north-east of Brazil (Marcondes, personal communication). The crossing results described above are summarized in Figure 1. These apparently confusing results are now explained by the fact that the males of *Lu. longipalpis* possess a pheromone gland beneath the pale spots and it is probably these semiochemicals that determine the reproductive compatibility of different siblings.

(b) Male Tergal Spots: Morphology and Histology

Examination of the pale tergal spots using scanning electron microscopy revealed the presence of numerous 3.0–3.5 μ cuticular papules each with a central pore and consistent with a site of pheromone emission (Lane & Ward, 1984). The tissue beneath the pale spots was subsequently sectioned and examined, using electron microscopy (Lane & Bernades, unpublished data). A gland was found with highly vacuolated cells each of which is equipped with an apparently secretory end apparatus. Boufana *et al.* (1986) later showed that the gland has a developmental cycle which is correlated with the onset of pheromone production.

Male Pheromones

The male glands of 6 populations of *Lu. longipalpis* from different areas of Brazil were examined by hexane extraction, followed by gas chromatography and mass spectral analysis. Four populations from the north and north-east including both male phenotypes, contained a hydrocarbon of molecular weight 272 ($C_{20}H_{32}$) and a mass spectrum which suggest the presence of a diterpenoid. Two other populations, of the one-spot type from the north-east and the south contained a hydrocarbon of molecular weight 218 ($C_{16}H_{26}$) with a mass spectrum similar to the pheromone homofarnasene (Lane *et al.*, 1985; Phillips *et al.*, 1986).

Pheromone Activity

To demonstrate some biological activity for the pheromones found in the gland an olfactometer was made in which female response to hosts was compared with their behaviour when male sandflies were present. In 6 preliminary experiments using a "diterpenoid colony" from Morada Nova, 261/384 females responded to hamsters. Of these, 40 were attached to a hamster alone and 221 to a hamster in the presence of male sandflies.

Auditory Communication

Another preliminary series of experiments in collaboration with Dr B. Burnett of Sheffield University has shown that male and female *Lu. longipalpis* produce distinct polycyclic sound pulses. These may prove to be species specific, but it is not yet known how much individual variation there is or if the pulses have a role to play in sexual communication (Ward *et al.*, 1986).

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Part I.IV The Transmission of Leishmaniasis by the Bite of the Sandfly

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Introduction

Perhaps the first published mention of leishmaniasis being transmitted by the bite of sandflies was by Beuno (1764) who noted that uta, a cutaneous form of leishmaniasis in Peru, was said to be contracted by the bites of a sandfly, also called uta. The first experimental evidence of transmission by bite did not arrive until 167 years later when Shortt *et al.* (1931) transmitted *Leishmania donovani* to hamsters by the bite of *Phlebotomus argentipes*. The transmission of oriental sore to man by bite was proven by Adler and Ber (1941) and of kala-azar to man by Swaminath *et al.* (1942).

Since the 1930's, much interest has focussed on the transmission of leishmaniasis. In particular, there are differences of opinion and contradictory observations on the forms of the parasite deposited in the skin of the vertebrate host when an infected sandfly bites. The

Table 1.

Combinations of sandflies and leishmaniae in which proboscis infections have been seen.

