STUDIES ON LEISHMANIAE OF LIZARDS

BY

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A B S T R A C T

Lizards Mabuya striata were used for experimental study of the developmental stages and infectivity of three Leishmania isolates. The three isolates were:

LIZ/KEN/ICIPE 140	(unidentified lizard isolate)
MHOM/SU/5ASKH L. major	(identified human isolate)
LIZ/RC/244 L. adleri	(identified lizard isolate)

The two developmental stages of leishmania, promastigotes and amastigotes, were detected in the lizard tissues at different time intervals post-inoculation. Promastigotes were seen in the lizard tissues up to 7 hours post-inoculation. At 14 hours post-inoculation only amastigotes could be seen in the various tissues.

Amastigotes were detected in impression smears of the intraperitoneal fluid, liver and spleen of the lizards. They were also seen in histological sections of the liver, spleen and gut. The parasites were seen mainly in the macrophage cells of these tissues.

Infectivity of the above mentioned isolates to the lizards was measured by counts of the number of amastigotes seen per macrophage cell and the percentage of macrophage cells infected in both liver and spleen. The isolate L 140 was found to be more infective to the lizards

than L. major and L. adleri with a maximum number of 12.8 ± 6.0 amastigotes per macrophage cell and maximum percentage of 60 macrophage cells infected.

The isolates L 140 and L. major showed a similar pattern of infectivity. In both cases infectivity decreased with time suggesting a trans ient infection to the lizards. The isolate L. adleri, which is a typical lizard Leishmania, showed a different pattern where infectivity increased with time indicating a natural infection to the lizards.

Inoculation of L 140 and L. adleri to mice showed that L. adleri had a transient infectivity to the mice where parasites could not be detected after the 5th week post-inoculation. L 140 isolate showed more persisting infectivity to the mice and amastigotes could be seen up to the 12th week post-inoculation.

Experimental infection of four species of sandflies, namely Sergentomyia ingrami, S. schwetzi, S. clydei and S. bedfordi, with Leishmania was carried out by feeding them on lizards inoculated with the isolates L 140, L. major and L. adleri. Two of the 120 flies fed on lizards inoculated with L. adleri, were found to harbour promastigotes. These were S. schwetzi and S. ingrami. None of the flies fed on lizards inoculated with L. major or L 140 was found to be infected.

The ultrastructure of the isolate L 140 showed that its characteristics are more comparable to those of mammalian leishmania.

The comparative study of the three isolates L 140, L. major and L. adleri showed that L 140, isolated from a wild lizard, is more related to L. major than to L. adleri and could be of a mammalian origin. Hence if lizards can harbour mammalian leishmania they can then act as reservoir hosts or incidental hosts and play an important role in the epidemiology of human leishmaniasis.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. The Genus Leishmania:

Leishmania is a genus of the class Zoomastigopherea and order Kinetoplastida of the flagellate family Trypanosomatidae which has both vertebrate and invertebrate hosts. A feature of Leishmania parasites is that they have two developmental stages which were indicated by Hoare and Wallace (1966) in relation to the position of the flagellum as:

i) "amastigote", has no external flagellum

ii) "promastigote", the flagellum arises near a kinetoplast infront of the nucleus and emerges at the anterior end.

A recent classification of the genus Leishmania was done by Laison and Shaw (1979) based on the mode of development in the gut of the invertebrate host which is the sandfly. The leishmaniae were divided into three sections as follows:

a) Hypopylaria, which are primitive species of Leishmania. The infection in the sandfly host moves to a posterior position in the intestine and becomes established in the hindgut. Reservoir hosts are restricted to lizards and so far only old world species are recorded. Parasites in the vertebrate may be in promastigote and/or the amastigote

form, in the blood or viscera. Transmission presumably takes place when lizards eat infected sandflies. Representatives of this section are Leishmania agamae and L.ceramodactyli.

b) Peripylaria, are leishmaniae that maintain an obligate hindgut development in their sandfly hosts but which in addition have now developed an anterior migration to the foregut. This section included some lizard parasites of the old world but is mainly dominated by members of the Leishmania braziliensis complex which are limited to mammalian hosts of the western hemisphere. There is very little information on the form of the parasite in the lizard hosts, but possibly both promastigote and amastigote stages occur. In mammals the parasites are found only in the amastigote form, in macrophages of the skin, viscera or blood. Transmission of the leishmaniasis of mammals is inoculative, following the bite of the infected sandfly. That of the lizard parasites may also be by bite, but definite proof is still lacking. All known species of this section which infect mammals are accidental and pathogenic parasites in man, causing a variety of cutaneous and/or

mucocutaneous leishmaniasis. The peripylaria contain Leishmania adleri and L. tarentolae in lizards and Leishmania braziliensis complex in man which include L. braziliensis guyaensis and L.braziliensis panamensis

c) Suprapylaria which includes leishmaniae that are restricted to the midgut and foregut. Reservoir hosts are domestic and wild animals of both the Old World and New World, Amastigotes are found in macrophages of the skin, viscera or blood, and transmission is by bite of the various sandfly vectors. Included in this section are the two groups of New and Old World parasites of Leishmania mexicana complex and Leishmania tropica complex which when infecting man, these two groups produce a variety of relatively simple and easily curable cutaneous lesions, but they are also capable of causing incurable "diffuse cutaneous leishmaniasis" (DCL). Also included in this section are Old and New World parasites of Leishmania donovani complex which are known for their viscerotropism and resulting high pathogenecity in man. Leishmania mexicana complex in mammals of the New World include L. mexicana mexicana, L.mexicana amazonensis, L. mexicana pifanoi, L. mexicana aristedesi, L. mexicana

subspecies and L. enrietti. Also in the New World mammals Leishmania hertigi complex include L. hertigi hertigi and L. hertigi deani. Leishmania donovani in mammals of the Old World include L.donovani and L. infantum while in mammals of the New World it includes L. shagazi. Finally Leishmania tropica complex in mammals of the Old World included L. tropica, L. major and L.aethiopica.

2. Sandflies

2.1 The vector:

About 600 species and subspecies of the subfamily Phlebotominae are known from various parts of the World (WHO, 1984). In Kenya 38 species were reported between 1956 and 1984 (Kaddu, in press). Most species are assigned, according to Theodor's (1970) system of classification, to three genera Phlebotomus and Sergentomyia in the Old World and Lutzomyia in the New World.

