

**SYSTEMATICS AND BIOLOGY OF PHLEBOTOMINE SANDFLIES OF  
THE VISCERAL LEISHMANIASIS FOCI OF NORTHERN PAKISTAN.**

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**Dedicated to my wife Sakieba Arif, My daughters Amna Arif and Ayesha Arif and my Parents for their great patience and constant encouragement throughout this study.**

## ABSTRACT

The aim of this study was to incriminate the vector (s) of visceral leishmaniasis in Northern Pakistan. Two contrasting disease foci were studied: Azad Jammu & Kashmir (AJK) and Northern Areas (NA) through monthly sampling.

Studies on the systematics defined morphologically variable species of the subgenera *Phlebotomus (Larroussius)* and *P. (Adlerius)*. Morphological characters of all the species belonging to these subgenera were intensively studied. The species belonging to the subgenus *Phlebotomus (Larroussius)* were identified as *P. major*, *P. keshishiani*, *P. kandelakii burneyi* and *P. spA*. and those belonging to subgenus *Adlerius* as *P. hindustanicus* and *P. salangensis*. Species belonging to other subgenera: *P (Phlebotomus) papatasi*, *P (Paraphlebotomus) sergenti* and *P (Paraphlebotomus) alexandri* were easily identified.

The longitudinal entomological studies were carried out in Bagh district (Rehra village) in AJK and Chilas district (Hudur village, 1200m and Thor village, 1700m) in Northern Areas between April 1991 and November 1991 and in the same months in 1992. Sampling was done using CDC-light traps, sticky-paper traps and mouth aspirators. In addition, a general survey was also undertaken at higher altitudes in Northern Areas and AJK.

A total of 9656 *Phlebotomus* sandflies were collected (8797 during the longitudinal studies and 859 during the general survey). Nine species of *Phlebotomus* were found, *Phlebotomus (A) salangensis* for the first time and a new species *P. (Larroussius) sp.A*. The species composition and relative abundance of species differed within and between the two areas. In Northern Areas *P. papatasi* (35.75%) followed by *P. sergenti* (19%) were dominant at the lower altitude of Hudur village but *P. keshishiani* (29.66%) was most abundant at the higher altitude of Thor village, whereas in AJK, *P. hindustanicus* (64.62%) was predominant.

Species differed in their seasonal abundance. In Northern Areas *P. papatasi* and *P. sergenti* showed peak activity in June and July and *P. keshishiani* in August. In AJK *P. hindustanicus* showed a clear peak in June preceding the monsoon. The activity was found to be positively correlated with temperature in Northern Areas and negatively correlated with rainfall in AJK.

The dominant anthropophilic species included *P.papatasi* at lower altitudes (Hudur village), *P.keshishiani* at higher altitudes (Thor village) in Northern Areas and *P.hindustanicus* in AJK. Most biting occurred around midnight between 2200-2400 hours with a second peak between 0400-0600 hours in Northern Areas. None of the sandflies were found biting human volunteers in AJK. Blood-meal analysis revealed that the dominant species *P.papatasi*, *P.sergenti*, *P.keshishiani* and *P.hindustanicus* have a range of hosts mainly human, bovines and dogs. Monthly sampling with dog baited traps also showed *P.keshishiani* and *P.hindustanicus* as the dominant species attracted to the potential reservoir host (dog).

Females of *P.keshishiani* (754), *P.salangensis* (36), *P.alexandri* (15) and *P.hindustanicus* (301) were dissected for natural infections with *Leishmania* promastigotes and squash blots probed by *Leishmania infantum* specific DNA probe (LUCA D2 200 BP). Only *P.hindustanicus* was found to be naturally infected with *Leishmania infantum* (parasites identified by DNA hybridization) in AJK. This species is the principal vector involved in the transmission of VL in AJK .

From ecological studies it is concluded that the transmission of visceral leishmaniasis takes place at higher altitudes in Northern Areas and that *P.keshishiani* is the vector.

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## CHAPTER 1

## INTRODUCTION

Phlebotomine sandflies (Order:Diptera; Sub-order:Nematocera; Family Psychodidae) are small (3-5 mm), delicate, hairy flies with long slender legs. The thorax is convex and wings are lanceolate and covered with hairs. When at rest the wings are directed upwards in V fashion. At present 700 species of sandflies are known both from the Old and the New World (Lane, 1993) but only about 70 species are thought to be involved in disease transmission.

Phlebotomines are responsible for transmission of several human diseases, namely, leishmaniasis, bartonellosis, and sandfly fever viruses in many countries of the World. In terms of the geographical distribution and diversity of vectors the most important disease transmitted by the bite of sandflies is leishmaniasis, which exists in several forms (Lainson, 1982). The control of sandflies is an important part of the prevention of diseases and must be directed against those species that transmit the disease. A knowledge of sandfly systematics, exact delimitation of species, and information on their population biology is therefore important to understand disease epidemiology and for devising necessary control measures.

**1.1. THE LEISHMANIASES.**

The Leishmaniasis are a group of parasitic diseases caused by morphologically similar parasites in the genus *Leishmania* (Order Kinetoplastida, Family Trypanosomatidae) and transmitted by the bite of phlebotomine sandflies. Vector species belong to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New

World.

The leishmaniases can be broadly separated into two categories on epidemiological grounds: 1) Anthroponotic, with man as the main source of infection and transmission occurring mainly in settled communities, or 2) Zoonotic, with domestic or wild animals as the main source of infection. Clinically, the disease occurs in several forms, ranging from simple cutaneous ulcers (e.g. caused by *Leishmania major*) through disfiguring mucocutaneous form (caused by *Leishmania brazilienses*) to fatal visceral infection (caused by for example *Leishmania donovani*). Although clinical manifestations in Man had been the principal criterion on which the disease and its epidemiology was studied, with the use of sophisticated biochemical methods for identifying the parasites it was found that clinical criteria were not always reliable predictors of the infecting parasite and now the key to studying the disease is the accurate identification of parasites. For example, dry cutaneous lesions in the Old World were previously considered to be caused by *Leishmania tropica* but now it is clear that similar lesions are also caused by *Leishmania infantum* and *Leishmania major*. To date some 21 species of *Leishmania* are known to be pathogenic to humans.

### 1.1.1. Disease incidence.

Leishmaniasis has been considered to be a serious public health problem in many countries of the world and can have an adverse economic and social impact. The disease is documented to be currently prevalent in 82 countries (21 in the New World and 61 in the Old World). The annual incidence is estimated at some 600 thousand new clinical cases, officially reported, with a global prevalence of 12 million cases and



a population at risk of approximately 350 millions (Desjeux, 1992). In an another study by Ashford *et al.*, (1992) visceral leishmaniasis is known to occur in 47 countries, with approximately 200 million people at risk, and 100,000 cases annually. Cutaneous leishmaniasis occurs in 61 countries, with almost 200 million at risk and 300,000 cases annually.

### 1.1.2. Geographical distribution.

Leishmaniases occur in most tropical and sub-tropical parts of the world: southern North America, most of South and Central America, the Mediterranean Basin, East and North Africa, the Caspian littoral, the Arabian peninsula, the Persian Gulf, the Indian sub-continent, China and the southern Former Soviet Union (FSU).

In the Old World visceral leishmaniasis (VL) caused by *Leishmania infantum* occurs sporadically from the Mediterranean area through the Middle East and Central Asia to North China. It is usually associated with rocky areas and mainly affects children. In contrast, the major VL focus in northeast India, Bangladesh and Nepal involves young adults living on the plains and the causative agent is *Leishmania donovani*. In East Africa two areas are affected, Kenya and Ethiopia, and the Southern Sudan (*Leishmania donovani*)

Cutaneous leishmaniasis (CL) in the Old World is mainly found in arid-regions and occurs in North Africa, the Mediterranean littoral, the Middle East to northwest India, Central Asia, and East Africa (Lane, 1993).

### 1.1.3. Reservoirs.

Important reservoirs of zoonotic cutaneous leishmaniasis in the Old World include *Rhombomys opimus* (FSU, Iran, Northern Afghanistan), *Meriones libycus* (USSR), *Psammomys obesus* (Saudi Arabia, Libya and parts of Israel), *Arvicanthis niloticus*, *Mastomys (=Praomys) erythroleucus*, and *Tatera gambiana* (Afrotropical region). *Leishmania tropica* is considered to be strictly anthroponotic as transmission is believed to occur only from man to man. In some countries however, dog (USSR, Iran, Iraq and Afghanistan) and *Rattus rattus* (Iraq) have also been suspected to be the reservoir hosts but this is still controversial; in subsequent studies several authors failed to find *Leishmania* in these (Aljeboori and Evans, 1980) as well as many other wild mammals (Bettini *et al.*, 1980; Pozio *et al.*, 1982).

For *Leishmania infantum* the dog is the main domestic vertebrate host, although in some areas of the Old World rodents (*Rattus rattus*, *R. norvegicus*) and wild canids (*Vulpes vulpes*, *Nyctereutes procyonoides*, *Canis aureus*, and Cats) have also been incriminated or suspected as wild reservoirs (Ashford, 1977; Ashford and Bettini, 1987).

### 1.1.4. Vectors.

The vectors of leishmaniases in the Old and the New World have been comprehensively reviewed (Young and Lawyer, 1987., Killick-Kendrick, 1990). Briefly, species of *Phlebotomus* are vectors in the Old World and *Lutzomyia* in the New World.

The relationship of *Leishmania* and sandflies is specific. It is manifested in

differing susceptibilities of different species of sandflies to the particular species of *Leishmania*. This is well evident from the fact that in the Old World the vectors of *Leishmania infantum* are restricted to the subgenera *Ph.(Larroussius)* and possibly *Ph.(Adlerius)*, whereas those of *Leishmania donovani* are restricted to the subgenera *Ph.(Euphlebotomus)*, *Ph.(Paraphlebotomus)* and *Ph.(Synphlebotomus)*. The list of the proven vectors of visceral leishmaniasis in the Old World is indeed a short one. The only sandfly species which fulfil all the complex criteria of vector incrimination (Killick-Kendrick, 1990) include *P. aiasi*, *P. perniciosus* and *P. perfiliewi* which transmit *Leishmania infantum* and *P. alexandri*, *P. argentipes* and *P. martini* which transmit *Leishmania donovani*. Each of these species has been studied in depth and provides useful information for vector research in many other countries of the World. Before considering the detailed aspects of sandfly biology it is pertinent to review briefly the criteria of vector incrimination around which most of the sandfly studies revolve.

#### **1.1.4.1. Vector Incrimination.**

Incrimination of a sandfly as a vector is difficult as many criteria have to be satisfied before a species can be unambiguously incriminated, as discussed in depth by Killick-Kendrick, (1990). Among the essential criteria are the demonstration i) that the fly is anthropophilic and ii) the repeated isolation and identification of the same species of *Leishmania* from the sandfly as found in patients. Additional supporting observations include: i) demonstration that the sandfly commonly feeds on reservoir(s) host (s), ii) that it is present in the places where the *Leishmania* and the disease it

causes are found, iii) that it supports a flourishing development of the parasites, and that the fly can transmit the parasites by bite.

Modern data on the basis of the above mentioned criteria has revealed 20 species of Phlebotominae which can be undoubtedly classed as the vectors of *Leishmania*, 12 species belong to the genus *Phlebotomus* and 8 species and subspecies, to the genus *Lutzomyia*.

#### 1.1.5. Life cycle of *Leishmania* in the sandfly and vertebrate hosts.

The life cycle of *Leishmania* in both the vertebrate and invertebrate hosts has been comprehensively reviewed (Molyneux and Killick-Kendrick, 1987). Briefly, *Leishmania* has two forms in the life cycle; an amastigote in the reticuloendothelial system of the vertebrate host and a promastigote in the gut of the sandfly vector.

##### a) Sandfly vector.

Sandflies are pool feeders, having mouth parts adapted to cut the skin of the vertebrate host (Lewis, 1978). The behaviour and life cycles of different species of *Leishmania* in the sandfly are not uniform. When a sand fly feeds on the vertebrate host the blood is sucked up into the midgut by the muscular movements of the pharynx and cibarium. If the amastigotes are in the skin (dermis) or peripheral blood they are taken up with the blood meal into the midgut.

The amastigotes are released from the macrophages when host cells are ruptured during the feeding process or broken by cibarial teeth (Lewis, 1975). Involvement of cibarial teeth in the cell rupture for the release of parasites is however

not evident in *Lutzomyia longipalpis* and therefore Molyneux and Killick-Kendrick (1987) suggested that the rupture may occur when the swollen infected macrophages are exposed to trauma during ingestion of blood meal. After they are released from the cells the parasites then divide one or more time before transforming into promastigotes (Killick-Kendrick, 1979, Lainson *et al.*, 1987). The initial stages of development after the ingestion of amastigotes in the blood meal have rarely been seen.

Shortly after engorgement, the blood meal is encased by a peritrophic membrane secreted by the abdominal mid gut (Gemetchu, 1974) and the parasites are confined by this membrane for the next 72 hours (Killick-Kendrick *et al.*, 1974). As digestion proceeds, the membrane breaks up and free living promastigotes escape either through the broken membrane or via the anterior end into the mid gut and either attach to the mid gut wall by inserting the flagellum between the microvilli (Killick-Kendrick *et al.*, 1974) or attaching by hemidesmosomes to cuticle in the hindgut. Within the midgut a rapid multiplication of promastigotes takes place in the suprapylarian species during the digestion of blood meal before the anterior movement of parasites into the thoracic mid gut. The division of promastigotes in sandflies is principally by binary fission (Molyneux and Killick-Kendrick, 1987; Walters *et al.*, 1989b).

Promastigotes of varying morphology are found in the thoracic and abdominal mid guts and the stomodeal valve. Based on the varying morphology the terms nectomonad (long and slender, electron-dense, attached promastigotes) and haptomonads (broad, electron-lucid promastigotes with modified flagella capable of producing electron dense plaques called hemidesmosomes inside the sheath associated

with attachment to cuticular the intima of the stomodeal valve) ~~were~~ adopted (Killick-Kendrick *et al.*, 1974, Warburg *et al.*, 1986., Lawyer *et al.*, 1987, Walters *et al.*, 1989a, b).

In the cuticular part of the foregut other than the mouth parts (oesophagus, pharynx and cibarium), the commonest morphological form is the paramastigote which differs from the promastigote by having the kinetoplast beside the nucleus. Paramastigotes are round or oval in shape and much less motile than the promastigotes. Like haptomonads they attach to the cuticular intima of the foregut of the fly by the tip of the flagellum within which hemidesmosomes are formed (Killick-Kendrick *et al.*, 1977a, 1988; Walters 1989 a, b; Killick-Kendrick, 1990 . Perhaps the greatest recent advances in the studies of *Leishmania* in the sandfly is the confirmation of the production of so called metacyclic promastigotes, the sandfly forms which already appear to be adapted for life in the vertebrate host (Sacks and Perkins, 1984; 1985). Striking features separating metacyclic forms from others in the sandfly

are high motility, lack of attachment to sandfly tissue, and a small body size relative to the long free flagellum (with a length about twice that of the body) (Killick-Kendrick, 1990 b).