Parasite	Sandfly	Reference
<i>L. major</i>	<i>P. papatasi</i>	Present work Beier <i>et al.</i> (1986)
<i>L. major</i>	<i>P. langeroni</i> *	Beier <i>et al.</i> (1986)
<i>L. tropica</i>	<i>P. sergenti</i>	Adler <i>et al.</i> (1938)
<i>L. donovani</i>	<i>P. chinensis</i>	Feng and Chung (1939)
<i>L. infantum</i>	<i>P. perniciosus</i>	Adler and Theodor (1931; 1935)
<i>L. infantum</i>	<i>P. ariasi</i>	Rioux <i>et al.</i> (1979; 1984)
<i>L. infantum</i>	<i>P. major</i>	Adler <i>et al.</i> (1938)
<i>L. chagasi</i>	<i>Lu. longipalpis</i>	Sherlock and Sherlock (1961)
		Deane and Deane (1962)
		Lainson <i>et al.</i> (1977)
		Stephenson and Ward (1968)
<i>L. mexicana</i>	<i>Lu. diabolica</i>	Lawyer <i>et al.</i> (in press)
<i>L. mexicana</i>	<i>Lu. shannoni</i> *	Lawyer <i>et al.</i> (in press)
<i>L. mexicana</i>	<i>Lu. sanguinaria</i> *	Christensen and Herrero (1980)
<i>L. mexicana</i>	<i>Lu. gomezi</i> *	Christensen and Herrero (1980)
<i>L. m. mexicana</i>	<i>Lu. ylephiletor</i> *	Strangways-Dixon and Lainson (1966)
<i>L. b. braziliensis</i> (?)	<i>Ps. wellcomei</i>	Killick-Kendrick (1979)
<i>L. b. guyanensis</i> (?)	<i>Lu. umbratilis</i>	Killick-Kendrick (1979)
<i>L. b. panamensis</i>	<i>Lu. sanguinaria</i>	Hertig and McConnell (1963)
<i>L. braziliensis</i> (?)	<i>Lu. longipalpis</i> *	Cætho <i>et al.</i> (1967)
<i>L. braziliensis</i> (?)	<i>Lu. renei</i> *	Cætho <i>et al.</i> (1967)

*Not a known vector of the parasite.

controversy has centred around the question of whether it is essential for the life-cycle to culminate in the production of infective, metacyclic forms in the proboscis of the fly or whether transmission can be accomplished by the regurgitation of parasites from parts of the alimentary tract posterior to the mouthparts. Related questions are concerned with mathematical modelling of the circulating of the parasites in nature. For this, there is a need to be able to estimate the period from the time a sandfly takes an infecting feed until the life cycle of the parasite has progressed to a point when the bite is potentially infective. In addition, it is important to be able to judge the likelihood of an infected fly transmitting parasites each time it feeds. From the immunologist's point of view, work towards a vaccine would be accelerated if the antigenic nature of the forms against which man should be protected were fully known. Finally, a better understanding of the mechanism of transmission could help in attempts to create laboratory models of predictable transmission by bite.

In this paper, a review is given of observations on the forms which leave the proboscis in the act of biting in the light of (i) the recent unequivocal demonstration of the infective forms which arise both in cultures (in the stationary phase) and in the midgut of the sandfly and (ii) an hypothesis on ways in which promastigotes

interfere with the feeding behaviour of an infected sandfly.

Proboscis Forms in Sandflies

Promastigotes in the proboscis have been seen in 18 combinations of *Leishmania* species and vector (Table 1). Descriptions of the parasites suggest that they have a characteristic behaviour and morphology. In fresh preparations of the mouthparts, the parasites are small, unattached promastigotes which rapidly swim up and down the lumen.* Promastigotes in other parts of the alimentary tract of sandflies, which, like the mouthparts, are lined with a cuticular intima (ileum, pylorus, stomodæal valve, oesophagus, pharynx and cibarium) attach to the chitin by modified flagella within so-called hemidesmosomes (from Killick-Kendrick *et al.*, 1974, 1977a,b,c; Killick-Kendrick, 1979). In contrast, the forms in the proboscis do not form hemidesmosomes (Killick-Kendrick & Molyneux, unpublished observations) and are never attached.

The morphology of proboscis forms of all species of *Leishmania* appears to be similar. We have seen numerous promastigotes of a Saudi Arabian strain of *L.*

*Personal observations on *L. major* in *P. papatasi*, *L. infantum* in *P. ariasi*, *L. b. (?) braziliensis* in *Psychodopygus wellcomei* and *L. b. (?) guyanensis* in *Lu. umbratilis*.

major (HOM/SA/82/RKK) in the mouthparts of an experimentally infected specimen of *P. papatasi* from a colony established with females collected at the Al Hassa oasis, Saudi Arabia. The female had engorged on a lesion on the foot of a BALB/c mouse 12 days before the dissection and was maintained at 30°C. A solution of sucrose was provided *ad libitum*. A smear of the parasites in the proboscis was made and fixed in methanol; it was then stained in Giemsa's stain (pH 7.2).