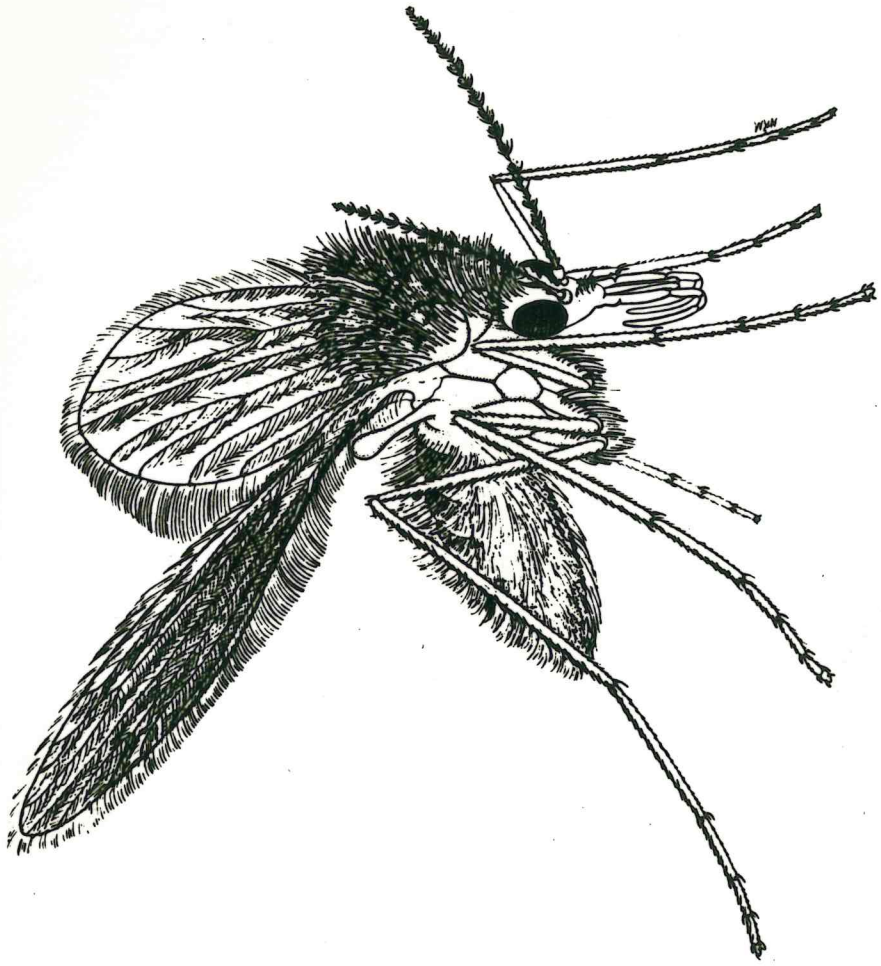
Most of the study on the sandflies in relation to human leishmaniasis is designed to find out which species are vectors, from animal to animal, from animal to man or from man to man.

This is often very difficult except in parts of the Old World with only one commonly occurring man-biter. So far 70 species of sandflies have been incriminated as vectors. It is necessary to study both extrinsic and intrinsic factors and when a sandfly is recognized as a vector it is necessary to consider quantitative and qualitative relations between Leishmania and the insect host. The relation of some sandfly species with Leishmania varies according to locality. Some sandflies may transmit leishmaniasis to man and yet have negligible significance, not being habitual vectors (Riox et al., 1968). Furthermore the importance of some vectors alters with environmental changes. There are biological factors acting as barriers between some sandfly and Leishmania. One of the factors that influence sandflies distribution is the altitude, (Lewis, 1971). Another factor is the annual fluctuations in precipitation and temperature which affect populations of sandflies and some, if not all, vertebrate reservoir hosts, (Lewis, 1971). The type of soil is also among the factors that affect sandflies in particular areas. For instance, in the USSR, the distribution of gerbil barrows, where sandflies are found, is determined by the nature of soil and the height of the water table. These barrows have an important influence on the distribution of various species of sandfly (Saf'janova and V'jukov, 1967). Hence only careful field studies

can determine the vector(s) of a given Leishmania. It remains to be seen to what extent some of these barriers depend on the behaviour of the mammalian and/or insect host. Nevertheless the specificity of Leishmania infection is usually more restricted in the invertebrate host than the vertebrate host, the ecological requirements of certain sandflies can determine the leishmania they can transmit (Lewis, 1971). It is known that all or most of the Old World vectors of human leishmaniasis belong to the genus Phlebotomus. Sandfly vectors of Leishamania tropica belong,mostly to the subgenera Synphlebotomus, Lavvoussius, Adlerius and Euphlebotomus (Lewis, 1971). In the New World most vectors of Leishmania mexicana amazonensis, L. mexicana mexicana, L. braziliensis panamensis and L. braziliensis guyanensis belong to the subgenera Nyssomiyia, while the vectors of L. braziliensis braziliensis belong to the subgenera Psychodopygus. (WHO, 1984).

2.2 Biology of Sandflies:

The sandfly may be characterized by its small size, long, thin legs and antennae, and its thick vesture of hairs and scales. At rest the wings are held back, upward and outward over the body. The head is set beneath the anterior end of the relatively large, convex mesontum giving the insect a humpbacked appearance.



The proboscis is long and relatively thick extending straight down and direct at the substratum. (Fig.1).

The male and female sandflies differ externally in their mouthparts and genitalia. The male mouthparts lack the mandibles and the remaining stylets are weaker than those of the female. Little is known of the mating behaviour of sandflies in nature. Males which do not take blood-meals, apparently may be attracted to hosts on which the females are feeding, (WHO, 1984).

The life cycle of a sandfly goes from the egg through four larval stages, or instars, before pupating and then hatching to an adult. A female sandfly lays 50-100 eggs at each oviposition. Adults emerge from pupae 7-10 days later. Depending upon temperature and larval diet, the average time from egg-laying to the emergence of adults ranges from 35-60 days. There have been few studies on the diapause of sandflies. Paleanetic species of sandflies apparently overwinter as fourth instar larvae. In addition there is evidence that at least some tropical species undergo diapause as eggs during periods of dryness or perhaps, of excessive rain. The duration of the genotrophic cycle in a genotrophically concordant sandfly, corresponds to the time from one blood-meal to the next. Information on the actual length of the gonotrophic cycle in nature is not available yet (WHO, 1984).

Sandflies generally breed in soil that is rich in humus and must be damp. Techniques employed in searching for larval breeding sites include the use of emergence cages and the examination of soil samples to find larvae (WHO, 1984). Resting sites of adults are known for few species of sylvatic sandflies, they include tree-holes and trunks. Peridomestic species rest on walls and at hot times of the day they retreat into cracks and crevices. In the tropics, many species of sandfly rest in the air shafts of the termite hills (Minter, & Wijers 1963). Such microhabitats can lead to much localization of sandflies and consequently of microfoci of leishmaniasis.

2.3 Leishmania in the Sandfly:

Phlebotomine sandflies are known to transmit leishmaniasis. Transmission generally occurs by bite of a female fly (WHO, 1984). The development of the parasite in the fly begins with the fly taking a blood-meal from infected vertebrates. Amastigotes taken up with the blood-meal appear to divide one or more times and then become transformed into highly active elongated promastigotes. Intensive division of these forms follows either in the abdominal midgut or in the hindgut. The parasites then migrate forward into the head of the fly and

colonize the pharynx. From this site, small highly mobile forms move into the mouthparts and are deposited in the skin of the next animal upon which the fly feeds. The duration of the cycle in the fly varies from 4 to 18 days according to the species of Leishmania, prolonged by low temperature or shortened by high temperature.

3. Leishmania in Mammals:

In man and other mammals leishmanias are intracellular parasites which attack the mononuclear phagocytes, macrophage cells. These cells are the first line of the host cellular defence against invading microorganisms. Leishmania parasites persist inside these cells, multiply and destroy them to invade others, (Hommel, 1978).