Temperature seems to have a significant effect on the development of *Leishmania* in sandflies not only on the degree of infection, but also on the capacity to migrate anteriorly and thus be transmitted efficiently (Killick-Kendrick 1979). The speed of development increases at temperature above 10°C. Rioux *et al.*, (1985) studied the effect of temperature (10, 15, 20 and 25°C) on development of *Leishmania infantum* in *Phlebotomus ariasi* and found that raising the temperature increased the

proportion of sandflies infected and speeded up multiplication of the parasites. From 15°C upwards the attachment of parasites to the walls of the stomodeal valve appeared to be encouraged.

The time require for the completion of the life cycle appears to vary with species of *Leishmania* e.g. *Leishmania infantum* can develop in 6 days in *P.perniciosus*, (Killick-Kendrick & Rioux, 1981) but take at least 14 days in *P.ariasi*, and *Leishmania mexicana* species can develop fully and be transmitted by the bite of an infected sandfly as early as 4 days after the infective blood meal (Ward *et al.*, 1977).

#### b) Vertebrate Host.

In the vertebrate host *Leishmania* exists in the amastigote form (without flagellum). After the initial infection of the mammal by metacyclic promastigotes from the infective sandfly they are phagocytosed by the cells of reticuloendothelial system, mainly macrophages where they are transformed into amastigotes within hours (Molyneux and Killick-Kendrick, 1987). The precise duration from the time when the promastigotes are inoculated and phagocytosed is evident from the study of Cheng *et al.*, (1981). Using sandfly derived promastigotes they found that macrophages from mouse peritoneal exudate became infected 4-8 hours after contact with promastigotes, and amastigotes began to appear after 24 hours.

Within the susceptible macrophage, the amastigotes begin to divide by binary fission which continues until a large number of parasites occupy the parasitophorous vacuole and the cell eventually bursts. The liberated amastigotes are ingested by other

macrophages, consequently resulting in the spread of infection within the mammalian host. The infection however tends to be benign and inapparent in the major reservoir species, but in others it can lead to destructive lesions of the skin, or pathological changes in the internal organs which may prove fatal (notably man, dog and equine) (Molyneux and Killick-Kendrick, 1987).

## 1.2. SYSTEMATICS AND BIOLOGY OF PHLEBOTOMINE SANDFLIES.

### 1.2.1. Brief history of sandfly systematics.

The first Phlebotomine sandfly was described by Bonanni (1691) in Rome, Italy, and nearly 100 years later Scopoli (1786), first described the first member of this group of Diptera as *papatasi*. The genus of the sandflies *Phlebotomus* was established by Rondani & Berte 1840

The comprehensive study of sandflies began in 1906, with the description of *Phlebotomus duboscqi* a new species from East Africa (Neveu-Lemaire, 1906). By 1910 a further 15 species of sandflies were known (Annandale, 1910). At this time Newstead (1911) placed the then known sandfly species into the Family Psychodidae, Sub-family Phlebotominae and Genus *Phlebotomus*, sub-dividing the last into groups with erect hairs (Type species-*Phlebotomus papatasi*) and recumbent hairs (Type species-*Phlebotomus minutus*).

The history of sandfly systematics can be separated into two periods according to the type of characters used to differentiate and classify species (Theodor 1948, Perfil'ev 1968). Briefly in the first period the taxa were distinguished according to external structures with particular emphasis on measurements and ratios. Thus



Newstead (1911) divided four Maltese species into two groups whose abdominal tergites had either recumbent or erect setae, and the classification of Franca & Parrot (1921) was based on the structure of the male genitalia, and on wing venation indices and other external measurements, which became known as 'Phlebotometry'. The second period followed the publication of Adler & Theoder (1926). Instead of phlebotometry, characteristics of the inner structure were used as a basis for the discrimination of females, namely those of structure of the spermathecae, the buccal cavity (or cibarium) and the pharynx in several Old World species. A detailed review on both the above mentioned periods is comprehensively covered by Perfil'ev (1968).

Morphological characters have been extensively employed to distinguish many species of sandflies. One noticeable advancement in this context is the more recent use of scanning and transmission electron microscopy in morphological studies which have significantly contributed to our understanding of the functional role of certain structures and some have been used taxonomically e.g. mouth part sensilla have been studied extensively by Lewis (1975) with light microscopy. These sensilla have been used taxonomically by Davidson (1986) and Lewis and Dyce (1984). Detailed examination of some traditional characters has also provided a useful tool for identification of closely related species. For example Léger, *et al.*, (1983) employed the basal section of spermathecal ducts for distinguishing females of Mediterranean species of the sub genus *P. (Larrousius)*.

Perhaps one of the outstanding achievements in the systematics of sandflies is the recent advancements made at biochemical and molecular level (for review see Lane, 1985; Ward, 1990). The introduction of such techniques as isoenzyme

electrophoresis, analysis of cuticular hydrocarbons, semiochemical identifications, and DNA probes, though still in a preliminary stage, will no doubt contribute substantially in the future to the taxonomy (including genetics) of Phlebotomine sandflies at both the specific and infra-specific level. From the control point of view this is an important aspect of sandfly biology and disease epidemiology.

The classification of the genus *Phlebotomus* and of the subfamily Phlebotominae has been discussed in depth by Abonnenc (1972), Fairchild (1955), Lewis *et al.*, (1977), Theodor (1948; 1958), Artemiev (1980) Parrot (1951), and others. Taxonomists have suggested several systems of the classification of sandflies, but uniformity has not been achieved and therefore the higher classification of sandflies is still in controversy and there is no universally accepted system (Lane, 1993). Some authorities considered sandflies to be a separate family (e.g. Abonnenc & Leger, 1976, Lewis, 1973 ; Perfil'ev, 1966) mainly following Rohdendorf (1964), but most retain sub-family status within the Psychodidae (Fairchild, 1955; Lewis, 1978, 1982; Theodor, 1958). In a publication Lewis *et al.*, (1977) proposed a stable classification of the phlebotomine sandflies which is mainly the modification of Theodor's system (Theodor, 1948) based on practical criteria; with the Phlebotominae composed of five genera: *Warileya* (2 sub-genera); *Phlebotomus* (10 sub-genera); *Sergentomyia* (7 sub-genera); *Brumptomyia* and *Lutzomyia* (26 sub-generic taxa with 19 species unplaced). This approach has been generally accepted in the literature.

### **1.2.2. Biology of phlebotomine sandflies.**

The main interest to study the biology of phlebotomine sandflies is

due to their ability to transmit leishmaniasis and certain viruses to humans. The fundamental aim is to incriminate the vector species, and to determine their distribution, population dynamics and behaviour in the field. Such information provides useful estimates regarding disease transmission and is of inestimable value for devising successful control strategies especially, where vector control needs to be undertaken. The biology of phlebotomine sandflies have been a subject of several excellent reviews (Lewis, 1971, 1974; Ward, 1985; 1990; Killick-Kendrick, 1978, 1979; Lane, 1993). Some of the key aspects of sandfly biology in general as well as in relation to disease epidemiology will be briefly outlined here:

#### **1.2.2.1 Some key aspects of sandfly biology.**

##### **a) Colonization.**

Many important aspects of sandfly biology has been the outcome of successful colonization (for review see Killick-Kendrick *et al.*, 1991). One of the most remarkable achievements has been understanding the life cycle of *Leishmania* in these insects (Killick-Kendrick *et al.*, 1974; Molyneux *et al.*, 1975) and transmission mechanisms. Also, it has provided reasonable details on the immature stages which are difficult to obtain in nature.

##### **b) Life history and breeding sites.**

Based on information gathered from laboratory rearing of various species of sandflies, the female lays between 30-70 (Lane 1993) and some times up to hundred eggs (Ward, 1985) in a single oviposition but the average is usually 40 eggs. The eggs

are laid singly and are elliptical in shape with distinct patterns as observed by SEM (Ward & Ready, 1975; Zimmerman *et al.*, 1977; Lane & El Sawaf, 1986; Endris *et al.*, 1987; Fausto *et al.*, 1992). These patterns have also been used successfully in some studies for differentiating between morphologically indistinct females. There are four larval instars which actively feed on rich organic detritus and larval development takes 30-60 days dependent upon species, prevailing temperature and food supplies. The pupa is inactive and is characterized by the retention of the larval skin. After 7-10 days, the pupa darkens and the newly formed adult emerges. The period from oviposition to adult eclosion is 20 to 40 days, but up to several months in diapausing species. During periods of adverse climate such as prolonged cold periods, some species undergo diapause as fourth instar larvae (e.g. *P. ariasi* in the Mediterranean Basin and *P. longipes* at high altitudes in East Africa).

Information on the larval breeding sites is still obscure. Important reviews in this context are those of Hanson (1961), Bettini (1989) and Killick-Kendrick (1987b). In nature larvae are generally found in damp soil, humus or leaf litter, the forest floor in tropical forests, rodent burrows in deserts, soils in animal pens and dens, caves and presumably in termite hills and cracks under rocks.

### **c) Feeding habits.**

Most sandflies are crepuscular and nocturnal in their biting habits. Both sexes feed on sugars but only females feed on blood.

Sugars are an important nutrient source of sandflies (Killick-Kendrick, 1978) and it enhances the chances of an infected fly transmitting *Leishmania* (Smith, *et al.*

1941; Swaminath, *et al.*, 1942; Shortt, 1945; Killick-Kendrick, 1979, 1980). The presence of glucose, fructose, and sucrose has been detected in the crops of both the Old and New World sandflies (Lewis & Domoney, 1966; Killick-Kendrick, 1979; Young *et al.*, 1980). In nature sugars are obtained either from aphid honey dew e.g. by *P. ariasi* (Killick-Kendrick & Killick-Kendrick, 1987) or by piercing stems and leaves of different plants e.g. by *P. papatasi* (Ashford, 1974; Schlein *et al.*, 1986; Dinesh & Dhiman, 1990).

Only females suck blood, using nutrients for ovarian development and subsequent oviposition. Most of the sandflies are gonotrophically concordant (ovaries develop with the digestion of a single blood meal). Autogenous females are however an exception e.g. *P. papatasi*, they do not require a blood meal for egg maturation. Feeding takes place on the exposed parts of the host and sandflies feed by creating a small pool and injecting a potent vasodilating peptide into the wound (Riberio *et al.*, 1989). Blood is taken directly into the midgut. Liquids taken by other means (e.g. sugars) are directed first to the crop for sterilization and then to the mid-gut (Schlein *et al.*, 1986).

Oviposition takes place 4-5 days after the blood meal. The highest parous rates occur towards the end of the sandfly season, when incidently, sandfly infection rates are at their highest and transmission most intense. Unfortunately it is not easy to determine with accuracy how many times an adult female has laid eggs. The residual secretions in the accessory glands have been used for differentiating between parous and nulliparous females but are not very reliable. At present the established method for searching for follicular relics in the ovarioles still needs to be developed (Ready

*et al.*, 1984).

**d) Mating, dispersal and longevity.**

Mating behaviour in sandflies is less well studied than in many other insects. The general observation is that male sandflies are strongly attracted to a host and mate with blood seeking females. This phenomenon has been observed in *Lutzomyia vexator occidentalis* (Chaniotis, 1967) and *Lutzomyia longipalpis* (Ward, 1986; Santos *et al.*, 1991). Recently Lane *et al.*, (1990) observing mating behaviour of *P. argentipes* in nature, found swarms of males on the host forming a mating lek in which individuals are spaced regularly and there is a considerable competition for position manifested by continuous jostling whenever a new individual arrives in the array. Pheromones (Ward, *et al.*, 1986) and sound communication by male sandflies (Ward *et al.*, 1988) to attract females for courtship and mating are also evident.

Knowledge of the dispersal of vector species is of epidemiological significance as it determines the degree of contact between the vector and its mammalian host. Little is known of their long range movements, although they can fly up to 2.2 km. (Killick-Kendrick, *et al.*, 1984) over a period of few days in open habitats.

Epidemiologically, an extremely important aspect of sandfly biology is their longevity in nature, which has been little studied. The only information available is for *P. ariasi* and *P. argentipes*. Mark-release-recapture studies have shown that *P. ariasi* infected with *Leishmania infantum* can survive in nature for 29 days, which could allow 3-4 gonotrophic cycles (Killick-Kendrick, 1978). The only study on the life expectancy of phlebotomine sandflies is that of Dye *et al.*, (1987). They calculated

mean life expectancy for *P. ariasi* as 1.54 (SE 0.04) ovarian cycles in nature.

#### **1.2.2.2. Essential aspects of sandfly biology in relation to disease epidemiology.**

Sandfly biology in relation to disease transmission has been discussed in depth by Lewis and Ward (1987). Briefly, to understand the epidemiological cycles and the development of control strategies it is essential to acquire information on population densities, biting rates, anthropophilic habits (food preferences), natural infections and host-vector contact. These aspects are briefly discussed as below:

##### **a) Seasonal changes in sandfly densities.**

Studies on seasonal changes in sandfly densities are of vital importance for determining the most effective time to implement control measures (Killick-Kendrick, 1978; Lewis & Ward, 1987) and also in incriminating a vector by determining the possible and most likely period of disease transmission.

The changes in population densities of phlebotomine sandflies have been widely studied in different geographical regions. Generally in temperate zones such changes are associated with temperature and sandfly adults are present only in the summer (Lewis, 1974). Here transmission may be most intense at the end of the season when the maximum number of sandflies are parous (Guilvard *et al.*, 1980). Most species in temperate regions have one generation per year, and consequently a single peak of activity and transmission, but the same species can have two or three generations per year in a climatically more favourable areas. In tropical regions, flies can flourish in the wet or dry season throughout the year (Lewis, 1974) but the

seasonal changes have generally been associated with rainfall (Ward *et al.*, 1973). Several anthropophilic species can be present in any one area, each with its own annual cycle of activity and potential for parasite transmission.

Numerous studies have been carried out to observe seasonal fluctuations in sandflies in various countries of the World. In the Oriental region phlebotomine sandflies are most abundant before or immediately after the monsoon usually with two peaks of abundance in a year representing two generations (Lewis, 1978; Srinivasan & Panicker, 1992a; Dhanda *et al.*, 1983; Dhanda & Modi, 1971; Mitra, 1956; Napier & Smith, 1926).

In Mediterranean areas adult sandfly activity coincides with the season when people seek relaxation in rural areas. *Phlebotomus perniciosus* is considered as the vector of visceral leishmaniasis caused by *Leishmania infantum* in many territories of the Mediterranean area, being distributed throughout the western mediterranean. The annual fluctuation density of this fly in Tunisia (Croset, *et al.*, 1970); Algeria (Parrot, *et al.*, 1933); USSR (Petrisceva, 1962); and Italy (Biocca, *et al.*, 1977; Maroli & Bettini, 1977) shows two main peaks, one in June and another in September which suggest the existence of two generations. On the contrary, in southern France the density trend of *P. ariasi* and *P. perniciosus* is monophasic (Rioux & Golvan, 1969) being attributed to the short warm season.