The promastigotes were not in division nor were they attached to the chitin of the mouthparts. Measurements of 50 consecutively seen parasites showed them to be monomorphic. The total length ($\pm$ standard deviation (SD)) of the 50 promastigotes was $30.6 \pm 2.75\mu$; the body length ($\pm$ SD) of the same parasites was $10.1 \pm 0.89\mu$ and the length ($\pm$ SD) of the flagellum was $20.5 \pm 2.16\mu$. At the widest point, the body measured $1.0\text{--}1.2\mu$. The posterior end was narrow and pointed. The depth of the flagellar pocket was $1.5\text{--}2.5\mu$ and the kinetoplast was a little less than 1.0μ in diameter. The nucleus was generally oval in shape measuring $2.5\text{--}3.5\mu$ in the longitudinal axis. It lay slightly posterior to the centre of the body. There were no stained inclusions or vacuoles in the cytoplasm (see Figure 1).

Adler and Ber (1941) measured promastigotes seeping from the site of a bite of a specimen of *P. papatasi* experimentally infected with "*L. tropica*" (probably *L. major*). The length of the body was $6.5\text{--}13.0\mu$ and that of the flagellum $13\text{--}24\mu$. In a description of promastigotes of *L. infantum* in the proboscis of *P. perniciosus*, Adler and Theodor (1931, p486) gave measurements of $4.7\text{--}10.0\mu$ for body length; the flagellum was usually much longer than the body. Shortt *et al.* (1926) described the most anteriorly positioned promastigotes of *L. donovani* in the cibarium of *P. argentipes* as "small elongated forms with a well developed flagellum". This description fits the proboscis forms of *L. infantum* in *P. ariasi* (personal observation) and, presumably, is characteristic of the stages of most, if not all, species of *Leishmania*.

There is mounting evidence that the small, active unattached promastigotes are the only forms in the sandfly which are infective to vertebrates (Sacks & Perkins, 1984; Sacks *et al.*, 1985; Franke *et al.*, 1985). Although it has been suggested that these proboscis forms probably arise from paramastigotes in the pharynx of the fly (Killick-Kendrick, 1979), it is far more likely that the major, possibly sole, site of development is in the midgut. Promastigotes from the midgut are commonly infective and the short promastigotes must, therefore, often be in that part of an infected sandfly. They have been found in the midguts of *P. perniciosus* infected with *L. infantum* and, moreover, it was shown that proboscis infections were commonest in sandflies with "short forms" in the midgut (Adler & Theodor, 1931).

The factors controlling the change from non-infective to infective promastigote in the midgut are not known.

Sacks and Perkins (1984) suggested that the change was perhaps a response to "adverse growth conditions when the digestion of the bloodmeal was nearing completion". The stimulus might be the waste products of the large numbers of promastigotes which are usually present at the same point of digestion. This is one interpretation of the observation that infective stages arise *in vitro* after suspending promastigotes for 4–6h in spent culture medium (Sacks *et al.*, 1985).

Infective promastigotes develop early in the midgut infection and differ in a number of ways from promastigotes which are not infective. It has been shown (i) that infective stages of *L. major* and *L. m. amazonensis* can arise in the midguts of *P. papatasi* and *Lu. longipalpis*, respectively, as early as 3 days after an infecting bloodmeal* (Sacks & Perkins, 1984, 1985); (ii) that some surface carbohydrates of non-infective forms differ from those of infective forms (Sacks *et al.*, 1985); (iii) that, whereas uninfected promastigotes in the logarithmic phase of division are complement sensitive, those in the stationary infective phase are complement resistant (Franke *et al.*, 1985); (iv) that short stationary phase promastigotes *in vitro* can, unlike other forms, survive in macrophages (Franke *et al.*, 1985); and (v) that, by Western blot analysis and immunoprecipitation of surface labelled organisms, an antigen, which is absent from the surface of forms which are not infective, has been demonstrated on infective promastigotes (Sack *et al.*, 1985). These observations support the opinions of early workers (Shortt *et al.*, 1926; Adler & Theodor, 1931, 1935) that there is one particular form of promastigote in the sandfly (and in cultures) which is adapted for life in the vertebrate.