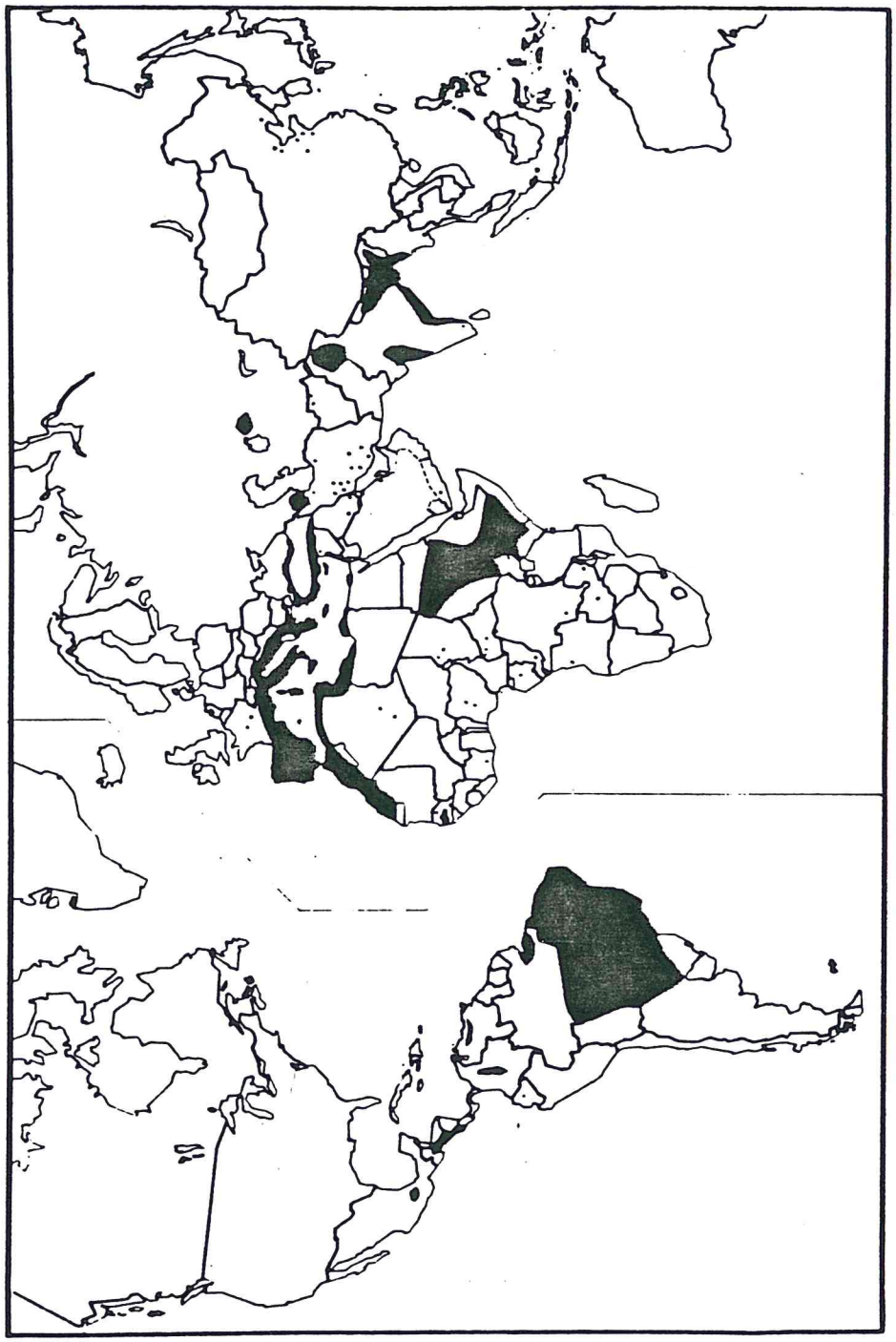
Some animals act as reservoir hosts for Leishmania parasites and some as incidential hosts. A reservoir may be defined as the ecological system in which the parasite population is maintained indefinitely. Usually there is one reservoir host for a given Leishmania species in a particular area. Other mammals in the same biotope may become infected, these incidental hosts do not normally play any role in the long-term survival of

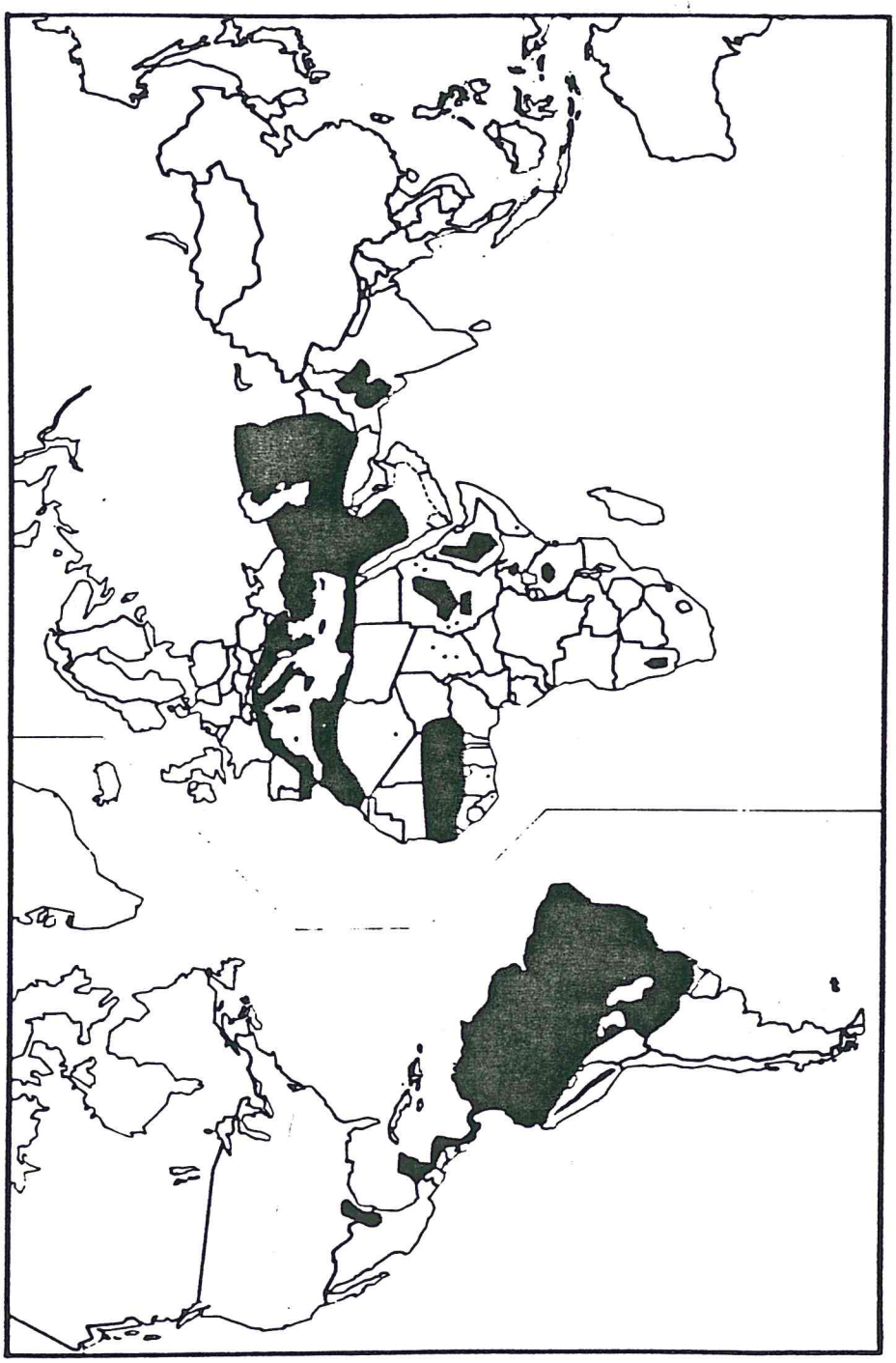
the parasites population. An incidental host may occasionally bring the parasite from its enzootic bitope into closer contact with man and thus become a source of human infection (WHO, 1984).