In central Asia the sandfly season begins when the average day temperature reaches 18-20°C. It varies according to altitude and latitude and may last 10 weeks (June-August) with one generation, or 8 months (April-November) with three generations. In some places there are two peaks of abundance corresponding to two



generations (Lewis, 1974). In north and north-east China adult sandfly activity is in the mid or later half of June (Zahar, 1980) whereas in north-west China, most similar to northern Pakistan, the peak activity is in the mid or later half of July with a unimodal curve.

In most part of Saudi Arabia phlebotomine sandflies are scarce or absent from late November or early December till the end of January to February or even early March except in the low lands where sandflies occur throughout the year (Büttiker & Lewis, 1983). In lowland areas the seasonal distribution has been observed to be bimodal whereas in the highlands it is unimodal (Al Zahrani *et al.*, Unpublished). More or less the same situation is found in other middle eastern countries such as Iraq (Abul-Hab & Baghdadi, 1972; Abul-Hab & Mehdi, 1970); Jordan (Kamhawi, 1989); Egypt (Beier *et al.*, 1986) and Iran (Nadim, *et al.*, 1977).

#### **b) Host vector contact.**

The ability to identify natural hosts of blood-sucking insects is an integral part of many ecological investigations, and it is of paramount importance in epidemiological studies especially when insects are of medical or veterinary importance (Service *et al.*, 1986). Feeding habits of sandflies are important to determine the degree of anthropophily and zoophily in the disease foci (Killick-Kendrick, 1978) which gives important information regarding preferred hosts and also point to a possible reservoir host (Bray, 1982).

Knowledge regarding the host range of sandflies is rather limited (Lewis, 1974; Killick-Kendrick, 1978; Mutinga *et al.*, 1984, 1990; WHO, 1990). Like other

haematophagous insects, sandflies feed on a wide variety of hosts. Some feed primarily on cold blooded animals, while others prefer mammals and/or birds. In general, females of the genus *Phlebotomus* feed on mammals and birds and those of *Sergentomyia* on reptiles and amphibians (Lewis 1974). Several peridomestic and mammal feeding sandflies (e.g. *P.papatasi*, *P.sergenti* and *Lutzomyia longipalpis*) also feed on poultry.

Various methods have been used to determine the feeding habits of sandflies. These include human biting catches, blood meal analysis and animal baited catches.

#### **i) Human biting catches.**

This is the classic method by which human biting of sandflies and other blood sucking insects has been measured (Hoogstraal & Heyneman, 1969; Gomez *et al.*, 1990). In practice human biting catches are obtained by quantifying the number of vector species biting exposed skin per unit time. However because of the risk of acquiring infections during biting, landing catches are generally preferred. The method however, provides accurate information on host vector contact and the time when humans are at maximum risk of acquiring infections. Studies carried out in different countries e.g Italy (Maroli *et al.*, 1984); France (Brooks, 1985); Ethiopia (Foster *et al.*, 1972, Ashford, 1974, Gebre-Michael, 1992); India (Srivinivasan and Panicker, 1992b); Iraq (Abul-Hab *et al.*, 1988) and Egypt (El Said *et al.*, 1986) showed different peaks of biting activity of various sandfly species at certain hours of night suggesting the degree of human vector contact as well as the time when humans were at maximum risk of acquiring infections.

**ii) Blood-meal analysis.**

This is another important method for studying the feeding habits of sandflies and in determining the preferred host. Identification of blood meals have mainly involved the use of immunological methods, recently reviewed by Pant, *et al.*, 1987. Until recently, the blood-meals of sandflies were mainly analyzed using the Precipitin ring test and to a limited extent by counter current immuno-electrophoresis. The former method was however found to be of limited value for identification of blood meals in sandflies due to the small size of the blood-meal (Guy, *et al.*, 1984). Alternatively, enzyme linked immunosorbant assay (ELISA) was adopted and found to be more practical for the identification of blood meals because of its higher specificity (Burkot *et al.*, 1981). This method has been successfully used for the identification of blood meals in *Culicoides* (Blackwell *et al.*, 1994) and also phlebotomine sandflies (Ngumbi *et al.*, 1992).

Blood meal analysis of various sandflies species has provided useful information on the anthropophilic and zoophilic index of these species, which is an important observation from the epidemiology point of view. An outstanding example of such a work was a publication from India (Das *et al.*, 1976) which showed that *P. argentipes* which appeared to have been largely controlled by spraying against mosquitoes, had re-acquired man biting habits. On the basis of which it was correctly predicted that this could lead to new epidemic of Kala-azar.

The preferred hosts of only a few known or suspected vectors of leishmaniasis have been studied using blood meal identifications. The notable contributions in the Old World have been from India (Mukopadhyay *et al.*, 1987, Dhanda & Modi, 1971).

Pandya *et al.*, 1972, 1985; Namita, *et al.*, 1991; Gosh *et al.*, 1990); Egypt (El-Sawaf *et al.*, 1989); France (Guy, *et al.*, 1984) and Kenya (Mutinga *et al.*, 1990, Ngumbi *et al.*, 1992).

### iii) Animal baited traps.

Apart from blood meal analysis, observations to discover which anthropophilic sandfly species feed on animal reservoir hosts have also been made with animal baited traps e.g. Disney traps and Tent traps (Killick-Kendrick, 1988). Host preference studies using animal baited traps have been successfully used by several workers (Johnson, *et al.*, 1993; Quinnell, *et al.*, 1992; Mutinga *et al.*, 1986; Christensen & Herrer, 1980; Killick-Kendrick *et al.*, 1977; Rioux & Golvan, 1969; Shaw & Lainson, 1968; Quate, 1964) to determine preferred mammalian hosts of the known and suspected vectors both in the Old and the New World.

### c) Natural *Leishmania* infections.

Repeated isolation of *Leishmania* indistinguishable from those causing human disease from anthropophilic sandflies not only provides strong evidence for incriminating vector species (Killick-Kendrick, 1990) but also serves as a basis for estimating the risk of infection at a given time and place (Young and Lawyer, 1987). The ideal method is to dissect large numbers of females for the presence of *Leishmania* promastigotes, which should be cultured and later identified by isoenzyme electrophoresis. Attempts to culture promastigotes especially in the field are not always successful. Alternatively, part of the infected midgut can be squashed on to nylon

membrane for later identification using *Leishmania* species specific DNA probes. Currently, the latter method does not provide evidence on whether the promastigotes were the same as isolated from man or reservoir host to the same resolution that isoenzyme electrophoresis does..

### **1.3. METHODS OF SANDFLY CONTROL.**

The control of phlebotomine sandflies and leishmaniasis has been discussed recently (Lane, 1991; Ward, 1985., Vioukov, 1987; WHO, 1988., Lane ,1993).

In view of the diversity of phlebotomine biology there is no one method of control either applicable or attainable in the different foci throughout the world. A comprehensive understanding of the transmission cycle is therefore essential before the feasibility and cost effectiveness of a control strategy can be assessed. Most examples of successful vector control are in places where the vector is endophilic and peridomestic.

The principal method used for the control of sandflies is the application of insecticides, sometimes in conjunction with environmental management. Due to the limited knowledge of larval breeding sites, control is limited to attacking the adults.

#### **1.3.1. Insecticides.**

Insecticide control of adult sandflies is only practicable where peridomestic transmission occurs in discrete and well populated communities. Thus, in cases where the disease is an anthroponosis or a peridomestic zoonosis, direct attack on sandflies by means of residual insecticides spraying of houses, barns and stables has proven

effective. This type of activity can be appreciated in the results obtained from antimalarial programmes that have greatly reduced visceral leishmaniasis (Kala-azar), oriental sore (*L.tropica*) and sandfly fever in India, Italy, Greece, USSR and Israel (Safjanova, 1971; Hertig, 1949; Tesh & Papaevangelou, 1977). Indoor residual spraying is a simple and cost effective method of controlling the endophilic vectors and can have a long lasting effect, depending on the insecticide, the surface treated, the dosage and the method of application (WHO, 1990). The most widely used insecticide against sandflies has been DDT. The role of spraying DDT against *P.argentipes* in Bihar, N.E India is well documented (Safjanova *et al.*, 1962, Pandya, 1983) particularly in relation to the epidemic of Kala-azar due to the cessation of antimalarial activities (Thakur, 1984). Similar beneficial effects of house spraying against mosquitoes have been claimed from Iran (Nadim & Amini, 1970 and Rashti & Nadim, 1975), Greece (Tesh & Papaevangelou, 1977), Pakistan, Tunisia and Israel (Young & Lewis, 1977). In addition to DDT, other insecticides such as BHC, dieldrin, malathion, acetoxon, and more recently synthetic pyrethroid e.g deltamethrin are also being used for sandfly control. Recommended doses for the application of DDT and Malathion are 1 or 2 g/m.sq and HCH is applied at 0.5g/m.sq (WHO, 1990). Information on susceptibility of sandflies to various insecticides is limited. The only report of sandfly resistance to DDT has been from N.E. India where *P.papatasi* survived exposure to 4% DDT in WHO test kits (Kaul *et al.*, 1978; Joshi *et al.*, 1979).

Where sandflies are exophilic, or bite away from human habitation insecticide control is often not viable. Attempts to control non-peridomestic sandflies by spraying

the resting sites have not been encouraging as for example efforts made to control *P.orientalis* in Sudan (Turner *et al.*, 1965) and *Lutzomyia* in Panama (Chanotis *et al.*, 1982), and Brazil (Ready *et al.*, 1985) were not successful. The use of insecticides to control sandflies by spraying their resting and breeding sites in conjunction with reservoir control though claimed to be more effective does not always seem to be ideal. Thus in Central Asia although the focus of Zoonotic Cutaneous Leishmaniasis (ZCL) in Turkmenia (Latyshev and Kryukova, 1941) was successfully eliminated (incidence rate dropped from 70-0.4%) by treating all burrows of the great gerbil with chloropicrine the main resting and breeding sites of the vector, yet similar measures carried out in other foci of ZCL in Turkmenia were not as successful (Safjanova *et al.*, 1962). Attempts to control the same sandfly/reservoir association in Iran using DDT dust in rodent burrows and poisoned bait to kill gerbils within a 300 metres radius of villages were also unsuccessful in reducing the incidence of disease (Seyedi Rashti and Nadim, 1973).

### **1.3.2. Ecological control.**

An efficient means of control is the exploitation of the habitat concomitantly rendering it inhospitable to the sandfly. This type of intervention has been widely used in natural foci of ZCL in the Central Asian deserts. Successful control of sandflies has been achieved by eradicating their predominant habitats, the burrows of great gerbil (*Rhombomys opimus*) either by ploughing up the burrows as in Uzbekistan (Faizulin, 1980) or by crushing them with heavy machinery as in Turkmenia (Ustimenko *et al.*, 1979., Zhogolev *et al.*, 1979). Other environmental management measures include

removal of rubble and garbage, blocking crevices in walls, deforestation and urbanization. The methods are claimed to be relatively simple and economical. Their use, however requires a thorough preliminary survey, precise mapping of all sandfly breeding and resting sites and several follow-up evaluations of control.

### **1.3.3. Personal protection.**

Personal protection measures include the use of repellents and bednets. A limited number of field studies have been carried out on the efficacy of these methods against sandflies. Bednets such as those used against mosquitoes do not provide complete protection as they can be easily penetrated by the sandflies; fine bed nets therefore have to be used and they are uncomfortable to sleep under in tropical climates (Lane, 1993). Impregnated clothing is not always effective, for example, the trials carried out in Panama showed that permethrin impregnated jackets failed to provide protection against four species of *Lutzomyia* sandflies (Schreck *et al.*, 1982). The use of permethrin-impregnated nets and curtains however seem to be successful in certain places in providing protection against endophilic sandflies (Maroli & Lane, 1989; Majori *et al.*, 1989; Maroli & Majori, 1991).

Repellents like Diethyltoluamide, Chlorodiethylbenzamide, Dimethyl Phthalate when applied to the skin can provide partial protection against the sandfly bites for only a limited time (Safjanova, 1963; Schmidt & Schmidt, 1969) as they are frequently lost through perspiration, specially during manual labour. The efficacy of these compounds in tropical and humid areas is therefore doubtful.



#### **1.3.4. Biological and Genetic control.**

The biological control of sandflies involving the use of pathogens or predators is still in its infancy and needs to be explored further before such an approach can be practically used.

In conclusion, current methods of interrupting the transmission through vector control are inadequate and need to be developed further. The development of biological and especially genetic techniques and their application in conjunction with the already established methods would be of great significance for future vector control and the interruption of disease transmission. Under the present circumstances however, it seems that a combination of two methods is the most important for sandfly control at present: (i) a chemical method with an auxiliary ecological procedure to control anthroponotic and visceral zoonosis leishmaniasis and (ii) an ecological method with the auxiliary use of chemicals to control Zoonotic leishmaniasis, at least in the Old World.

### **1.4. LEISHMANIASIS IN THE ISLAMIC REPUBLIC OF PAKISTAN AND NEIGHBOURING COUNTRIES.**

#### **1.4.1. LEISHMANIASIS IN THE ISLAMIC REPUBLIC OF PAKISTAN.**

Three types of leishmaniasis are found in Pakistan. Zoonotic cutaneous leishmaniasis is mainly prevalent in the south-western region, anthroponotic cutaneous leishmaniasis in the central region and visceral leishmaniasis in the north-eastern region. The last form is mainly endemic in the two administrative districts. Northern Areas and Azad Jammu & Kashmir (Figure 1.1). The disease status up to 1986 has been

reviewed by Munir *et al.*, (1989).