Infection by the Bite of the Sandfly

The characterization of the infective stages does not answer the question of whether it is essential for a sandfly to have parasites in the proboscis before it can transmit the infection, or whether promastigotes from other parts of the alimentary tract are regurgitated in the skin during the act of feeding (Sacks & Perkins, 1985). There are, however, three observations on the forms likely to be deposited in the skin of a vertebrate which suggest that the only parasites put in are the short metacyclic forms. Promastigotes have been recovered from (i) the proboscis of infected sandflies by feeding them with a fine capillary; (ii) the fluid oozing from a bite of an infected sandfly; and (iii) the fluid on which infected sandflies had fed through a membrane (Adler & Theodor, 1929, 1931, 1935; Adler & Ber, 1941). In all these observations, short promastigotes indistinguishable from those seen in the proboscis of infected sandflies were the only morphological form seen. Had parasites been regurgitated from the

*Infective forms probably do not arise in the midgut before 3 days. Parrot and Donatien (1927) inoculated mice with large numbers of promastigotes of *L. major* from midguts of *P. papatasi* with 34–48 hour-old infections with negative results.

Figure 1. Metacyclic promastigotes of *Leishmania major* in a stained smear from the proboscis of an experimentally infected specimen of *Phlebotomus papatasi*; day 12.

stomodæal valve or midgut, the majority of the parasites would presumably have been large promastigotes, some probably in division, but none was reported.

On the other hand, among accounts of laboratory transmissions by bite with 20 different combinations of parasite and vector (see Table 2), there are some which are difficult to reconcile with the idea that infections of the proboscis are essential if transmission by bite is to succeed. Lainson *et al.* (1977) first saw promastigotes of *L. chagasi* in the probosces of experimentally infected *Lu. longipalpis* 14 days after an infecting bloodmeal (21 flies dissected between days 5–17). Transmissions to hamsters by the bite of the same batch were, however, achieved on days 7, 11 and 13 (also 14 and 17). The results of the examination of the dissected flies raise the possibility that transmission may have been by the bite of flies with no parasites in the proboscis. However, there were short, active promastigotes in the pharynxes of some of the flies (Figure 1(d) of Lainson *et al.*, 1977) which were like the forms now proven to be infective to vertebrates. Stephenson and Ward (1986) also transmitted *L. chagasi* to hamsters by the bite of *Lu.*

longipalpis but only rarely saw very few parasites in the probosces of the flies.

Pozio *et al.* (1985) experimentally transmitted *L. infantum* by the bite of *P. perniciosus* and noted that none of the sandflies which infected rats had parasites in the proboscis or pharynx. In other work with the same parasite and fly, none of the 300 females had parasites anterior to the stomodæal valve. The authors prudently pointed out that these observations require confirmation by experiments in conditions which were more natural than those in the laboratory.

The only information on the complete life-cycle of a *Leishmania* in a sandfly in nature is in an unpublished paper cited by Killick-Kendrick (1979) who summarized the results of mark-release-recapture experiments with *P. ariasi* after engorgement on dogs naturally infected with *L. infantum*. Of sandflies which lived long enough, 60% developed infections in the pharynx; of these, one in ten could be expected to have infections in the proboscis on any one day (i.e. 6% of infected females surviving >16 days; see Killick-Kendrick & Rioux, 1981). The bite of a fly recaptured 22 days after an

Table 2.
Laboratory transmissions of *Leishmania* species by the bite of experimentally infected sandflies

