interest due to insemination failure, or the successful repulsion of males by females. Clearly, however, the MN x LC couplings lasted considerably longer and in this case perhaps the females are less successful in repelling the advances of the males.

In the second series of crosses, apparently homozygous 1 spot individuals from Sobral were matched with apparently homozygous 2 spot individuals of the opposite sex from the same location. Once again it appears as if the two forms of *Lu. longipalpis* are almost totally sexually isolated, either behaviourally and/or mechanically. The ratios of the pure 1 spot, 2 spot and mixed batches of adults reared from eggs from wild caught females were almost identical to those observed from females mated in a mixed cage in the laboratory (wild : lab, 1 spot 17:15, 2 spot 23:20, mixed 5:3). It would appear therefore that there are insemination barriers and possibly assortative mating occurring in the laboratory and in the field. The laboratory hybrid crosses indicate that wild females which produce phenotypically mixed males are either hybrids or have mated with one. However, it does seem that with the reproductive barriers between the two forms that hybrids must be selected against, resulting in little or no genetic introgression in the sites where the two forms are sympatric. To date, the pure laboratory lines of the Sobral 1 and 2 spot *Lu. longipalpis* isolated for the crosses and colonies have remained distinct for tergal colouration during 3 generations.

In crosses between allopatric populations of *Lu. longipalpis* (Lapinha cave and Marajó island) with phenotypically similar tergal spot markings there are again almost complete insemination barriers. Total reliance on male tergal colouration to identify sexually isolated populations of this sandfly is therefore not possible.

In summary it appears that the 1 spot Sobral form of *Lu. longipalpis* is different from the 2 spot form; that the 2 spot Morada Nova form is different from 1 spot Lapinha cave flies and that 1 spot Marajó island also differs from 1 spot Lapinha cave *Lu. longipalpis*. We do not yet know however, if the 1 spot Sobral form is sexually compatible with either Marajó or Lapinha populations (Fig. 5).

We conclude that there are at least 2 different sexually isolated forms of *Lu. longipalpis* which may represent members of a species complex. Until further crossing experiments and morphological studies on field material are carried out it would seem unwise, however, to assign these to specific taxa. Similar systematic problems have been encountered in other organisms. For example the mussels *Mytilis edulis* and *M. gallopro-*