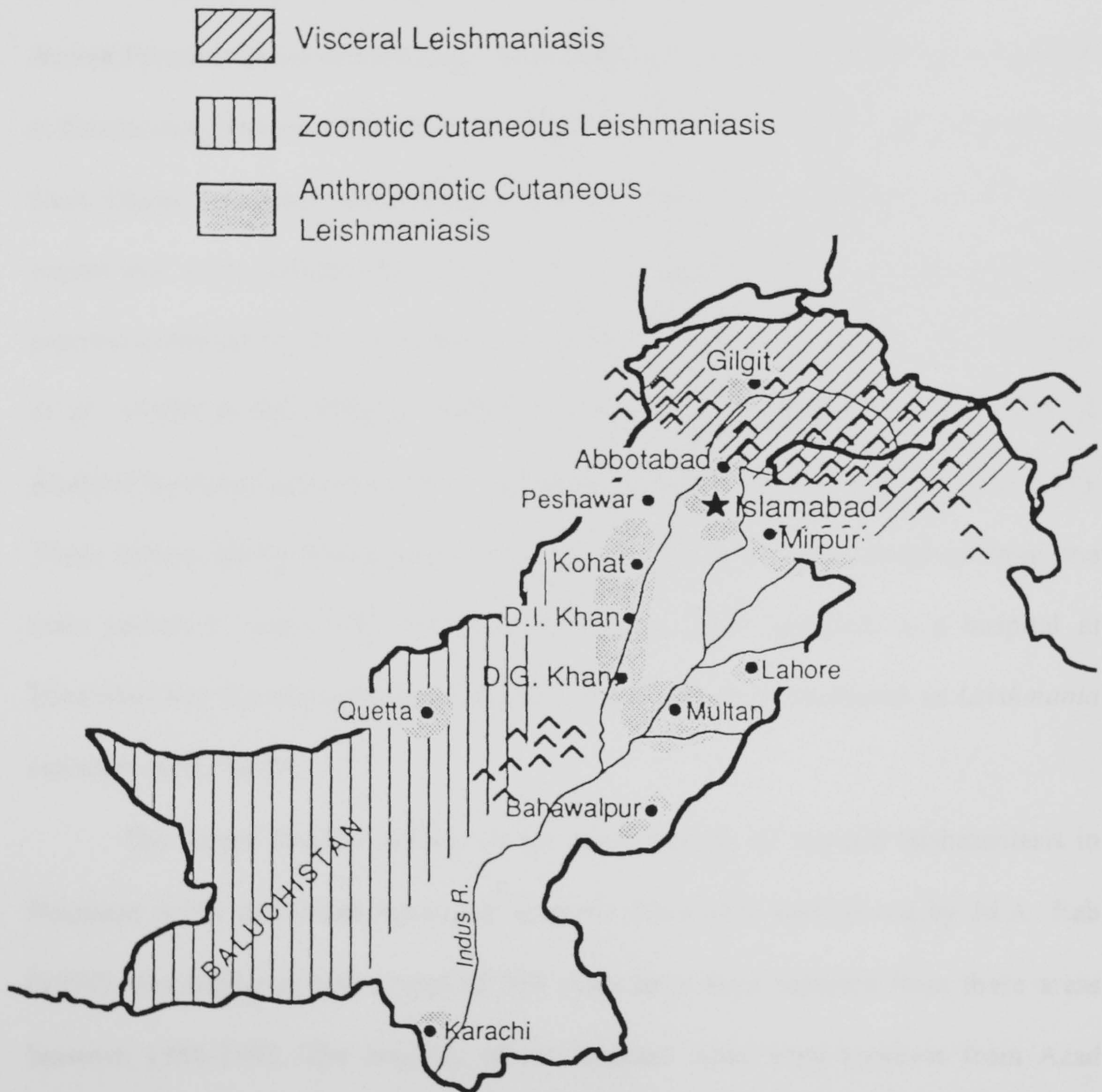
#### **1.4.1.1. VISCERAL LEISHMANIASIS.**

Visceral leishmaniasis was first reported in Pakistan from Baltistan by Ahmed *et al.* (1960). In 1962, Ahmed and Burney, published detailed accounts of their investigations in this area. According to the authors most of the cases came from Kuru, Gwadi and Keris villages situated in the Khapulu valley along the river Shyok at altitudes between 2300 and 2600 metres above sea level. A total of 23 patients were diagnosed clinically and on the basis of parasitological and serological findings. Serology was done using the complement fixation test and the aldehyde test. Most of the cases were children between 4-6 years age group.

As a result of the above survey intensive control measures were undertaken, involving DDT spraying and mass treatment of cases. The follow up study in 1964 did not reveal any cases. In 1974 new disease foci were discovered in the Kharmang valley and a total of 25 cases were recorded which were diagnosed on clinical, parasitological and serological (complement fixation test and indirect fluorescent antibody technique) grounds. The subsequent survey in 1975 revealed only 2 cases from Perkuta (Mehdiabad) and Ghasing in the Kharmong valley (Burney *et al.*, 1979). In 1979 once again an extensive survey of all Baltistan was undertaken and seroepidemiological assessments made but no cases were found (Burney *et al.*, 1981). Based on this survey it was thought that the disease had been eliminated, but the

Figure 1.1

# Geographical distribution of Leishmaniasis in Pakistan and AJK



picture changed when fresh cases of VL started appearing again in the early nineteen eighties, not only from Baltistan but also from Azad Jammu & Kashmir, an area previously not known to be endemic. As a response further studies were carried out.

Saleem *et al.*, (1986) published a record of 15 cases of VL diagnosed at the Armed Forces Institute of Pathology, Army Medical College, and the Military Hospital at Rawalpindi, between 1983-85. Nine cases were from Azad Jammu & Kashmir, two from Chilas, one from Abbottabad (NWFP) and three from Rawalpindi. All cases except one were children under 10 years of age. Diagnosis was confirmed on bone marrow examination. The subsequent seroepidemiological studies carried out by Rab *et al.*, (1989) in 280 children residing in the endemic villages of Azad Jammu & Kashmir however did not reveal a significant level of antibodies (*IFAT titre 1:40*). These authors also cultured parasites from a bone marrow biopsy obtained from one male patient (2 years old) from Murree (Rawalpindi) admitted in a hospital at Islamabad and the parasites were typed by isoenzyme characterization as *Leishmania infantum sensu stricto*.

The recent detailed studies on the epidemiology of visceral leishmaniasis in Northern Areas and Azad Jammu & Kashmir have been carried out by M.A. Rab (1994). According to him a total of 240 cases have been reported from these areas between 1984-1992. The majority of the reported cases were however from Azad Jammu & Kashmir. Between 1990-92 a further 15 isolates of *Leishmania* were made from humans; 11 from bone marrow, 3 from skin and 1 from spleen. All these isolates were identified on enzyme electrophoresis as *Leishmania infantum* (LON49, MON1).

### a) Vector studies.

The first attempts to study vector species were those of Ahmed *et al.*, (1960) and Ahmed and Burney (1962). They collected a total of 191 sandflies from Baltistan, of which 105 were from Gawadi and 86 were from Keris at an altitude of 2600 metres above sea level. These species were identified as *P.chinensis longiductus*, *P.major*, and *P.kandelakii*. The last species was subsequently described as a new subspecies by Lewis (1967), as *P.kandelakii burneyi*. All the flies recovered from patient's houses were dissected for natural infections but none was found positive. These remained the only vector studies from the visceral leishmaniasis foci until the present studies. It may be mentioned that *P.argentipes* has never been recorded from the visceral leishmaniasis areas in Pakistan. Nothing is known about the biology of phlebotomine sandflies in Pakistan.

### b) Reservoir studies.

Earlier studies carried out to discover the animal reservoir involved in the transmission of VL were those of Ahmed & Burney, (1962) and Rab *et al.*, (1989). None of the animals (dogs and rodents) examined in these two studies was found infected. Recently, Rab (1994) examined a large population of dogs from Azad Jammu & Kashmir, Chilas and Abbottabad and successfully cultured parasites from the lymph nodes of these animals. A total of four isolates were made and identified by enzyme electrophoresis as *Leishmania infantum* s.s (MON1, LON49) which is the same as found in humans.

#### 1.4.1.2. CUTANEOUS LEISHMANIASIS.

Both anthroponotic and zoonotic cutaneous leishmaniasis caused by *Leishmania tropica* and *Leishmania major* respectively, are endemic in Pakistan. Strain typing has not been done but the two types were differentiated on clinical grounds (dry or wet lesions). Recently however, *L.major* and *L.tropica* have been identified from patients with CL in different parts of Baluchistan (Personal communication from M.A. Shahid DESTO laboratories, Karachi).

##### a) Zoonotic cutaneous leishmaniasis.

The distribution records indicate the existence of zoonotic cutaneous leishmaniasis all over the country, being widespread over a large area in Baluchistan province. The information regarding CL in Baluchistan is summed up by Munir *et al.*, (1989). The incidence is high among children in the indigenous population, whereas the adults are generally immune as a result of contact with parasites during their childhood. The non-immune adults, including military personnel and labourers, are at a high risk of acquiring infections.

Nothing is known about the reservoir(s) and vector(s) of disease. On the basis of its proven status as vector elsewhere, (including Iranian Baluchistan) *P.papatasi* is strongly suspected as the vector of disease, although the vectorial role of *P.salehi* cannot be ruled out. A number of wild rodent species has been reported from Baluchistan which includes *Meriones spp.*, *Tatera spp.*, *Mus musculus* and the great desert gerbil *Rhombomys opimus* (Burney and Lari, 1986; Rab *et al.*, 1986)

### b) Anthroponotic cutaneous leishmaniasis.

ACL has been recorded mainly from urban localities, especially in southern Punjab. The disease is highly endemic in Multan (Burney and Lari, 1986). There was a sharp decline in the disease as a result of anti malaria spraying (mainly DDT) in the 1960's, but an increase in the number of cases has been noted more recently in North Western Frontier Province (NWFP) and Baluchistan and even around Islamabad and Rawalpindi (Rab, 1994). The main cause appears to be the mass migration of Afghan refugees from Afghanistan. Disease foci are also present in Gilgit town in Northern Areas and in parts of Azad Jammu & Kashmir. *Phlebotomus sergenti* is the most likely vector involved in disease transmission.

#### 1.4.1.3. SANDFLIES STUDIES IN PAKISTAN.

The first and only complete inventory of sandflies from Pakistan is that of Lewis (1967) which also includes general notes on the bionomics and the relation of sandflies to human disease. The following *Phlebotomus* species were identified, the names of which have been updated according to Lewis (1978).

<u>Sub genus</u>	<u>Species</u>
<i>Phlebotomus</i>	<i>papatasi</i>
<i>Paraphlebotomus</i>	<i>alexandri; nuri; sergenti</i>
<i>Larroussius</i>	<i>kandelakii burneyi , keshishiani; major major.</i>
<i>Adlerius</i>	<i>longiductus</i>
<i>Euphlebotomus</i>	<i>argentipes</i>

*Anaphlebotomus*

*colabaensis*.

In addition ten species of *Sergentomyia* were recorded.

## **1.4.2. VISCERAL LEISHMANIASIS IN COUNTRIES AROUND PAKISTAN.**

### **1.4.2.1. China**

#### **a) Disease history and present status.**

Kala azar has been reported from China since 1900. The disease was a major public health problem in the country before 1950, and was practically eradicated in 1951 following integrated control measures (Wang, 1985).

Based on various reviews (Chung, 1953; Wang & Wu, 1959; Zahar, 1980; Leng, 1982; 1988a Wang, 1985; Guan, 1991) the situation of kala azar in China can be summarised as follows: The disease was endemic in north China, in a band stretching from the east coast provinces of Liaoning, Hebei and Shandong westwards along the inner Mongolian border, Gansu province to Xinjiang Uyhur Autonomous Region sharing a border with Afghanistan and Russia. In east China the disease foci were located in low alluvial plains at an altitude less than 50 metres above sea level whereas in endemic areas in north-west China such as Gansu and Xinjiang disease was present at altitude above 1000 metres (up to 2300 metres in Gansu province) above sea level.

According to surveys made in 1951, the average rate of infection in different provinces was 10-50 per 10,000, and the estimated total patients were about 0.5 million. In east and north China the disease was mainly found in children between 5-10 years of age, whereas in north-west China children below 5 years of age were



mainly affected. As a result of control measures, the incidence of VL declined from 3500 cases per million in 1951, to less than 1 per million in 1961.

Nevertheless, cases reappeared again in 1968-1975 in the suburbs of Beijing and other endemic foci (Xu, 1988). During 1982-83 sporadic cases were reported from the mountainous and desert areas of the north-west, including Xinjiang, Gansu, northern Shaanxi, Nei Mongol, Shanxi and Sichuan (Wang *et al.*, 1983). A significant rise in incidence of disease was noted in the provinces of Sichuan and Gansu, from where a total of 1133 cases were recorded between 1985 and 1989 (Li and Guan 1990). Out of these, 75 percent of the children were under the age of 5 years and there were more males than females.

At present there are two epidemiological types of visceral leishmaniasis in China with three nosogeographical entities: the anthroponotic type which occurs in the plain as well as desert regions of eastern China, and the zoonotic type which is mainly endemic in the dry and cold mountainous and hilly regions of central and north-western China (Guan, 1991; Desjeux, 1991).

i) Anthroponotic form in the plain regions of eastern China.

Here VL is still under control and few cases have been notified since the 1960's. Sporadic residual cases have been reported since 1970. It was mainly a disease of humans and adults were mainly affected. Post kala-azar dermal leishmaniasis (PKDL) has been observed in some patients. *Phlebotomus chinensis* which is strictly peridomestic and endophilic in this area is the most likely vector.

ii) Anthroponotic form in the desert regions of eastern China.

In the reclaimed desert regions of eastern China sporadic cases have been

reported among the non immune population of migrants. In the dry desert region, 92% of cases were among children usually under 2 years of age with no adult case. Whereas in the stony desert 90% of cases have been reported from among children below 10 years along with a few adult cases. There is no evidence of PKDL.

In the dry desert region of Ejne Banner, Inner Mongolia, only sporadic cases have been reported and the epidemiological situation is not completely known. Lympho-glandular leishmaniasis has been reported to occur frequently in adults migrating to this area from non-endemic zones. Regarding vectors, *P.wui* (exophilic) is considered to be the suspected vector in the dry desert region and *P.alexandri* (exophilic) in the stony desert region. Two isolates from *P. alexandri* were identified by monoclonal antibodies and more recently by isoenzyme analysis as *L.donovani*. No animal reservoir host has so far been identified.

### iii) Zoonotic VL.

This form occurs mainly in the hilly and mountainous endemic areas of central and north-western China where cases have been described at an altitude of 2,334 metres above sea level. The disease is common in young children, as most of the cases were children under 10 years. In endemic mountainous regions in north-west China, children below 5 years are mainly affected (Chung and Chang, 1986). *Phlebotomus chinensis* is the main sandfly vector. Resurgence of VL in these areas is considered to be due to the fact that *P.chinensis* is partly sylvatic and exophilic and therefore not effectively controlled. Most of the reported cases are from Gansu province (200 cases/year since 1985) and children below 5 years of age are mainly affected. Results of leishmanin skin tests predict the continuity of transmission (Positive among all age

groups). Several isolates from dogs and patients have been typed as *Leishmania infantum* (Desjeux, 1991).

In recent years new infections have occurred in the deserts of Xinjiang and western Inner Mongolia. In these foci infected animal reservoir hosts have not yet been identified (Guan, 1991).

b) Sandfly vectors.

A total of 31 species *Phlebotomus*, and 19 species of *Sergentomyia*, have been recorded from China (Wang, 1985).

*Phlebotomus chinensis*, *P.longiductus*, *P.wui* and *P.alexandri* have been incriminated as the vectors of visceral leishmaniasis in various parts of China.

*Phlebotomus chinensis* is the main species in all endemic foci except the autonomous region of Xinjiang and inner Mongolia (Chung, 1953). Natural infection of these flies with flagellates of *Leishmania* has been known since 1936. Experimental studies with hamsters also indicated high infection rates in this species (Hoeppli, 1940).