Parasite	Sandfly	Vetebrate infected by bite	References
<i>L. donovani</i>	<i>P. argentipes</i>	Chinese hamsters	Shortt <i>et al.</i> (1931), Napier <i>et al.</i> (1933), Smith <i>et al.</i> (1936, 1940)
<i>L. donovani</i>	<i>P. argentipes</i>	Golden hamsters	Smith <i>et al.</i> (1941).
<i>L. donovani</i>	<i>P. argentipes</i>	Mice	Smith <i>et al.</i> (1940)
<i>L. donovani</i>	<i>P. argentipes</i>	Man	Swaminath <i>et al.</i> (1942)
<i>L. donovani</i> (?)	<i>P. chinensis</i>	Chinese hamsters	Feng and Chung (1941), Ho <i>et al.</i> (1943), Chung <i>et al.</i> (1)
<i>L. donovani</i> (?)	<i>P. smirnovi</i>	Golden hamsters	Dergacheva and Strelkova (1985)
<i>L. donovani</i> (?)	<i>P. longiductus</i>	Golden hamsters	Dergacheva and Strelkova (1985)
<i>L. infantum</i>	<i>P. ariasi</i>	Dog	Rioux <i>et al.</i> (1979)
<i>L. infantum</i>	<i>P. perniciosus</i>	Rat*	Pozio <i>et al.</i> (1985)
<i>L. chagasi</i>	<i>Lu. longipalpis</i>	Golden hamsters	Lainson <i>et al.</i> (1977), Stephenson and Ward (1986)
<i>L. major</i> (?)	<i>P. papatasi</i>	Man	Adler and Ber (1941)
<i>L. major</i>	<i>P. papatasi</i>	Gerbils	Kryukova (1941)
<i>L. major</i>	<i>P. duboscqi</i>	Mice	Beach <i>et al.</i> (1985)
<i>L. mexicana</i>	<i>Lu. anthophora</i>	Golden hamsters	Endris and Young (in Young and Perkins, 1984)
<i>L. mexicana</i>	<i>Lu. diabolica</i>	Golden hamsters	Lawyer and Young (in press)
<i>L. mexicana</i>	<i>Lu. shannoni</i>	Golden hamsters	Lawyer and Young (in press)
<i>L. m. mexicana</i>	<i>Psychodopygus</i> sp. †	Man	Strangways-Dixon and Lainson (1962, 1966)
<i>L. m. mexicana</i>	<i>Lu. longipalpis</i>	Golden hamsters	Coelho and Falcão (1962)
<i>L. m. mexicana</i>	<i>Lu. renei</i>	Golden hamsters	Coelho and Falcão (1962)
<i>L. m. mexicana</i>	<i>Lu. cruciata</i>	Man	Williams (1966)
<i>L. m. amazonensis</i>	<i>Lu. longipalpis</i>	Golden hamsters	Killick-Kendrick <i>et al.</i> (1977c)
<i>L. m. amazonensis</i>	<i>Lu. flaviscutellata</i>	Golden hamsters	Ward <i>et al.</i> (1977)
<i>L. m. amazonensis</i>	<i>Lu. furcata</i>	Golden hamsters	Ryan <i>et al.</i> (1986)
<i>L. garnhami</i>	<i>Lu. townsendi</i>	Golden hamsters	Scorza and Anez (1984)

*Immunosuppressed *Rattus rattus*.

†See Ready and Lainson (1982) and Williams (1983).

infecting feed gave rise to leishmaniasis in a dog (Rioux *et al.*, 1979); the fly was dissected after the infective bite and, in addition to parasites in the midgut and pharynx, a few small active promastigotes were seen in the proboscis. Others must have been deposited in the skin of the dog.