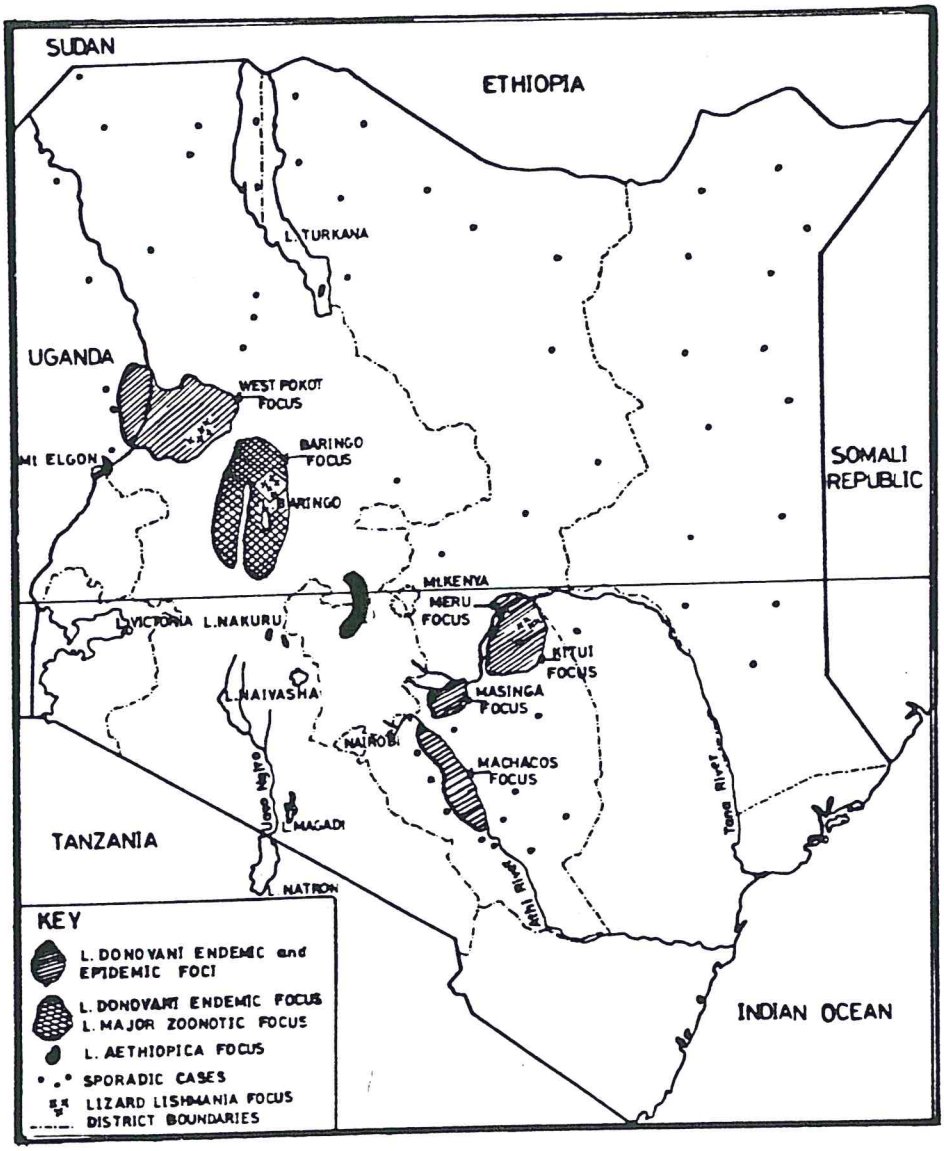
4. Geographical Distribution of Human Leishmaniasis:

The area of distribution of leishmaniasis is determined by the distribution of its nosogeographical forms, these being determined by the composition of the parasitic system (parasite-vector-host) and by environmental conditions, (Lysenko, 1971).

Geographical distribution and prevalence of leishmaniasis is subject to alter due to environmental and other changes which may eliminate the disease in some highly endemic foci or, conversely, may increase its prevalence in others. Furthermore, in many countries, leishmaniasis is not a notifiable disease or, if it is, many cases are not reported (WHO, 1984). The known worldwide distribution of the leishmaniae that infect man is given in Figures (2) and (3). Figure (4) shows the distribution of leshmaniasis in Kenya.



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BARINGO FOCUS
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MERU FOCUS
L. VICTORIA
L. NAKURU
KITUI FOCUS
L. NAIVASHA
MASINGA FOCUS
NAIROBI
MACHACOS FOCUS
Uaso Nyiro
L. MAGADI
TANZANIA
Tana River
L. NATRON
Athi River
INDIAN OCEAN
KEY
L. DONOVANI ENDEMIC and EPIDEMIC FOCI
L. DONOVANI ENDEMIC FOCUS L. MAJOR ZOONOTIC FOCUS
L. AETHIOPICA FOCUS
SPORADIC CASES
LIZARD LISHMANIA FOCUS
DISTRICT BOUNDARIES

5. Lizard Leishmania

5.1 Evolutionary Trend

Hoare (1948) postulated that the lizard leishmaniae provide convincing evidence of a transitory stage of evolution in which an invertebrate parasite has become adapted to life in a vertebrate host. Both this worker and Baker (1965) felt it reasonable to assume that although promastigote forms have been reported from the intestine of reptilian vertebrates (Bayon, 1915, Leger, 1918; Wenyon, 1921; Franchini, 1921) they do occur more rarely in vertebrate than in invertebrate hosts and that if they are genuine intestinal promastigotes they have been acquired secondarily, first, for example, via the ingestion of infected arthropods by ancestral saurians and subsequently by their adaptation to the intestinal environment within the vertebrate host. It seems reasonable to suppose that subsequent invasion of the blood and other tissues took place, and in most Leishmania species of lizards the parasite has become restricted to the blood and viscera.

An early view of a possible evolutionary route towards the development of mammalian leishmaniae was given by Minchin (1908),

Mesnil (1918), Leger (1918) and later by Lavier (1943), Hoare (1948) and Cameron (1956). Their veiws were rejected by the majority of protozoologists who believed that the haemoflagellates arose from intestinal flagellates of invertebrates, without an intervening phase in the gut of vertebrate. Based on the known life-cycle of various species, Hoare (1948) had advanced a working hypothesis by which evolutionary events could be traced. Leishmania chamaeleonis, the most primitive of the lizard leishmanias, is restricted to the intestine and cloaca of chameleons in the promastigote form. The subsequent stage is represented by L.henrici of iguanas in which promastigotes are found in the cloaca but have also invaded the blood in small numbers whereas L.hemidactyli promastigotes appear to inhabit the blood in large numbers thus providing a route by which cyclical development in a phlebotamine can occur if it bites an infected lizards. Additional evidence to support this hypothesis has been shown by the ability of promastigotes of L. adleri, L.gymnodactyli and L.tarentolae to form amastigotes when inoculated into worm-blooded animals. Presumably, a similar event took place in nature and the mammalian genus Leishmania arose, i.e. the parasites became phagocytosed by the vertebrate mononuclear phagocyte and intracellular multiplication occurred, a phlebotomine vector ingested these forms from the mammalian skin, transformation to

the promastigote stage took place in the insect gut and cyclical development was completed by their anterior migration to the mouthparts of the vector. L.adleri also provides an example of a saurian Leishmania which has undergone this later evolutionary step in the vector Sergentomyia clydei (Heish, 1958).