In east and north-east China adult activity commences in the mid or latter half of May, reaching a peak in the mid or later half of June, and completely disappearing in the second half of August (Zahar, 1980). In north-west China the peak activity is in the mid or later half of July. According to Wang (1985) in east China *P.chinensis* is mainly anthropophilic and usually found in human dwellings, whereas in hilly mountainous areas it becomes a wild or semi-wild, chiefly zoophilic species, occasionally feeding on man.

Recent reports of natural infections of *P. chinensis* with *Leishmania donovani* are from the mountainous regions at an elevation of 2200 metres (MOPH, 1989). This species has been reported as the sole vector of disease in Gansu and Sichuan provinces (Li and Guan 1990).

The vector *Phlebotomus longiductus* is the only widely distributed species in Xinjiang province in domestic and peridomestic situations feeding both on cattle and man. In the deserts of Xinjiang and inner Mongolia, *P. wui* has been implicated as the major vector of disease. The sandfly is inactive at temperatures below 16°C (Hsiung

*et al.*, 1979). Its activity extends from early May to late September with two peaks, one in June and the other in August. Being a wild species it seldom enters houses except at night and rests and breeds in the burrows and lairs of wild animals. In nature, 1.7-6.0 percent of the flies have been found infected (Wang, 1985). The sandfly fauna of Xinjiang is of particular importance, because this province borders northern Pakistan in the north west. *Phlebotomus alexandri* is the main vector in the stony desert region of east China but the only information on the bionomics of this fly comes from the study made by Guang-Hua *et al.*, (1963) in Kansu province. The sandfly season occurred from early June to the later part of August or the middle of September. The highest peak occurred within the first 10 days of July.

The sandfly fauna of the Aksu region of Xinjiang revealed differences in four landscape zones. In mountainous areas covered with brown calcareous soil and in an ancient area with whitish oasis soil, *P. longiductus* represented 91.1% and 92.5% of the sandfly population. In the stony desert area with brown desert soil, *P. alexandri* accounted for 91.5% of the catches. In the dry desert area covered with scrubby

meadow soil, *P.wui* represented 60.1% of the sandflies and neither *P.chinensis* nor *P.alexandri* were found in this area. Based on the results of experimental infections of these flies and the natural infection of *P.alexandri* all were implicated as vectors of Kala-azar in Aksu region (Guan *et al.*, 1986a).

#### c) Reservoir studies.

Based on information from various reviews (Chung, 1953; Zahar, 1980; Leng, 1982; Wang, 1985), canine infection was mainly prevalent in the hilly mountainous regions of the northern parts of China, particularly in the north west and north east. Of 139,095 dogs examined between 1951 and 1959, the infection rate varied from 14.4- 204 per 10,000 (Wang, 1985). The data reported by various authors on the rates of infections in dogs has been summarized by Xu (1988).

A large number of wild animals including rodents and carnivores has been examined from north-west China, but none showed *Leishmania* infection, except *Rhombomys opimus* from which a non pathogenic species, *Leishmania gerbilli* was isolated (Wang, *et al.*, 1983 ). Recently a racoon dog *Nyctereutes procyonoides* from the Beijing suburbs was found infected (Xu, 1982) and the parasites subsequently identified as *Leishmania infantum* by isoenzyme electrophoresis (Xu *et al.*, 1984; Xu, 1988).

#### 1.4.2.2. Islamic Republic of Iran.

Visceral leishmaniasis unlike cutaneous leishmaniasis is not common in Iran (Nadim *et al.*, 1988). The disease occurs sporadically throughout the country, except in the southern part of Baluchistan, neighbouring Pakistan where it is absent. Majority

of cases are from mountainous areas in the south west.

a) History and present status.

The status of VL in Iran has been comprehensively reviewed (Nadim *et al.*, 1978; Zahar, 1980). A total of 120 cases of VL were reported in Iran up to 1978 infecting nomads from the south-western part of the country. The disease is most common in children below 10 years of age and in males. Nasab and Shirazi (1980) analyzed the clinical presentations of these patients, and reported a considerably high mortality rate in infants. The death rate was found to be 30.5%.

Currently, visceral leishmaniasis is mostly endemic in the provinces of Fars in the south (< 50 cases/Yr) and the districts of Meshkinshahr and Moghan in the south west (< 20 cases/Yr) (Nadim, 1988). Most of the cases in the Meskinshahr focus are children under 9 years old. Parasite isolates have not been typed either from humans or animals, however on epidemiological investigations the disease seems to be zoonotic and the parasite<sup>is</sup> probably *Leishmania infantum*.

b) Sandfly vectors.

The sandfly fauna of Iran has been thoroughly studied (Adler *et al.*, 1930; Pervomaiski, 1948; Lewis, 1957; Lewis *et al.*, 1961; Theodor & Mesghali, 1964; Nadim *et al.*, 1971; Javadian & Mesghali, 1975 ). A check list of 20 *Phlebotomus* and 14 *Sergentomyia* species from Iran is given by Javadian and Mesghali (1975).

Species recorded in areas where cases of visceral leishmaniasis were reported include: *P. major*, *P. halepensis*, *P. chinensis brevis*, *P. kandelakii*, *P. keshishiani*, *P. tobbi*

and *P.wenyoni* (Nadim, 1978). Natural promastigote infections were not seen but *P.major* has been strongly suspected as the vector in the infected areas because its distribution coincided with that of the human cases and also this species was found in the same locality in the Caspian area where a confirmed case of VL was detected and an infection in a jackal was discovered in 1979. However, this does not preclude the possibility of other *Phlebotomus* species belonging to subgenera *Adlerius* or *Larrousius* (especially *P.halepensis*) being involved in disease transmission. Nadim (1988) mentions the possible role of *P.alexandri* as a vector of VL in Khuzistan. Studies on vector bionomics from VL foci are not available.

c) Reservoir studies.

The animal reservoir seems to be wild and domestic Canidae: jackals, foxes and dogs have been found infected in various parts of Iran (Nadim, 1988).

Out of a total of 20 jackals and 10 foxes shot, parasites were detected in bone marrow and spleen smears of one jackal (*Canis aureus*) in the eastern part of the Caspian area and in a fox (*Vulpes vulpes*) in the north eastern part of the country. Based on the fact that these animals were shot far from any CL area, the author assumed that infection in both the animals was due to *Leishmania infantum* (Nadim *et al.*, 1978). Following this, Hamidi *et al.*, (1982) examined 161 jackals and 100 dogs from northern Iran. Parasitological examination of smears from bone marrow, liver, spleen and skin of these animals, revealed amastigotes in 4 jackals and 3 dogs. Serological (IFA) tests gave an antibody titre of 1:160 in 6 jackals (48 tested) and, 1:320 in 6 dogs (34 tested).

### 1.4.2.3. Jammu Kashmir (India).

Information on the prevalence of visceral leishmaniasis in Jammu and Kashmir are scarce. The only reports available are those of Jacob and Kalra (1951) who found 12 cases of VL among army personnel and Khan *et al.*, (1975) who recorded two VL cases from local people.

#### a) Sandfly fauna.

Jacob and Kalra (1951) captured sandflies from houses and animal quarters, using aspirators. Nine species were collected including four *Phlebotomus* species. These, in order of abundance, are:

<i>Phlebotomus major:</i>	Recorded in four villages of Jammu and 11 villages of Kashmir at an altitude of 1100-6800 feet(320-2100m.)
<i>Phlebotomus papatasi:</i>	Recorded in four villages of Jammu and five villages of Kashmir at an altitude of 2200-5400ft.(160-1640m.).
<i>Phlebotomus sergenti:</i>	Recorded from one village in Jammu and four villages in Kashmir at an altitude of 5600- 10500 ft.(1700-3200m.).
<i>Phlebotomus chinensis:</i>	Recorded from one village in Jammu and three villages in Kashmir at an altitude of 5600-10000 ft.(1700-3000m.).

The adults are found from April to October, with a peak in July and August.



Kaul and Shetty (1983) surveyed three districts of Jammu region, during July and August, and captured 191 sandflies belonging to the genus *Sergentomyia* only. However, they compiled a revised check list of sandflies based on the studies of Jacob and Kalra, 1951; Lewis, 1978; Mitra, 1951; Kulkarni, 1978. and Mitra, 1953). Following is the regional record of the species belonging to the genus *Phlebotomus*:

Region	Altitude(metres)	Sandfly Species
Jammu	314-1830	<i>P. (Adlerius) longiductus</i> , <i>P.(Larroussius) major major</i> , <i>P.(Phlebotomus) papatasi</i> , <i>P.(Paraphlebotomus) sergenti</i> .
Kashmir	1380-2100	<i>P. longiductus</i> , <i>P. major</i> , <i>P. papatasi</i> , <i>P. sergenti</i> .
Laddakh	2740-3200	<i>P. longiductus</i> , <i>P. sergenti</i> .

No information is available on either disease, vector(s) or reservoir(s) in the area.

#### 1.4.2.4. Afghanistan.

Visceral leishmaniasis, unlike cutaneous leishmaniasis, is not very common in Afghanistan. A total of 21 cases of VL have so far been being diagnosed from different areas of Afghanistan (Desjeux, 1991). However, nothing is available in the literature on the origin of these cases and reservoirs. Detailed studies on the vectors of disease and information on their biology is also lacking. Phlebotomine sandflies from different areas of Afghanistan have however been identified and listed, but these efforts were only to solve taxonomic problems rather than search for disease vectors. The sandfly fauna has been described by Artemiev (1974a,b, 1976a,b; 1978)

According to the updated list of the sandfly fauna of Afghanistan (Artemiev, 1978) a total of 42 sandfly species belonging to genus *Phlebotomus* and *Sergentomyia* have been recorded. The maximum number of species was in the subgenera *Phlebotomus* (*Adlerius*). A detailed list of species is given in chapter 5 (Table 5.1) of this thesis.

## CHAPTER 2

### OBJECTIVES.

The general objective of this study was to determine the vector(s) of visceral leishmaniasis in northern Pakistan.

**More specifically :** to determine

1. the sandfly fauna of the disease foci, and the exact identification of the *Phlebotomus* species and their distribution.
2. the seasonal and relative abundance of the *Phlebotomus* species.
3. which species bite humans.
4. which species are naturally infected with *Leishmania*.
5. which sandflies bite potential reservoir hosts.

## CHAPTER 3

### DESCRIPTION OF STUDY AREAS.

Sandfly surveys were made throughout northern Pakistan but most of the entomological studies were confined to two areas where visceral leishmaniasis occurs: Northern areas of Pakistan (Diamir district, Chilas area with Hudur and Thor villages) and Azad Jammu and Kashmir (Bagh 1991 and November 1991 and March 1992 and November 1992. The following is a brief description of the two foci:

#### 3.1. GENERAL DESCRIPTION.

##### 3.1.1. NORTHERN AREAS OF PAKISTAN.

The Northern Areas cover a total area of 72,496 square kilometres with an estimated population of 573,724 (Census Report, 1981). The population density is calculated to be 21 persons per square kilometre.

The region lies between 71° E longitude and 32° and 37° N latitude. It is bounded on the north-west by the Chinese Xinjiang province and Afghan Pamir with the former Soviet Union close behind it. On the southern side is the Indian-held Kashmir and Laddakh (Figure 3.1). The area is administratively divided into five districts; Gilgit, Ghizar, Diamir (Chilas) Skardu and Ghanche. The last two form part of Baltistan (Figure 3.1).

Towering mountains with snow clad peaks including Nanga Parbat (8190m third highest peak in the World), deep river beds and narrow valleys are characteristic of this region. The three most important mountain ranges of Central Asia, namely the north-western Himalayas, the Karakorum and the Hindukush meet here. Of these

FIGURE 3.1

Map showing the Northern Areas of Pakistan



mountain ranges the major part of the Karakorum lies within Pakistan and form a link between the northern Himalayas in the east and the Hindukush and Pamir in the west. In Pakistan, the northern boundary of the Himalayas constitutes the hill ranges of Kashmir, Hazara and across the river Indus, includes hills of the eastern Swat and Peshawar valleys.

The climate changes abruptly with elevation of the land. At an altitude between 800-1200 metres, the climate is warm and subtropical whereas it is cool and temperate between 1200-2400 metres, turning to cold and temperate between the altitudes of 2400-3660 metres. Beyond 3600 metres, the climate is mainly arctic. The mean monthly temperatures and humidity and rainfall in some areas are shown in figure 6.3, 6.7 & 6.12. Average annual precipitation (Fig 6.14) is low and the maximum precipitation recorded is 30mm.

Although the mighty Indus flows through the region, it is not of much direct help to the local population except for rare places such as Skardu and Chilas where living on the Indus banks is possible. People are therefore confined to the narrow valleys and mountain slopes where glacial water is at hand for drinking and irrigation. Although the large population depends upon agriculture, suitable land is very limited.

Families are large and in winter most of the members stay in the village, but in summer a large number of people migrate with their flocks of goats, sheep and cattle, to higher altitudes, where green pastures are readily available following the melting snow. This migration lasts for nearly five months. They gradually return with the onset of winter to join their family members left behind. The course of such migration is well defined in the entire region.

Traditionally, local people burn wood in houses as their main source of fuel and wood is also used in construction. A good share of the timber revenue is indeed paid to the local population and divided according to ancient rules; meadows and forests traditionally being considered as a common property. Rapid depletion of these forests is therefore happening which is not compensated by an equivalent expansion of agricultural land.

In all the upper reaches of the Indus, narrow alluvial fans spill down the occasional cracks in the mountain rocks. Good soil is rare here and perched on these mud platforms the villages have to fight an endless battle against erosion by wind and climate. Appalling storms are common. Topographical elements such as steep slopes, glaciers, weathered rocks and deep gorges are some of the natural hazards responsible for the slow development in these areas along with deforestation and lack of education.

#### **i) Vegetation.**

At lower elevations scrub forest consists of broad leaved vegetation, mainly Oak (*Crucis hylex*), Willow, Poplar and crus (bunni). Occasionally pine trees are found, as are Chinar, Walnut and wild olives. At higher elevations forests of tall pine and Deodar trees grow at height between 2000-3000 metres. Between 4000-5000 metres the vegetation is alpine scrub, comprised of grass and numerous small plants. The vales in between the mountains are rich in fruit trees, such as apricot, almond, walnut, chestnut, mulberry, peaches and grapes.