There are other observations illustrating the high efficiency of transmission by bite. Adler and Ber (1941) transmitted "*L. tropica*" (probably *L. major*) to four volunteers by the bites of experimentally infected *P. papatasi*. Of a total of 28 lesions, 20 were produced by bites or probes of only 9 sandflies and 27 by no more than 11 sandflies. In transmissions of *L. m. mexicana* by the bite of *Lu. cruciata*, Williams (1966) described how a single fly probed the skin of a volunteer 5 times giving rise to 4 lesions. In Kenya, a volunteer was bitten 11 times by one *P. duboscqi* infected with *L. major*; a lesion arose at the site of each bite (Beach *et al.*, 1984). *L. major* was transmitted by one sandfly (*P. duboscqi*) to five mice by consecutive probes (Beach *et al.*, 1985). Finally, Killick-Kendrick *et al.* (1985) recorded 11 lesions on the arm of a volunteer where a sandfly (*P. papatasi*) infected with *L. major* had probed 26 times. These and other similar observations show that, when conditions are right, transmission by bite is remarkably efficient.

Multiple probing is common with infected sandflies and it has been suggested that this is caused by the presence of parasites in the head of the fly where they are presumed to interfere with the function of sensilla (Killick-Kendrick *et al.*, 1977a; Beach *et al.*, 1985). Killick-Kendrick and Molyneux (1981) published stereoscan photographs of two sorts of sensilla; one kind, like a thorn, was on the ventral plate of the labrum and the other, with a wide base, was on the roof of the cibarium (Lewis, 1984). Engorgement is presumed to be controlled by such chemo or mechanoreceptors. If the presence of promastigotes interfered with their function, a female attempting to feed might not "know" that she was successfully engorging. She would then withdraw her proboscis and try again. A malfunction of sensory responses could well interfere with the control of the pumping of blood, possibly resulting in the deposition of promastigotes from parts of the alimentary tract posterior to the mouthparts into the skin of the vertebrate. The parasites most likely to be regurgitated are unattached promastigotes in the cibarium or pharynx (Shortt *et al.*, 1926; Lainson *et al.*, 1977). Killick-Kendrick *et al.* (1977a) pointed out the evolutionary advantage to *Leishmania* if the presence of the parasites changed the feeding behaviour so that a fly probed many times, thus enhancing the chances of the parasite being transmitted to a vertebrate host.

Summary and Conclusions

Infective forms of *Leishmania* first arise in the midgut of an infected fly three or more days after an infecting

feed. They are small promastigotes with a long free flagellum. They neither divide nor attach to the wall of the alimentary tract and are highly motile. These stages are the only parasites seen in the proboscis of the sandfly and are undoubtedly deposited in the skin when the sandfly bites. The ability of morphologically identical forms separated from cultures to survive in macrophages and to resist complement supports the assumption that metacyclic promastigotes in the fly are adapted for life in the vertebrate. Transmission by bite is highly efficient and the infectivity of these small forms in the fly appears to be high.

The feeding behaviour of infected flies is disturbed by the presence of parasites. The pumping action during feeding may then not function properly and infective forms posterior to the mouthparts could perhaps be deposited in the skin of the vertebrate. It is therefore concluded that, although the weight of evidence supports the notion that transmission is normally by the deposition in the skin of highly motile infective forms from the proboscis, a vertebrate may also acquire an infection from a fly with no parasites in the mouthparts.

In a mathematical model of the transmission of leishmaniasis, it can be reasonably assumed that a fly capable of transmission will deposit an infective dose of promastigotes each time it feeds. Furthermore, if the time to the appearance of parasites in the proboscis is known, it can also be assumed that an infected fly of that age will transmit. It cannot yet be said unequivocally that the same fly would be unable to transmit on an earlier day.

In spite of the new information about the infective forms of *Leishmania*, an understanding of the mechanism of transmission has progressed little since 1926 when Shortt wrote: "the direct presumption would be that the elongate free forms, which are the most anteriorly situated, are the most likely to be transferred to the wound and so lead to infection of the vertebrate host. It is possible, however, that a more massive dose might be inoculated during the active manipulation of the mouth parts of the fly in feeding by the detachment of a portion of the main flagellate growth at the more posterior part of the buccal cavity. This question . . . must at present be left unsettled" (Shortt *et al.*, 1926).

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