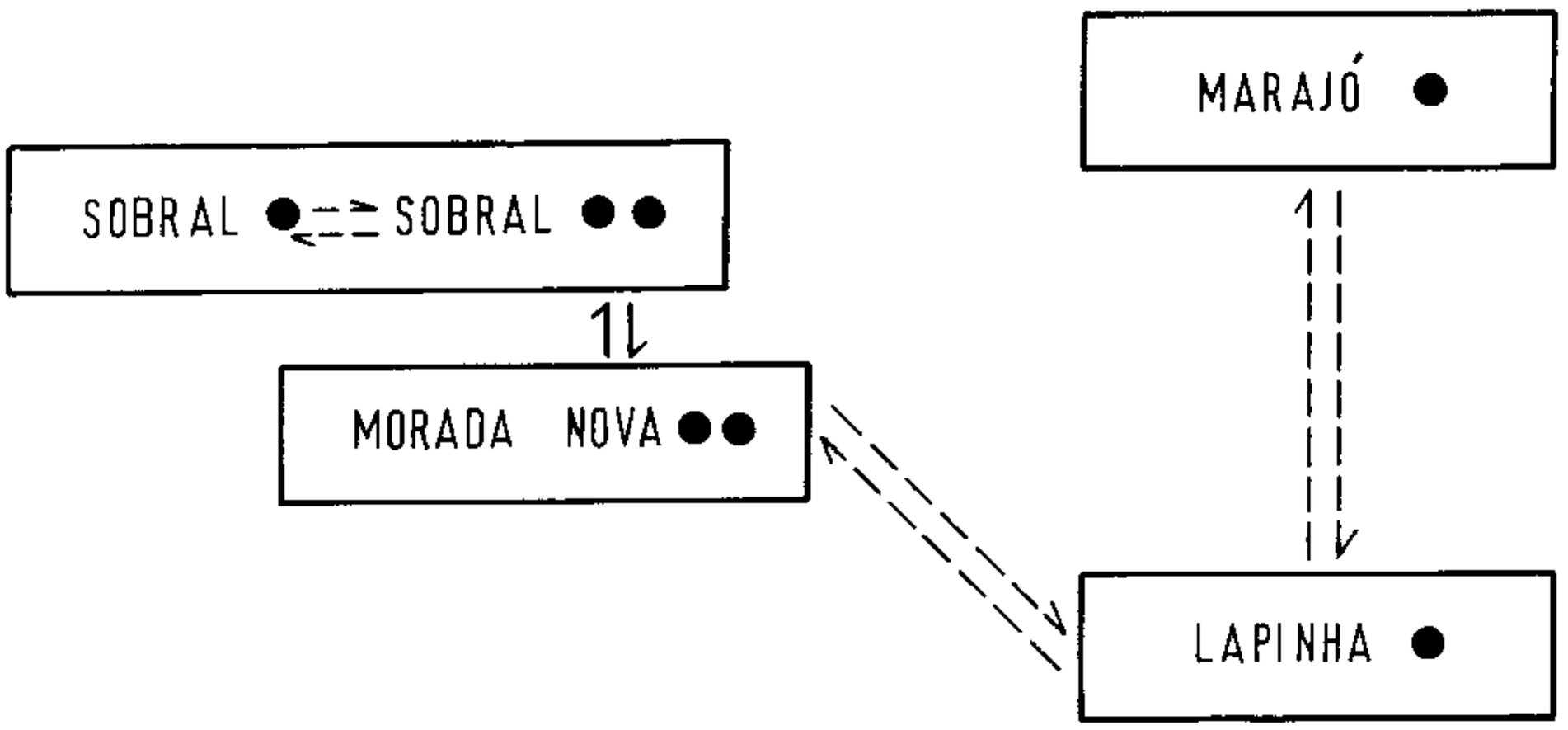

Fig. 5 – The crossing relationships between 5 stocks of *Lu. longipalpis* from Brazil. (– – – – – – = reduced insemination, ———— = normal insemination), ● = male flies with a pair of pale spots on tergite 4 and ● ● = male flies with similar pale spots on tergites 3 and 4.

vincialis show only slight morphological differences, they occur sympatrically in some areas and yet produce up to 19% of fertile hybrids in some localities (Gosling, in press).

In addition to the taxonomic significance of the present findings it may be that the forms identified have behavioural and/or physiological differences influencing the transmission of *L. donovani chagasi*. Visceral leishmaniasis occurs from Mexico to Argentina, with about 87% of human cases recorded in Brazil, in particular from the north eastern states (Ward, 1977a). Our observations to date, indicate that the 1 spot form of *Lu. longipalpis* has a wide distribution including Mexico, Venezuela, Colombia and Brazil, whilst the 2 spot form is restricted to the eastern side of Brazil from Ceará state almost to the Paraguayan border. Furthermore during our work in Ceará it was noted that *Lu. longipalpis* in houses was highly anthropophilic. In contrast observations in Colombia with Dr. A. Morales during 1979 showed that only the 1 spot form was present and that it rarely entered houses or bit man. In conclusion it is tempting to suggest that the 2 spot form of *Lu. longipalpis* may be a more important vector of *L. d. chagasi* than the 1 spot form and that its restricted distribution is perhaps linked to the focality of human cases. It is to be hoped that future observations on the relative distribution, abundance and behaviour of the two forms will answer some of these outstanding questions.

RESUMO

Os machos de *Lu. longipalpis* ocorrem em duas formas distintas: uma com um simples par de manchas claras no 4º tergito e a outra com um par adicional de manchas caracterizadas no 3º tergito.

Cruzamento entre linhagens obtidas no laboratório das duas formas, originárias de áreas alopátricas e simpátricas no Brasil, demonstrou que quase todos os machos falharam em inseminar fêmeas heterólogas. Observou-se também falhas na inseminação entre populações alopátricas de *Lu. longipalpis* com as mesmas características de manchas indicando a existência de duas ou mais formas num aparente complexo de espécies. A possibilidade de *Lu. longipalpis sensu latu* representar mais que um simples taxon é discutida e a relevância destes encontros nos futuros estudos epidemiológicos do kala-azar é considerada.

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