### 3.1.2. AZAD JAMMU AND KASHMIR.

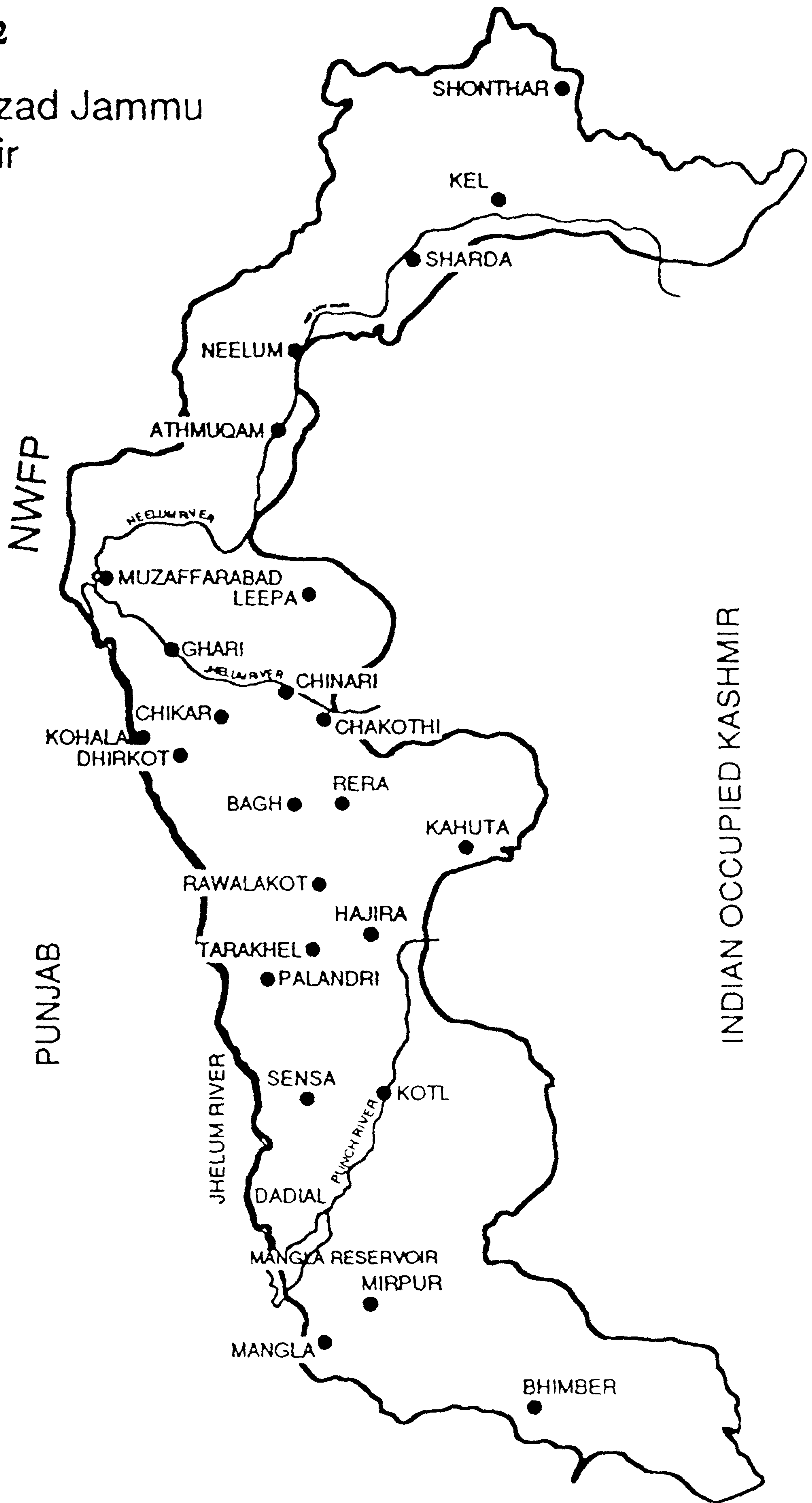
Kashmir's first appearance on the earth surface was in the form of a volcanic island 100 million years ago. It passed through various evolutionary phases and finally during the Eocene age both Kashmir and Tibet became very dry following the slow emergence of the Himalayas as the peninsular tectonic plate moved northwards into the main asian plate.

The Azad Jammu and Kashmir was formerly part of the State of Jammu and Kashmir. Its total area is estimated to be 5134 square kilometres. The region lies between longitude 73-75° and latitude 33-36° and is sandwiched between Pakistan on the west and the Kashmir valley on the east (Figure 3.2). The southern part, except for the plains of Punjab, mainly comprised of sub-himalayan Siwalik foothills of hilly terrain between 1500-2600 metres above sea level. In the north it borders Northern Areas. The topography is strictly mountainous with valleys and plains at some places. Azad Kashmir has different climates. The north and north-western mountains are extremely cold in winter while the summer months of April to September are very pleasant. On the other hand, the plains of Mirpur, Kotly and Bhimber are extremely hot in summer and cold and dry in winter. The monsoon starts from July and ends by the end of September. Average rainfall is approximately 134 mm. and floods are not uncommon. Heavy rains cause floods and land slides every year, many main bridges connecting this area with Pakistan are seriously damaged and it becomes difficult to approach this area. The area was under heavy floods during part of this study (August and September 1992) due to the heavy rains and appalling storms numerable pine trees were dislodged from the hill tops and the sliding logs



**FIGURE 3.2**

**Map of Azad Jammu & Kashmir**



took away with them many precious lives and dismantled many houses, roads as well as important bridges.

The main rivers are the Jhelum and Neelum. The Jhelum river comes from Verinog and the Neelum from the upper areas of the Neelum Valley (Indian held Kashmir). There is another small river called the Poonch river which flows through Kotli district and also comes from the mountains of Indian held Kashmir and joins the river Jhelum at Mangla.

Azad Kashmir comprises five administrative districts namely Muzaffarabad, Poonch, Kotli, Mirpur and Bagh. The population, according to the estimates of the Expanded programme of Immunization (EPI) of Pakistan, were 2,653,698 in 1992. The literacy rate is 28% and about 90% of the population is rural. The area agricultural economy mainly depends upon seasonal rains with maize, wheat and rice as the main crops. According to the Agriculture Department, the total area under crops is 591,067 acres. Apples, apricots, peaches, walnuts, plums, pears, cherries, strawberries, bers and citrus are grown in different parts of the area.

### **i) Vegetation**

The region is blessed with valuable forests covering 553297 hectares which is 42% of the total area of Kashmir and 3% of Pakistan. Forests are rich in high quality timber, wild life, medicinal plants and green pastures. Of the trees, Deodar (*Cedrus libanis*) is the most valuable species: it grows between 1500-2700 metres. Between 1800-3000 metres, blue pine is abundant and above 3000 metres dwarf rhododendrons and junipers are found. *Pinus longifolia* can be found between 500-

1700 metres, but not above it. Besides there are many other local trees at lower elevations.

### **3.2. Wild life of Northern Pakistan.**

In Northern Pakistan some of the rarest species of wildlife exists. The alpine pastures at heights between 3500-5000m are inhabited by the Snow Leopard (*Panthera unica*), the Himalayan Ibex (*Capra ibex*) and the Musk deer. Below these elevations live the Markhor (wild goat) and two species of bears. The Brown bear (*Ursus arctos*) is mainly seen in the alpine meadows and the sub alpine scrub zones whereas the black bear (*Selenarctos thibetanus*) inhabits the hill sides. In AJK another species of leopard, *Panthera pardus*, is found mainly at lower altitudes and monkeys (*Macaca mukulata*) are also common. Among rare avian species are the snow partridge, *Lerwa lerwa* and the snowcock, *Tetraogallus himalayansis*, (Ram Chakor). Both species are found at heights above 3000 metres.

### **3.3. Local languages in Northern Areas and Azad Jammu & Kashmir.**

Two languages are commonly spoken in Northern Areas: Shina and Burushaki. Shina is the main language in Gilgit, Ghizar and Diamir districts whereas Burushaki is mainly spoken in Hunza and Nagar valleys. In Azad Jammu & Kashmir: Kashmiri, Punjabi and Urdu are the main languages spoken.

### **3.4. DESCRIPTION OF VILLAGES SELECTED FOR LONGITUDINAL ENTOMOLOGICAL STUDIES IN NORTHERN AREAS AND AZAD JAMMU & KASHMIR.**

Based on the evidence of reported cases of visceral leishmaniasis in recent years, two main study areas were selected. One was the district of Chilas in Northern Areas and other Bagh district in Azad Jammu & Kashmir.

#### **3.4.1. NORTHERN AREAS.**

##### **3.4.1.1. Chilas district.**

Chilas, which is also the headquarters of the Dyamir district is situated on the upper reaches of the river Indus, under the shadow of the world famous Nanga Parbat. Dyamir was actually the name given to the Nanga Parbat Peak by the old people meaning heavenly mount. The Dyamir district includes the Tehsils of Tangir, Darel and Astore.

The town of Chilas lies at 74° 1' E and 35° 4' N at a distance of about 590 kilometres from Islamabad and 130 kilometres south of Gilgit along the Karakorum highway on the right bank of river Indus at a height of about 1200 metres above sea level. The district includes the sub divisions of Darel and Tangir. To the west and south of Chilas is the Kohistan district of NWFP. The area is characterized by a rugged terrain and high mountains. Human habitation has only been possible because of the wide dry soil slopes locally called Das. In between the intrusive ranges, which are broken by flood torrents, the Das spreads for miles and miles, making several terraces of the receding river Indus. As the area is closed by the high mountains on the north and the south, Chilas is cut off from the rains, making the area extremely dry (mean

annual precipitation being 14.4mm.), hot (maximum temperature 39.6°C) in the summer and cold in winter (0.6°C) (Figures 6.2 & 6.4). A strong westerly wind blows in the afternoons from the direction of Kohistan and as a result river sand is blown, lifted and spread over the soil as a thin layer giving the appearance of a vast desert. It is these sandy deposits that meet the eye every where.

The hilly ranges around Chilas are a part of territory uplift that threw up the Himalaya to their present height, enclosing many older rocks. The outer layers of these hills is granite which have numerous faults and hollows created by earth movements.

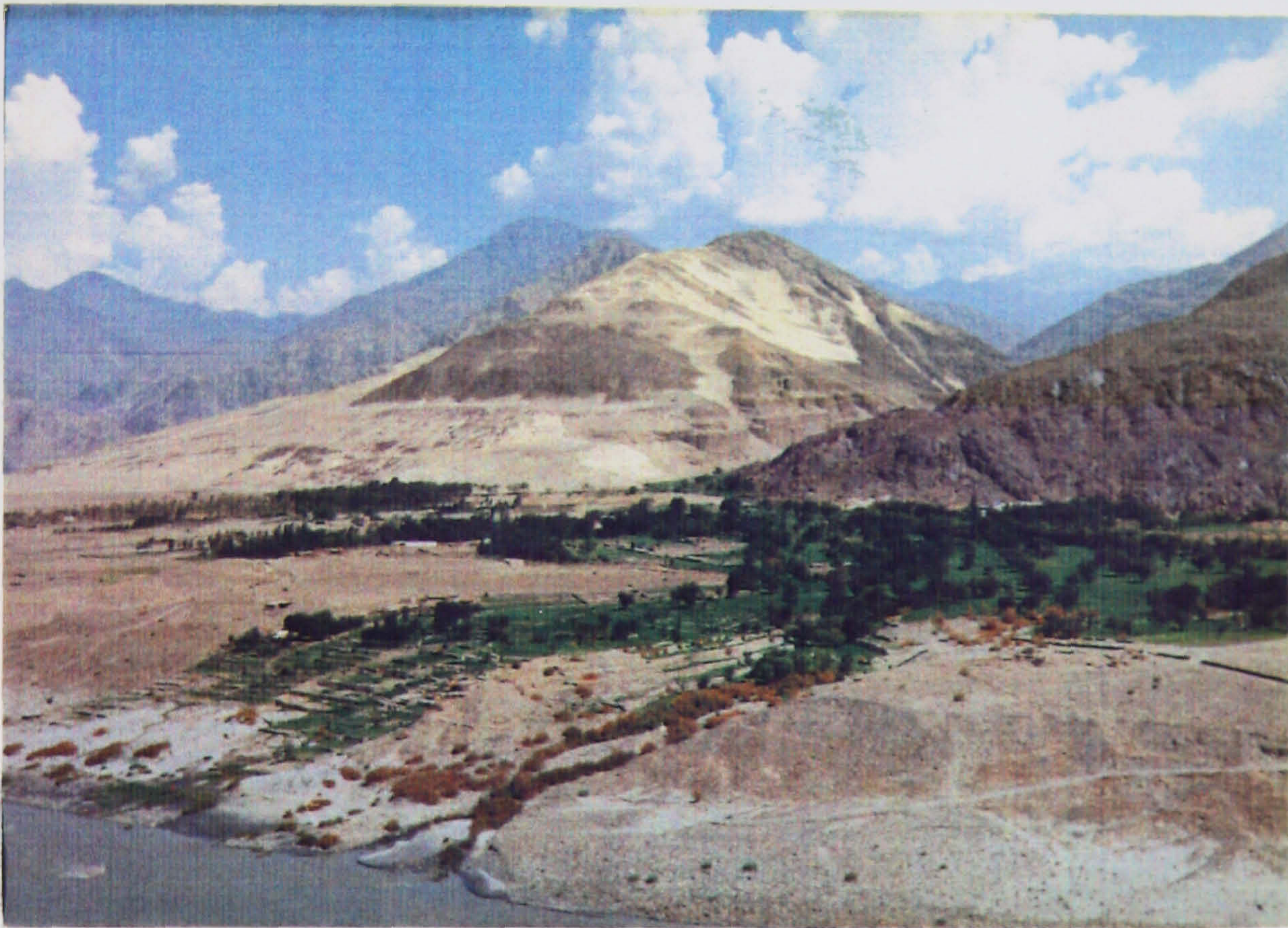
Within Chilas, two sites were selected for detailed studies. Hudur village (for longitudinal sampling) and Thor village (summer sampling only).

#### **i) Hudur village.**

Hudur village is situated on the right bank of the river Indus at a distance of about 15 kilometres west of Chilas town, at an altitude of 1200 metres above sea level (Figure 3.3). It spreads east-ward up a narrow valley for miles, alongside a glacier stream known locally as Hudur Nala, partly cultivated and partly barren. A modern suspension bridge connects the village with the main Karakorum highway. On either side of the village are big mountains of the Karakorum range. The terrain is rough and rugged and the soil has a characteristic sandy layer, accumulated from the strong winds during the summer. Water from Hudur Nala is used for drinking and irrigation.

The total population of Hudur is about 6000. The literacy rate is extremely low. All are Suni muslims, strictly religious and conservative; the women observe strict purdah and this makes studies in houses very difficult. Generally the people are

**Figure 3.3**



**Hudur valley: A view of the hudur valley situated on the right bank of river Indus. In the back ground are the Karakorum mountains.**

tough and hard workers. Polo is played and watched with great interest here and people are also good hunters.

A considerable percentage of the people live a nomadic life. Since the area is extremely hot in summer which dries out the vegetation, the majority of the population therefore move to high pastures as along with their animals by the end of May, leaving behind a few individuals to look after the crops. The people then return to the low land by the middle to the end of September. Domestic animals include cattle, sheep, goats, donkeys, mules, horses, and dogs. Buffalo are rare and dogs are mainly kept as guard dogs.