5.2 Species of Lizard Leishmaniae

The natural occurrence of more than ten species of lizard leishmaniae have been described and were entirely confined to the Old World. Four of these species were described in Africa, Table (1).

One of the first reports of reptilian promastigotes was that of Sergent et al (1914), who obtained cultures of typical Leishmania promastigotes from 16% of NNN media (Novy, McNeal and Nicolle's media) inoculated with either blood or organs of the gecko, Tarentola mouritanica in Algeria.

Work to investigate the natural infection of Leishmania promastigotes in geckoes was carried out in Turkmenian SSR by Sahsuvarli (1934), Zmeyev (1936), Khodukin and Sofiev (1940), Latyshev & Pozyvaj (1937), Latyshev (1949), Popov (1941), Andrusko & Markov (1955) and later Belova (1971). Table (2) summarize their findings.

Table 1: Leishmania species in lizards of Africa

(Adapted from Garnham, 1971)

Species of Leishmania	Vertebrate host	Suspected vectors	Region	Author
L. tarentolae	*Tarentola mauritanica*	*S. minuta* *S. minuta minuta*	North Africa	Wenyon, 1921
L. chamaeleonis	*Chamaeleon punnilus*	unknown	Egypt & Uganda	Wenyon, 1921
L. adleri	*Latastia long icaudata*	*S. clydei*	Kenya	Heisch, 1958
L. hoogstraali	*Hemidactylus turcicus*	*S. clydei*	Sudan	McMillan, 1965

Table 2: Species of lizard examined for promastigote infection in the Turkmenian SSR (Adapted from Belova 1971)

Family	Species	No. of specimens examined	No. of specimens with promastigotes
Agamidae	Phrynocephalus interscapularis	926	17
	Agama sanguinolenta	683	41
	Phrynocephalus mystaceus	99	1
	Agama caucasica	39	0
	Phrynocephalus raddei raddei	20	2
	Phrynocephalus helioscopus	4	0
Lacertidae	Eremias velox	354	11
	E. intermedia	277	3
	E. guttulata guttulata	169	3
	E. lineolata	113	5
	E. grammica	88	2
	E. scripta scripta	4	0
Gekkonidae	Gymnodactylus caspius	907	139
	Teratascincus scincus	101	1
	Gymnodactylus russowi	3	0
	Crossobamon eversamani	4	0
Anguidae	Opisaurus apodus	13	0
	Mabuya aurata	7	0
Varanidae	Varanus griseus	4	0
Scinciidae	Eumeces taeniolatus	1	0
	E. schneideri princeps	2	0

5.3 Life cycle and Transmission:

A review on the life cycle of lizard Leishmania was given by Wilson & Southgate (1979). Their review reflected the fact that lizard Leishmaniae have been the subject of very few morphological studies, either within the vector or the vertebrate host. Lizard Leishmaniae are known to live predominantly as promastigotes, which are similar morphologically to those of the mammalian species within the cloaca, intestine or blood of the vertebrate host. In most species the parasite is found exclusively in the blood where it may also assume an amastigote form. Avakyan as cited by Garnham (1971), had noted a striking ultrastructural difference between promastigotes of the reptilian and mammalian species. In the former the subpellicular tubules lie 58-67 nm part whereas in the latter they are only 35-42nm apart. The difference in the spacing of these organelles between reptilian and mammalian species has been confirmed by Lewis (1975); who used promastigotes of L.agamae, L.hoogstraali, L.adleri and L.mexicana and found a mean seperation of 45.6nm and 26.5nm respectively.

The amastigote form has never been described in detail and has been seen only on rare occasions in reptiles. The presence of amastigote form of saurian species of Leishmania was described for

the first time by Shortt & Swaminath (1928). Six amastigotes measuring 2.5 um in diameter were found within a peripheral blood leucocyte and others were extracellular in the blood. Previously, the parasite had been demonstrated in the promastigote form only by Mackie et al. (1923) and named Herpetomonas hemidactyli. These typical Leishmania-like amastigotes provided the necessary evidence for placing the gecko parasite in the genus Leishmania, a taxonomic position into which it had already been placed by Wenyon (1926) on the ground that it underwent part of its life cycle in a vertebrate host.

Leishmania agamae was isolated by David (1929a) from cultures of the cardiac blood of Agama stellio in Middle East. Examination of stained blood films resulted in finding a single amastigote within a monocyte. Later Edeson & Himo (1973) examined Giemsa-stained blood films from A.stellio caught in the Bekaa and Roumich districts of Lebanon, and demostrated the presence of amastigotes in 8% of the lizards.

The other occasion where amastigote forms have been demostrated in the blood of a naturally infected lizard was during an epidemiological study by Rioux et al. (1969). They found amastigote forms of L.tarentolae in peripheral blood of geckoes

caught in the district of Banyuls-sur-mer Southern France. From 3 to 10 parasites were seen within individual monocytes and others were extracellular.

Following a review on the problem of transmission by Adler & Theodor (1957), and other workers it is generally accepted that infection of the phlebotamine host follows the ingestion of a blood meal, that multiplication of lizard Leishmania occurs within this host and that the promastigotes develop typically (Hertig et al, 1969), but not always (Adler, 1964), at the posterior station in the hindgut of the sandfly. Furthermore, such posterior development suggests that transmission to the saurian host is by ingestion of an infected sandfly.

In order to study transmission of lizard Leishmania, which has been poorly studied, it is important to know the behaviour of the parasites within the sandfly and the ability of the latter to feed on saurian hosts. Lainson et al. (1977) were unable to establish L.hoogstraali in Lutzomyia longipalpis, and suggested that either the parasites had lost their infectivity to the phlebotomine host or that the sandfly environment was unfavourable. David (1929b) & Adler & Theodor (1929b) found that L.agamae and L.ceramodactyli adopted a posterior position in the

sandfly Phlebotomus papatasi, Adler & Theodor (1935a) provided a conflicting evidence that the development of an experimental infection of L.tarentolae occurred at the anterior station in S.minuta. Parrot (1935), however, recorded the development of the same parasite at the posterior station of S.minuta minuta. However, Heisch (1958) described a parasite, L.adleri, from the blood of Latastia longicaudata from Kenya and presented evidence that natural vector of this parasite was S.clydei in which the promastigotes developed at the anterior station. It thus became clear that the position of the promastigotes in the invertebrate host does not provide a certain clue as to the vertebrate host. On the other hand evidence of the anterior development of a saurian Leishmania does indicate the possibility of transmission to the lizard by the bite of an infected fly.

Simic (1930) was first to report the ability of sandflies to feed on reptiles in Yugoslavia. Minter & Wijers (1963) stated that both S.cydei and S.schwetzi fed mainly on the blood of reptiles. These observations were confirmed by B. McMillan during studies in the Malakal area of the Sudan (Hoogstraal & Hyneman, 1969). Evidence was presented by Nadim et al (1968) and Sayed-Rashdi et al. (1971) that S.sintoni is probably the vector of a lizard parasite in the Iranian Turkmen Sahara. An

experimental evidence was also provided by Belova (1971) showing that three species of sandfly P.papatasi, P. causcasicus and S. sentoni were all able to feed on lizards.