The houses are constructed from stone and mud and therefore usually dark and dingy, they are surrounded by vegetation. Animals are usually kept close to the living quarters.

Maize is the main crop but wheat and animal fodder is also planted. Wheat is sown by the end of November and harvested by the third week of May, then maize is sown before the end of June and harvested by the end of October. The animal fodder, locally called shaftal, is sown as a supplementary crop along with maize. Vegetables are also grown locally at a subsistence level and fruits are abundant. The main fruits include: mulberry (May), apricots (end of May-early June), peaches and plums (early June), grapes (late July-August), almonds (end August), walnut (September) and apples (December).

## **ii) Thor Village.**

Thor village is about 40 kilometres from the main town of Chilas on the left

bank of river Indus, and is a 'highland' village at an altitude of about 1700 metres (Figure 3.4). People live along the stream which comes from a glacier. The total population of the entire Thor valley is estimated at 9000. The climate is cooler here compared to Hudur, especially at nights, although people do live higher than 1700 metres for logistic reasons it was not possible to sample the area above this height. The villagers are mainly farmers, and only a small percentage of them are involved in non-agricultural activities. The general life style is the same as described for Hudur village. The area is surrounded by high mountains with a rugged terrain and vegetation is sparse. Important trees are pines, walnut and buni. The main crops are maize, wheat and animal fodder, and fruits are abundant.

The houses are constructed from mud and rocks and are surrounded by vegetation. Despite the altitude, living rooms have windows. Animals are kept close to human quarters, and include sheep, goats, donkeys, cattle, poultry and dogs. Buffalo are rare .

### **3.4.2. AZAD JAMMU & KASHMIR.**

#### **3.4.2.1 Bagh District.**

Bagh district of Azad Kashmir is about 250 km. east of Islamabad to which it is connected by the Kohala bridge over the river Jhelum. The district is about 1200 metres above sea level and is moderately warm in summer (mean maximum temperature 32°C) and cold (0°C) in winter with occasional snow (Figures 6.12). The area is green and fertile with much vegetation (Figure 3.5). According to the census report of 1981 the total population of Bagh District was 228,405. Within this district,



**Figure 3.4**



**Thor valley: A view of Thor valley. This picture shows the study site at an altitude of 1700m. The valley is at a distance of about 25 kilometres west of Chilas. In the background are the western Himalayas.**

Figure 3.5



A view of a typical village in Azad Jammu & Kashmir in the Bagh district. The background hills represent the sub Himalayan range. The area is covered with dense green vegetation and rains are abundant.

Rehra village was selected because recent cases of visceral leishmaniasis were reported to Islamabad for treatment.

**i) Rehra village.**

The village is about 15 kilometres east of the main town of Bagh and is densely forested (Figure 3.5). Houses are distributed all over the rugged hills and the majority of houses are constructed from mud, stones and wood. Some of the houses have wooden roofs while others only thatched roofs. Almost all rooms are provided with windows. Animals are usually kept close to human quarters and include cattle, buffalo, sheep, goats, donkeys, mules, poultry and dogs. Although a large percentage of the dogs in the area are strays, a few are kept as guard dogs or pets.

A large stream passes through the village from which water is raised for irrigation. Spring water is mainly utilized as drinking water. The land nearest to the stream is used to grow rice. The surrounding hills have been terraced and planted with maize, wheat, rice with some oats and animal fodder. Maize is sown mid April-early May and harvested by the middle to the end of September. Animal fodder is also harvested along with the maize. Rice is sown by early April and harvested by the end of August and wheat is sown by the end of October and harvested by the end of November.

The total population of the union council (Sawan) is estimated to be 10217 (According to 1990 figures obtained from District Health Officer, Bagh).

### 3.5. DESCRIPTION OF SITES SELECTED FOR THE GENERAL SURVEY.

In addition to the longitudinal studies a general survey was also conducted to sample the sandfly fauna at higher elevations in Northern Areas and Azad Jammu & Kashmir.

#### a) Northern Areas.

In Northern Areas a general survey was conducted in the Khaphlu (Kuru & Keris villages) and Kharmang (Mehdiabad village) valleys in Baltistan. Cases of visceral leishmaniasis in Pakistan were first recorded from these valleys.

Baltistan is also known as Tibet-i-Khurd or little Tibet. The area is surrounded by high mountains of the Karakorum range. The principal town of Skardu lies alongside the river Indus at an altitude of 2400 metres above sea level. There are four main valleys in Baltistan. West of Skardu, along the river Indus is the Rondu valley and in the east along the Shyok river is the Shyok valley. In the north is the famous Shigar valley which is the gateway to the second highest peak in the world K-2. In the south-east is the Kharmang valley which leads into Ladakh valley in India. Most of the villages are situated at the junctions of rivers and mountain streams. The area has long winters from October to April and the real summer period is from June to mid August.

Principal crops in the area include wheat, millet and rapeseed. Fruit trees of mulberry, apricots, peaches, plums, apples, walnuts and almonds are abundant here. Grapes of varied types are plentiful in the lower valleys.

Conifer forests of Chir pine abound in Baltistan along the streams of Basho

and Rondu. Musk willow is abundant in Shigar and Skardu and junipers are also found along side streams.

All the three villages surveyed, Keris, Kuru and Mehdiabad are at an altitude of between 2300-2600 metres. The houses are mainly made of mud and stones and are dark and dingy not necessarily surrounded by vegetation. Usually the living quarters are raised from the ground level, the ground floor being used for domestic animals (Figure 3.6, 3.7). The area is extremely dry (Figure 3.14) with little rainfall and the humidity hardly rises beyond 45-50%. Dogs are rare in this area.

#### **b) Azad Jammu & Kashmir.**

For the general survey three villages were selected at elevations more than 1500 metres above sea level. Two villages (Gali Malot & Bees Bagla) were in the district Bagh and one village (Gam) was in Poonch district. Detailed area descriptions of the area have already been given above. Brief descriptions of the villages is as follows.

Gali-Malot (Figure 3.8) and Bees Bagla are situated at a distance of 20 kilometres north of Bagh at an altitude of 1700 metres and 1890 metres above sea level. Compared to lower elevations, temperature is low here in summer whereas winters are extremely cold with a heavy snowfall. House construction is typically as in rest of the Kashmir and the houses are scattered over the hills. Domestic animals include cattle, goats and buffalo and a large number of dogs. Animals are mainly kept very close to human habitations sometimes in adjacent rooms. The total population of Gali-Malot is estimated to be 15563 individuals (1990, figures, obtained from the

Figure 3.6



This picture shows Kuru village (Ghanche district) in Baltistan from where visceral leishmaniasis was first recorded. The village is at an altitude of 2550m.

Figure 3.7



A view of Mehdiabad village (previously known as Perkuta) in Baltistan. *Phlebotomus kandelakii burneyi* was first recorded from this village (Lewis, 1967).

Figure 3.8



Another view of habitations at higher altitudes in AJK. A view of Gali Malot in Bagh district at an altitude of 1690 m. This is the type locality of *Phlebotomus (Larroussius) sp.A*.



office of the District Health Officer, Bagh).

Village Gam in Banjosa (Figure, 3.9) is situated at a distance of 20 kilometres southeast of Rawlakot tahsil of Poonch district. The village is at an altitude of 1700 metres and houses are scattered between 1700 and 1850 metres. The total population of the village is 7115 (according to the estimates of the Assistant District Health Officer, Rawlakot). The climate, vegetation and house construction are the same as seen in Gali Malot. Dogs are abundant and other domestic animals including buffalo, cattle, sheep and goats are also kept close to human habitations.

Figure 3.9



This picture shows Banjosa village of Rawlakot district in Azad Jammu & Kashmir at an elevation of 1800m. A naturally infected sandfly, *Phlebotomus hindustanicus*, with promastigotes of *Leishmania infantum* was caught here.

## CHAPTER 4

### MATERIALS AND METHODS

#### 4.1. STUDY AREA.

Based on recent evidence of visceral leishmaniasis, two main study areas were selected. One was Chilas district in Northern Areas and other Bagh district in Azad Jammu & Kashmir. For longitudinal entomological studies two villages (Hudur and Thor) were selected in Northern Areas and one village in Azad Jammu & Kashmir (Rehra village). In addition, as a part of the general survey, sandflies were also sampled from Baltistan (Mehdiabad, Kuru, Keris villages) at an altitudes between 2300-2600 metres and from AJK at altitudes between 1500-1800 metres (Gali Malot, Bees Bagla, and Banjosa) above sea level. Full description of these sites are given in Chapter 3.

#### 4.2. SAMPLING REGIME OF PHLEBOTOMINE SANDFLIES.

Entomological studies were carried out in the Chilas area (Hudur and Thor villages) in the Northern Area and Bagh district (Rehra village), Azad Jammu and Kashmir between March 1991 and November 1991 and March 1992 to November 1992. In the winter months the adult sandflies were not present and in any case sampling could not be done during the months from January to February due to heavy snow, landslides and other adverse weather conditions which made the roads impassable.

The entomological studies were undertaken to investigate sandfly biology in the two geographically distinct foci of visceral leishmaniasis (AJK and Northern Areas) with particular reference to the sandfly fauna, the seasonal and relative

abundance, man-vector contact, animal-reservoir vector contact and natural infections.

The main objective was to find out which sandfly species is/are the vector(s) of visceral leishmaniasis in Northern Areas and Azad Jammu & Kashmir and secondly can these species be suppressed as a part of disease control programme.

#### 4.2.1. GENERAL SURVEY.

Apart from the longitudinal surveys, discussed below, sandflies were also sampled from Perkuta, Keris, Kuru (June) in Northern Areas and from Malot, Beesbagla (June), and Banjosa (July) in Azad Kashmir to obtain a complete picture of the sandfly fauna in northern Pakistan. In addition flies were also sampled from the Margella hills near Islamabad in September. Sampling for general survey was done only once during the peak sandfly season for three consecutive nights per locality. Flies were sampled from inside houses mainly using CDC miniature light trap and from outdoors using sticky paper traps and CDC light-traps. All the flies collected were identified to species excluding *Sergentomyia*. Dissections were also made for natural *Leishmania* infections.

#### 4.2.2. LONGITUDINAL ENTOMOLOGICAL STUDIES.

Two districts, Diamir (Chilas) in northern Pakistan and Bagh in Azad Kashmir were selected on the basis of the most reported cases of visceral leishmaniasis. Within these districts, three villages, two from Northern Areas and one from Azad Kashmir were selected as the origin of maximum number of visceral leishmaniasis cases in the recent past. Hudur village and Thor village (to sample the flies from higher altitude)

were selected in Chilas the headquarters of Diamir District and Rehra village was selected in Bagh District. Within each village, three houses were sampled with a paired wild-site about 400-500 metres away. Thus there were three replicates each for houses and wild sites in each area. Since in both areas (Northern Areas and Azad Jammu & Kashmir) all houses had a surrounding compound both inside houses and compounds were sampled to ensure that all flies potentially coming into contact with humans and dogs were sampled. Each village was sampled for three consecutive nights each month. The sampling in the two areas was staggered two weeks apart for logistic reasons. For the longitudinal studies, phlebotomine sandflies were collected using CDC light traps and sticky paper traps. The per night minimum number of traps placed per paired site (house + wild) were as follows:

TRAPS	INSIDE HOUSES	COMPOUNDS	WILD SITES	TOTAL/VILLAGE/NIGHT
CDC light traps	1	1	-	6
Sticky paper traps	-	25	25	150
Total/habitat/Night. (In three replicates)	3	75	75	-

### 4.3. SAMPLING METHODS.

#### 4.3.1. CDC light-traps.

A total of 6 CDC light-traps (John William Hock Co Ltd; Gainesville, Florida) were used per night, each equipped with a photosensitive automatic switch and a six-volt, heavy-duty, rechargeable dry-battery. Essentially the traps use downward suction which draws flies attracted to the light source into a collecting cage attached below

a fan. Two light traps were used per house/night in each village (one trap was set inside a house and other in the associated compound). The traps were set at height of about 2 feet above the ground and were operated from dusk to dawn (1800-0600hours) (Fig. A).

In the early morning, the CDC lighttraps were collected, taken to the field laboratory and sandflies transferred by mouth aspirator into glass tubes. After killing with chloroform, males and females were separated under the dissecting microscope. Females *Phlebotomus* were dissected for leishmanial infections (see below) and males were preserved in Berlese medium for later identification. Although *Sergentomyia* species collected during the study were also preserved, they were not identified to the species since they do not play any role in the transmission of leishmaniasis. All blood fed flies were separated and after removing the head and genitalia for identification the female abdomens were individually stored in labelled gelatine capsules containing a few dehydrated crystals of silica gel. These capsules were then stored in the refrigerator at 4°C. until blood-meal analysis.

The results of the light trap samples were expressed as the number of sandflies/trap/night. These data were used to determine the number of species present, their seasonal and relative abundance and infection rates with *Leishmania* spp. Blood meal identification was used to determine the range of hosts on which the sandflies fed and also the percentage of flies fed on humans.

#### **4.3.2. Sticky paper traps.**

Sticky paper traps were used for sampling around houses and in the 'wild'

areas. A total of 75 sticky paper traps (25 traps\compound\night x 3 replicates) were used in each sampling site (=village). Wild sites were sampled only during the period March 1992 and November 1992 in both the field sites. For sampling wild flies a total of 75 sticky papers (25 traps/wild site /night x 3 replicates) were used in each sampling site (village).

Sticky paper traps consisted of A4 size paper sheets coated with odourless castor-oil and were placed under large stones, rocks and or inside the holes and crevices. Sticky papers were set at dusk and collected early the following morning, usually at dawn. Sticky papers were used to determine the species present and abundance in the domestic as well as in the wild habitats; the results were expressed as the number of flies/paper/night.

#### **4.3.3. Human biting catches.**

Human biting catches are crucial in studying anthropophilic sandflies, especially to determine which commonly bite man, their diel rhythm, and relative and seasonal abundance (Lane,1993). Man biting catches were performed by two persons sitting inside a living room representative of the typical 'indoor' biting environment in the two villages, to simulate normal nocturnal human activity and probable natural contact with sandflies. A pair of baits/collectors sat on the floor or bed to collect sandflies biting each other by mouth aspirator using torch light. Collections were made continuously throughout the night between 1800 to 0600 hours at two hourly intervals for three consecutive nights/month in Thor and Rehra and due to time limitations two nights/month only in Hudur during the period March 1992 - November 1992. The two

hourly collections were pooled in disposable cups fitted with a nylon mesh, labelled with the time period and kept until the following day when identifications and dissections were made.

These collections were used to determine the species and numbers which regularly (or occasionally) came to bite humans, their relative and seasonal abundance, biting rhythm at night and natural infection rate with *Leishmania*. The results were expressed as mean number sandflies/two-man team/two hours.

#### **4.3.4. Dog-bait catches.**

These were important to determine the species of sandflies coming to feed on dogs which are the most important reservoirs of visceral leishmaniasis (*Leishmania infantum*) around houses in both the Northern Areas and Azad Jammu and Kashmir (Rab, 1994).