Ngoka & Mutinga (1978) in a survey of animal reservoir for kala-azar in Kenya have isolated Leishmania promastigotes from 59 out of the 219 different lizards caught. In their investigations of the suspected vectors of lizard leishmaniasis, Mutinga & Ngoka (1981) carried blood meal analysis for various species of sandflies collected in different parts of Kenya. Their results showed that Sergontomyia bedfordi, S. rodhiani, S.schweitzi, S. antennatus, S.affinis and S. africanus, fed mainly on reptiles and occasionally bite man.

6. Relationship of Lizard and Mammalian Leishmaniasis:

Studies on the relationship between lizard and mammalian leishmaniasis have been carried out by investigating the susceptibility of lizards to promastigotes of Leishmania species and also by investigating the susceptibility of mammals to lizards Leishmaniae. Kandelaki (1939) reported successful infection of 7 Agama caucasica in Georgia USSR with strains isolated from cases of visceral leishmaniasis. Khodukin & Safiev (1940) obtained

negative results in Bymnodactylus caspius inoculated with strains of Leishmania isolated from lizards and a dog. Experimental infections by Belova (1971) showed a considerable proof of a genetic affinity between reptiles and mammalian species of Leishmania. Further studies were also carried by Mohiudin (1959) who studied the behaviour of L.adleri in four species of lizards and found that three species, Mabuya striata, A.mutabilis and Lacerta viridis could be infected, while cultures of isolated mammalian strains of L.donovani and L.tropica failed to infect these lizards.

Belova (1971) had, by her findings, supported the work of Medina (1966, 1968) in Venezuela, who was able to infect seven species of Saurians with each of the four species of mammalian Leishmania i.e L.braziliensis braziliensis, L.m.pifanoi, L.m.mexicana and L.tropica. The lizards were inoculated intraperitoneally and became infected 4-63 days post-inoculation. In some hosts there was high percentage of hepatosplenic involvement and occasionally myocardial invasion was detected, the majority of lizards developed visceral amastigote infections and the subsequent intraperitoneal inoculation of this material into hamsters produced an almost 100% infection of the viscera from 10-39 days post-inoculation. However, in those lizards in which amastigote

infection of the liver could not be demonstrated microscopically, the inoculation of the saurian liver tissues into hamsters produced positive visceral infections in 65% of the animals and demonstrated the presence of cryptic infections in the reptilian hosts. Hemidactylus species could not be infected with any of the mammalian species of Leishamania, and in Gonatocles vittaties and Mabuya mabuya hepatosplenic infections could be obtained only by L.m.pifanoi and L.m. mexicana.

Phagocytosis of L.adleri promastigoes by lizard leucocytes was observed by Dollahon & Janovy (1973) and was found to be similar to that of mammalian leishmanias with mammalian macrophage cultures done previously under similar conditions by Miller & Twohy (1967) and Akiyama & Haight (1971).

In studying susceptibility of mammals to lizard leishmanias, Young & Hertig (1927) produced visceral followed by cutaneous lesions in the Chinese hamster (Cricetus griseus) as a result of inoculating, intraperitoneally, promastigotes of the gecko parasite, L.tarentolae. Manson-Bahr & Heisch (1961) obtained transient infections in man by inoculating five-day promastigote cultures of L.adleri into four volunteers. Belova (1971) carried out experiments to determine whether a variety of mammals (white

mice, golden hamsters and red-tailed gerbils), were susceptible to seven promastigotes reptilian strains in the Turkmanian USSR. Subcutaneous and intraperitoneal inoculations were made and the experimental animals were observed from two to three and a half months. The results were negative in all cases. However, Adler (1962) using a culture of L.adleri short ly after isolation, inoculated adult and baby hamsters and baby mice and obtained transient infections. It was also demonstrated by Belova (1971) that transformation of lizard promastigotes to amastigotes did occur in 2-4 hours following intradermal inculation of Leishmania promastigotes into mammals. The amastigotes then disappeared within 4 days post-inoculations and also any inflamatory reaction. These results by both Adler (1962) and Belova (1971) provided further evidence of a certain affinity between reptilian promastoges and mammalian leishmania .

The in vitro interactions of hamster peritoneal macrophages and promastigotes of L.adleri were examined by Scheiber (1972). He showed that the parasites were readily engulfed by the mammalian macrophoges and transformed to amastigotes. This was the first time that the in vitro intracellular transformation of lizard Leishmania promastigotes had been demonstrated in mammalian cells and is in agreement with the in vivo observation of Belova (1971); and Manson-Bahr & Heisch (1961).

It has been difficult, until recently, to identify Leishmania species, particularly those which originate from areas where two or more species are present. Serology was one of the methods used recently. Studies on the serological reactions of lizard leishmaniae and their antigenic relationships with human species of Leishmania are few. Adler (1962, 1964) found that L.adleri showed common antigens with L. donovani, L.infantum, L. braziliensis and L. tropica. It was also shown by McMillan (1965) that L. hoogstraali shared agglutinating antigens with Sudanese, Kenyan and Indian strains of L.donovani, L.infantum, L.branziliensis, L.mexicana and L.adleri, but was more closely related to the latter than any other species. These antigenic relationships provide further evidence to support the hypothesis that L.adleri and probably L. hoogstraali and L. tarentolae represent transitional stages in the evolution of Leishmania from a purely reptilian to mammalian parasites.

Cross immunity between the Leishmaniae of lizards and man was also used as a method to identify different Leishamania species. Cross reactivity in man between lizard and human species of Leishmania was first demonstrated by Latyshev & Kryukova (1953). They injected some volunteers with the gecko flagellate L.gymnodactyli. Following the discovery of L.adleri in Kenya by

Heisch (1958), Southgate & Oriedo (1967) found that many people living near a newly established focus of Kala-azar in the Voo locations of Kitui district in Kenya had positive leishmanin skin tests although they had never had Kala-azar nor vaccination against it. Southgate & Manson-Bahr (1967) showed that the same people were immune to the intradermal and subcutaneous inoculation of cultures of rodent strains of L.donovani. It was suggested that their immunity might have resulted from natural skin infections with L.adleri caused by the bites of sandflies which normally feed on lizards. A similar situation was reported in Sudan by McMillan (1965) who found that a new species of gecko Leishmania, L. hoogstraali had common antigens with L.adleri. This report could offer an explantion for Van Peenen & Deithein (1963) who recorded an unexplained positive leishmanin reactions in the same province of the Sudan. It is possible that these reactions have been produced by infection of humans with L.hoogstraali. Mutinga & Ngoka (1983) suggested that probably when man is inoculated with lizard Leishmania by sandflies, they may confer on him some immunity to human leishmaniasis.

Finally the relationships between lizard Leishmaniae and human disease epidemiology was also used by some workers as a tool

for species identification. Such studies were carried out by Lemma et al. (1969) in Ethiopia. Similar studies were also carried out by Imperato & Diakite (1969) and Imperato et al. (1970) in Mali. All those workers have reached the same conclusion that exposure of humansto non-human Leishmania species is responsible for the irregularity in the positive rates of infection in the endemic areas. Another report on epidemiology of human Leishmania in the Soviet Union by Belova (1971) concluded that lizard leishmania play little or no role in influencing the distribution of human infections.

7. Objectives of the Present Work:

The fact that Leishmania-infected lizards live in close association with sanflies and rodents and that certain sandfly species are both saurian and mammalian, including man, biters had drawn the attention to the possible relationship between reptilian and mammalian leishmanias. Hence the objectives of the present work are the following:-

1) Detection of the different developmental stages of Leishmania in the lizards.

2) Localisation of the parasites in the various tissues of the lizard.

3) Host-parasite relationship in lizard leishmaniasis.

4) Determination of infection rate of lizard-feeding species of sandflies.

5) Localization of the parasites in the sandfly gut.

6) Infectivity of lizard Leishmania to mammals.

7) Comparison of the three vertebrate Leishmania isolates.

One of the objectives was to study, in details, the presence of L 140 parasites in histological sections of the lizard tissues and their histopathological effect on the lizards and also the ultrastructure of the various forms of the isolate.

C H A P T E R II

GENERAL MATERIALS AND METHODS

1. Animals

1.1. Sandflies:

Rearing and breeding of sandflies is being carried out at the ICIPE Insectary where conditions are controlled to match, as far as possible, the natural habitat. The methods of colonization used was based on the works of Adler & Theodor (1935b), Hertig & Johnson (1961), Harwood (1965), Gemetchu (1971), Killick-Kendrick et al. (1976); Beach et al. (1983). The method can be summarized as follows :-

a) Eggs from each fly (if unidentified) or of each species (if identified) were transferred to a separate dish, counted, labelled and kept moist in an incubator with controlled humidity and temperature, 80% and 26°C respectively.

b) First, 2nd, 3rd and 4th instar larvae and the pupae were counted daily and they were fed on a mixture of liver powder, rabbit commercial food and rabbit faeces.

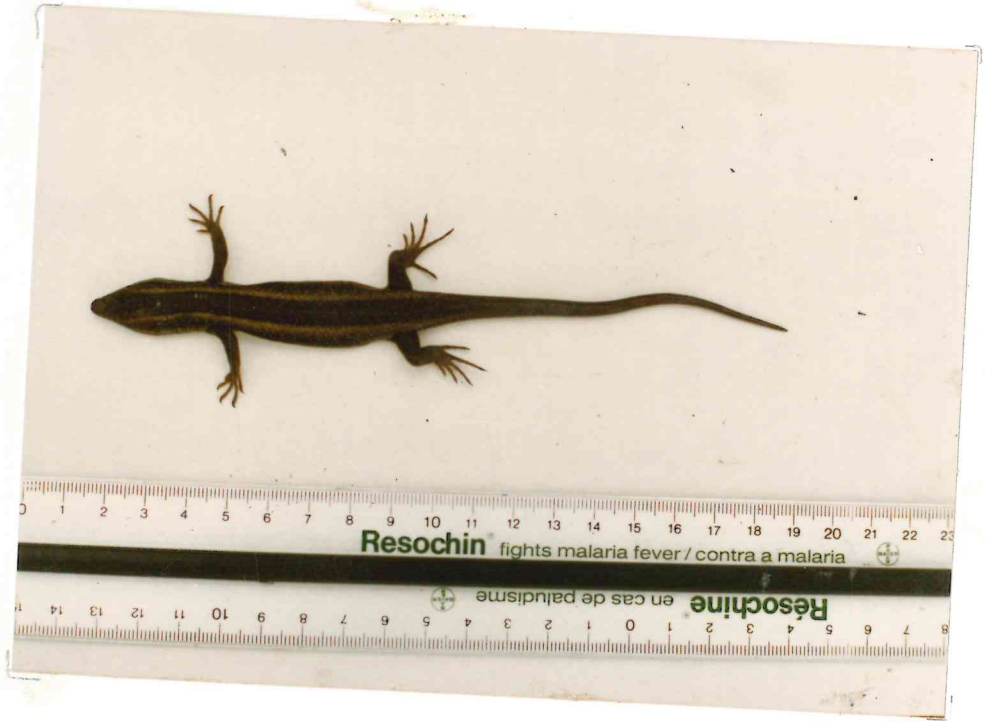
c) Adult flies emerging were transferred, each species in a separate cage and they were fed on hamsters or lizards.

The flies were offered apples for their sugar meal. All flies used for this experiment had not been blood fed previously.

1.2. Lizards:

Preliminary experiments during this work on susceptibility of the lizard Mabuya striata to infection with a lizard Leishmania isolate, gave positive results. Accordingly the lizard Mabuya striata of the family scincidae (Figure 5), was selected as the experimental subject for the present study.

Literature on the biology and behaviour of the species of the scincidae family showed that Mabuya striata give birth to their offsprings, (Bellairs, 1969, 1970). Rozouze Schults & Bolt (1975) were able to breed the Indonesian lizard Mabuya multifasciata in captivity. They found that females produce offsprings at the age of 13 months. Their study showed that it will take about a year to produce one generation of laboratory-bred Mabuya sp. Hence since this project was time limited, 180 wild lizards caught in a Leishmania-free area were used. The lizards were maintained in specially designed cages into which sunlight could penetrate and had some ventilation holes,(Figure 6). Some rocks and sawdust were put in each cage.


Resochin fights malaria fever / contra a malaria
Résochine en cas de paludisme

The lizards were fed on unlimited supply of laboratory-bred,adult and larvae houseflies and provided with water.

1.3. Mice:

Two hundred and twenty, four-week old, males, laboratory inbred white mice parasite-free (Figure 7) were used. The mice were maintained in groups of ten in a cage. Sawdust was used for bedding and the mice were fed on unlimited amounts of commercial food pelletes and tap water. The bedding was changed every other day.

2. Parasites:

Three isolates from ICIPE cryobank were used. These were:

LIZ/KEN/75/ICIPE 140	(unidentified lizard isolate)
MHOM/SU/73/5ASKH L.major	(identified human isolate)
LIZ/RC/84/244 L.adleri	(identified lizard isolate)

These isolates will be referred to as L140, L.major and L.adleri, respectively.

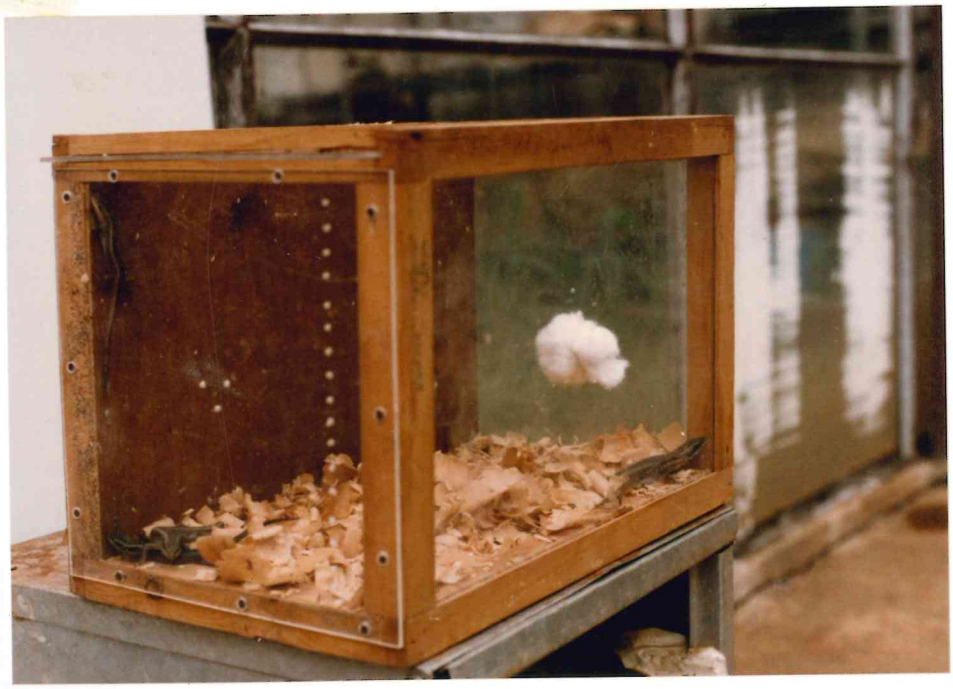
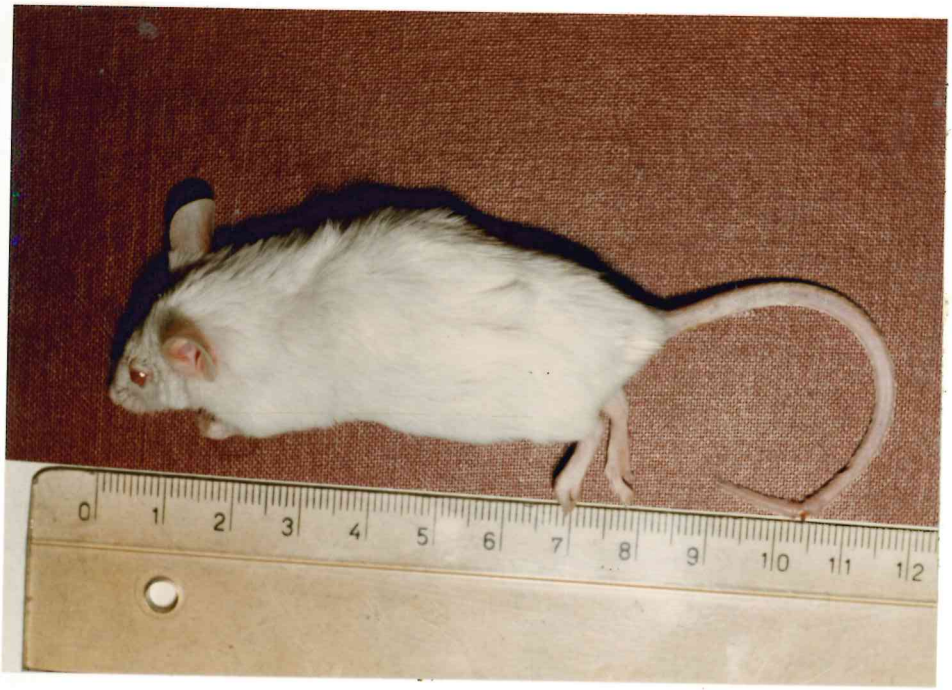


FIG. 7: White mouse used in the experiments.


0 1 2 3 4 5 6 7 8 9 10 11 12

2.1. Cultivation of Parasites:

The isolates were thawed aseptically and cultivated in Novy, McNeal and Nicolle's (NNN) medium. This culture medium was adopted from Tobie et al. (1950) and prepared according to the following procedure:

Base: Bacto-beef (Difco), 25g., infused in 500 ml distilled water in 56 C waterbath for one hour, heated to 80°C for 5 minutes to coagulate a portion of the proteins, and filtered into a separate flask containing 10g. Neopeptone (Difco), 10g Bacto-agar (Difco), and 2.5g NaCl. This was heated to boiling to bring into solution cooled to 50-60°C, and adjusted to pH 7.2 - 7.4 with NaCl. After autoclaving at 15lbs for 20 minutes at 120°C, the mixure was then cooled to 45-50°C. Defibrinated rabbit blood and antibiotic solution were then added. The blood is 10% by volume of the final medium. The antibiotic concentration was 200 IU penicillin and 400 ug/ml streptomycin. Five ml of the medium was dispensed in screwcap culture tubes, slanted and cooled overnight at room temperature. Overlay: approximately 3ml of sterile Locke's solution is added per tube immediately after the base has hardened. The following ingredients were included in the Locke's overlay: NaCl 8g, KCl 0.2g, $CaCl_2$ 0.2g, KH_2PO_4 0.3g, dextrose 2.5g and distilled water 1000ml.

After inoculation of the isolates in NNN medium they were examined daily. Examination was carried in a sterile hood. One lobe of the liquid phase was removed from each bottle and put on a clean slide, covered with a coverslip and examined under X40 power of a light microscope. When the number of parasites per field reached about 50 they were subcultured in RPMl-1640 liquid medium (Gibco) with 10% foetal calf serum added to it together with penicillin (200IU) and streptomycin (400 µg/ml).

2.2. Inoculum Count:

The inoculation size was estimated by means of counts made in a haemacytometer. The method used was modified from that described by Lumsden et al. (1973). Before counting serial dilutions of the promastigotes were made using 1% Formaline to immobilize the promastigotes. The number of promastigotes in one ml of media was calculated from the number of promastigotes counted in five square millimeters of the Neubaur counting chamber of the haemacytometer as follows:

Number of promastigotes/ml =
Total count x Depth factor x area factor x dilution factor x 1000.

Counts were made immediately before inoculation of animals.

3. Inoculation of Animals

3.1 Lizards:

Prior to inoculation, the lizards were examined for the presence of naturally occurring trypanosomatids. Blood was obtained from the eye of each lizard using a capillary tube (Figure 8). Blood smears were made in duplicate and bottles of sterile culture media (NNN/Locke's) were inoculated and examined for the presence of parasites.

Mabuya striata lizards were divided into three groups. Each group had 60 lizards which were inoculated with one of the isolates, namely, L 140, L.major or L.adleri. Each lizard was inoculated with approximately 1 x 10^6 - 2 x 10^6 promastigotes. Inoculation was done through intraperitoneal injection using 1 ml sterile disposable syringes, (Figure 9). After inoculation, the lizards were kept in groups of ten i.e. there were six groups for each isolate plus one group as control.

3.2 Mice:

Mice were inoculated with L 140 and L.adleri. Three groups of mice, each of ten mice, were used for each isolate. In each

case one group was injected with approximately 1 x 106 - 2 x 106 promastigotes through intraperitoneal route for each mouse. The third group was kept as control.

4. Detection of Infection:

Lizards and mice were sacrificed at different time intervals according to the experiment. They were dissected to expose the viscera, Figures 10, 11, 12 and 13. A cross-section of the spleen and liver tissues were blotted on Whatman No.1 filter paper to remove excess blood. The cut surface of the tissue was dabbed lightly on a clean slide. Two impression smears were made for each organ. The smears were air-dried, fixed for 10 minutes in absolute methanol and stained for 25 minutes in 10% Geimsa stain. Smears were examined under light microscope using x 100 objective lens with oil emersion.

Cultures of the spleen, liver and heart blood were made in NNN diphasic culture medium. Small portions of the spleen and liver were chopped into very small pieces using a sterile surgical blade. Some small pieces were then put in the culture bottles containing NNN medium. Cultures were examined daily starting on day 6 up to day 15 before they were considered negative.

Small pieces of the spleen, liver, heart and gut were preserved in 2.5% glutaraldhyde in sodium cacodylate buffer at 7.4 pH and placed in the refrigerator for histological and fine structural studies.

5. Feeding of Sandflies on Infected Lizards:

Different species of sandflies were put in cages especially designed for sandflies. A lizard which had already been inoculated with one of the Leishmania isolates was trapped in a wire mesh and put in the cage with flies (Figure 14). Fed flies were dissected under a dissecting microscope by pulling the gut of the fly and examining its various parts under X10 power of a light microscope. If no parasites could be seen under that power a coverglass was put and the gut was examined under X40 power of the light microscope.

FIG. 8: Bleeding of the lizard Mabuya striata from the eye using capillary tubes.