For this experiment, a dog was caged at night and the sand flies coming to it were intercepted with horizontally placed sticky papers. The experiment was run for 2-3 nights each month in each village between the period March 1992- November 1992 in both disease foci. Collections were used to determine the species and numbers which were attracted to dogs and their seasonal and relative abundance. The parallel sticky paper traps set around compounds for sampling domestic flies were used as the controls to compare the catches made on dog.

#### **4.4. Meteorological records.**

Meteorological data for the two areas were recorded daily for three consecutive



nights during the sandfly sampling for the periods March 1991-November 1991 and March 1992-November 1992. Records included maximum and minimum temperature (°C), read at 2400hrs, 0600hrs, 1200hrs, 1800hrs respectively from a maximum minimum thermometer; relative humidity (%RH) read from a humidity meter four times daily (2400hrs, 0600hrs, 1200hrs, 1800hrs). For the temperature and humidity mean values were calculated for the three days sampling per month. In addition, monthly average values for temperature, humidity and rainfall were also obtained from the government meteorological headquarters.

#### **4.5. De-oiling and mounting methods.**

All sandflies caught on sticky papers were removed with a fine needle, soaked in detergent for a few hours, washed in several changes of water and preserved in Berlese medium in tubes for later mounting and identification in the laboratory at NIH Islamabad.

In Islamabad, the sandflies were sorted under a dissection microscope into those belonging to the genus *Phlebotomus* and *Sergentomyia*. *Phlebotomus* were then mounted in clear Berlese medium (Lewis, 1973b) prior to identification. This medium was used because it possesses a good refractive index for revealing transparent structures like spermatheca and is quite useful for rapid processing large numbers of specimens as compared to Canada balsam which is a comparatively slow technique (Murray, 1993). Usually, 3-4 flies were mounted on the same glass slide under individual coverslips. For each specimen, the head was removed from the body and mounted ventral side uppermost in a thin film of medium to facilitate examination of

its pharynx at high magnification and body mounted laterally (Lane, 1986). After drying at 37°C the specimens were identified.

#### 4.6. Sandfly identification.

All sandflies belonging to the genus *Phlebotomus* were identified to species. Males were identified mainly from the characters of the external genitalia, antennal formula (especially in the case of species belonging to the sub-genus *Adlerius*) while females were identified by the morphology of the spermatheca, cibarium, shape and armature of the pharynx and the relative length of the antennal and palpal segments. Identification was mainly based on the keys of Lewis (1967, 1978, 1982). Wherever it was felt necessary, early descriptions of other authors such as Theodor (1958) and Artemiev (1978, 1980) were also consulted. Type and other specimens were also examined from the collections of the British Museum (Natural History) London.

#### 4.7. Dissection of phlebotomine sandflies.

All phlebotomine sandflies belonging to the sub-genera *Larroussius* and *Adlerius* caught by CDC light traps or human bait were dissected for natural infection with *Leishmania* parasites as follows (based on Rioux *et al.*, (1984) and Killick-Kendrick, 1987a). Other sandflies, *P.papatasi* and *P.sergenti* were not dissected. These flies were easily distinguishable on gross features and hence separated from the rest of the collection.

Pins mounted on small, hard, plastic sticks (the sticks are used in several immunological kits as applicators) were used for dissecting the flies. In the field

laboratory, 5-10 flies were removed from the cages with an aspirator, blown into a 10 ml. glass vial, killed with chloroform and then briefly wetted in 2 % detergent (PBS-TWEEN 20) (to remove superficial detritus and body hairs). They were then washed in three changes of sterile saline in small glass petri dishes and dissected individually. A single fly was removed from the petri dish with the help of a needle and placed in a drop of physiological saline in the centre of a glass slide. After removing the head, two microneedles, one held gently across the thorax, the other to nick the posterior three abdominal segments after which they were pulled away from the body to expose the entire gut. The gut was placed in a fresh drop of saline and examined for natural infections under 40X objectives of a compound microscope. If no flagellates were seen initially, the gut was flattened under a sterile coverslip and re-examined to detect a possible light infection.

Every effort was made to dissect the maximum number of flies on the day following collection. When flagellates were seen they were inoculated into culture media and the guts squashed on to a nylon Hybond membrane. Guts of negative flies were also squashed on to Hybond membranes for later DNA probing. After dissection, remains of the fly were mounted in Berlese medium for subsequent identification.

#### **4.8. Isolation of *Leishmania* from sandflies.**

When promastigotes were seen in a dissected gut preparation, their location within the gut was noted and the gut pricked with a sterile needle to release the promastigotes into the saline for culture. An additional one or two drops of the saline were added to the saline containing parasites after which all the saline was then

aspirated into a 1 ml. syringe and the contents injected into a bottle containing NNN-media with overlay solution. Inoculation was made with the aid of a flame to reduce the risk of contamination. The culture vials were 5 ml. bijou bottles containing a modified NNN-medium and Locks overlay solution prepared according to the following formula:

#### **Modified NNN medium**

Nutrient Agar	9.2 gm
Glucose (Dextrose)	0.6 gm
Sodium Chloride(NaCl)	2.4 gm
Distilled water	400 ml
Defibrinated rabbit blood	100 ml

#### **Locks overlay solution**

Sodium chloride(NaCl)	9.0 gm
Potassium chloride(KCl)	0.4 gm
Calcium chloride(CaCl <sub>2</sub> )	0.2 gm
Sodium bicarbonate(NaHCO <sub>3</sub> )	0.2 gm
Glucose (Dextrose)	2.5 gm
Distilled water	1000 ml

Gentamycine (200 ug/ml) and 5-fluorocytosine (500 ug/ml) were added to the liquid overlay solution (WHO, 1984b).

#### **4.9. Squash-preparations of sandfly guts on to the nylon Hybond membrane for DNA hybridization.**

All dissected flies including those with microscopically negative guts were squashed on to a nylon Hybond membrane to detect any infection. All squashes were

made 1.5 cm. from each other and coded to correspond with the individual dissected. Membranes were then stored in a large polythene envelope with silica gel and kept at room temperature till further analysis.

The squashed flies on the Nylon membranes were processed in the Molecular Biology laboratory at the British Museum (Natural History) under the supervision of Dr Paul Ready. The radio labelled probe [LUCA-D2 (200BP)] was used (Feinberg and Fogelstein, 1984). Briefly the method is as follows:

The samples bound to the membrane were lysed by layering the whole membrane onto Whatman 3MM papers soaked with denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 7 minutes; neutralization buffer (0.5M Tris. HCl pH 7.5, 1.5M NaCl) for 1 minute and again with denaturation buffer for 5 minutes. The membranes were then dried at room temperature for a few minutes and then baked between 3MM paper sheets in oven at 80°C for 2 hours.

#### **4.9.1. DNA Hybridization.**

##### **a) Prehybridization.**

After drying the membranes in an oven at 80°C they were washed into 2xSSC solution gently for 5 minutes on a shaking platform. The membranes were then layered on a clean glass plate, one on top of another separated by mular sheets. Air was removed from the layered membranes by rolling a 10 ml. pipette over them. They were then scrolled and inserted into a hybridization bottle containing 2xSSC solution. The membranes were unwound by rolling the stoppered bottle along the bench. 2xSSC was then discarded and prehybridization solution (50% Formamide; 10ml., 5xSSC,

5ml. 1% SDS, 2ml; 10x Denhardt's, 2ml; Analar water, 1ml.) prewarmed to 42°C was added. The membranes were then incubated in a oven at 42°C for 4-6 hours.

**b) Hybridization.**

For hybridization, radio-labelled ( $^{32}\text{P}$ ) DNA probe for *Leishmania infantum* (LUCA-D2, 200bp) was heated for 10 minutes at 95-100°C in a water bath and then quenched on ice for 10 minutes. To this was added 10ml. of hybridization solution (50% Formamide, 5ml; 5xSSC, 2.5ml; 1% SDS, 1ml; 5xDenhardt's, 0.5ml.). The pre-hybridization solution was drained from the hybridization bottle and the hybridization solution containing radio-labelled ( $^{32}\text{P}$ ) DNA was added. The membranes were left overnight at 42°C in a Hybaid oven.

**c) Post hybridization stringency washes.**

After hybridizing the membranes overnight, the probe was tipped away into its original container and kept at 4°C. Membranes were washed twice for 5 minutes, each in a Hybaid oven at 42°C with 2xSSC and 0.5% SDS. They were then removed from the hybridization bottle and washed with 400ml. 2xSSC, 0.5% SDS at 65°C in a water bath for 30 minutes. Once again this step was repeated and after the second wash, the membranes were then set in an autocard (used to expose X-Ray films) and exposed at 80°C for 1-15 days. Negative and positive controls were also run along with the tests.

#### 4.10. Blood meal analysis.

All blood fed flies caught in the CDC light trap were killed using chloroform. The head and terminalia were then cut off under the dissecting microscope and mounted in Berlese medium for identification. The rest of the body was stored in a gelatine capsule containing a few silica gel crystals according to the method described by Killick-Kendrick (1987). Each capsule was labelled with a number corresponding to the slide on which the head and terminalia were mounted. The capsules were then sealed in polythene bags with silica gel crystals and stored at 4°C till analysis.

The blood meals were tested using direct ELISA (test kit obtained from Dr. Alistair Voller's laboratory, London) against human, bovines and dogs. The objective was to determine the proportion of flies fed upon humans, dogs and bovines. Abdomens of the individual blood fed sandflies stored in the gelatine capsules containing silica gel were grounded individually with a disposable plastic pestle in 400 ul. phosphate buffer saline in Tween 20 (PBS/T20: 50ml PBS/T20 x20 solution + 950 ml distilled water). After grinding, the pestle was rinsed twice in PBS and it was then wiped dry with a clean tissue to avoid contamination.

The Eppendorf tubes containing eluted blood meals were kept at room temperature for 2 hours prior to testing. Each tube was labelled with a number corresponding to a sandfly specimen. All assays were carried out in 96-well flat bottomed PVC microtitre plates coated with host specific antibodies against human, bovine and dog. Reference serum (positive control) was diluted 1:500 in appropriate diluent provided with the kit (20 ul serum to 10 ml diluent). PBS as well

as positive reference sera of two hosts other than the one being tested were used as negative controls. The first four wells of the microtitre plate contained 100 µl, each of the positive and negative controls. In the remaining wells 100 µl of the test samples were added. The same specimens were tested against the other two host species on the same day. Briefly, the following steps were carried out: once the positive and negative controls and the test sera were transferred to the microtitre plate, the plates were then incubated for 60 minutes at room temperature and then washed three times with PBS/Tween 20. They were then tapped dry onto a paper towel after the third wash cycle. In the subsequent step 100 µl volume of the diluted conjugate (HRP) (20 µl Horse raddish peroxidase in 10 ml relevant diluent) was added to each well and the plate was incubated again for 60 minutes at room temperature and washed and dried as in the previous step. After the second incubation and washing, 100 µl of the chromogen substrate, o-phenylenediamine (OPD) (2 OPD tablets in 6 ml distilled water, 1 drop of 3 % Hydrogen peroxide) was added to each well and the plates were incubated in the dark for 15 minutes. The reaction was stopped with 1M sulphuric acid and the results read visually against the positive and negative controls. Positive samples gave a yellow/orange colour whereas the negative samples were colourless.



## CHAPTER 5

### SYSTEMATICS OF PHLEBOTOMINE SANDFLIES FROM VISCERAL LEISHMANIASIS FOCI OF AZAD JAMMU & KASHMIR AND NORTHERN PAKISTAN.

#### 5.1. INTRODUCTION

The Phlebotomine sandflies of Pakistan mainly belong to the Mediterranean and Central Asian sub-regions of the Palaearctic region and to the Indian sub-region of the Oriental region.

The zoogeography of Pakistan as it relates to sandflies has been discussed in detail by Lewis (1967). Briefly species with a largely mediterranean distribution are *P.papatasi*, *P.alexandri*, *P.sergenti*, *P.major*, *S.dentata* and *S.theodori*. Oriental species include *P.argentipes*, *P.colabaensis*, *S.punjabensis*, *S.babu*, *S.shorttii*, *S.bailyi*, *S.montana* and *S.hospitii*. Species with central Asian affinities are *P.nuri*, *P.kandelakii*, *P.keshishiani*, *P.chinensis*, *S.grekovi*, *S.palestinensis* and *S.pawlowsky*. All these species occur in or near the northern hills of Pakistan.

The phlebotomine fauna of Pakistan and the role of certain species in the transmission of leishmaniasis in the country have been little studied. The studies of Ahmed *et al.*, (1960) and Ahmed and Burney, (1962) were based on expeditions to Northern Pakistan (Baltistan) in an attempt to discover the epidemiology of visceral leishmaniasis. They collected a total of 191 sandflies, of which 105 were from Gawadi (8400 feet above sea level) and 86 from Keris (8300 feet above sea Level) and identified as *Phlebotomus chinensis longiductus*; *Phlebotomus major* and *Phlebotomus kandelakii*. The last was described subsequently as *Phlebotomus kandelakii burneyi* by Lewis (1967). Their Searches for sandflies in cracks, fissures, rodent burrows and tree holes, were unsuccessful as were attempts to locate the sandfly breeding places

in decaying vegetable dumps. Adult flies could be obtained only by night collections in houses. All specimens they caught in the houses of visceral leishmaniasis patients were dissected, but none had natural promastigote infections.

The first, and only complete inventory of the sandflies from Pakistan is that of Lewis, (1967). Ten species of *Phlebotomus* belonging to six subgenera (Table 5.1) and ten species of *Sergentomyia* were recorded.

Between 1967 and 1989 no further studies of Phlebotomine sandflies from this part of the world took place, possibly because visceral leishmaniasis was thought to have been eliminated as a consequence of the control efforts made during the 1960s. However,<sup>a</sup> recrudescence of the disease during the early eighties in northern Pakistan as well as the appearance of fresh cases from Azad Jammu and Kashmir and adjoining areas gave impetus for detailed studies on the epidemiology of visceral leishmaniasis and the sandfly vectors. The present study is the result.

## **5.2 THE FAUNA OF PHLEBOTOMINE SANDFLIES FROM NEIGHBOURING COUNTRIES**

While reviewing the Phlebotomine fauna of Pakistan it is worth briefly discussing the sandfly fauna from neighbouring countries including China, Iran, Afghanistan, and India especially Indian held Jammu Kashmir because this will put the Pakistan fauna in context. Table 5.1 shows the comparison of *Phlebotomus* species reported from Pakistan and neighbouring countries.

### **i) China.**

A considerable amount of work has been done in China on various aspects of

Table 5.1. *Phlebotomus* species recorded in Pakistan and neighbouring countries.

Subgenera & Species	Pakistan	China	Iran	Afghanistan	India
<b>1. <i>Adlerius</i></b>					
<i>angustus</i>	-	-	-	+	-
<i>balcanicus</i>	-	-	+	-	-
<i>brevis</i>	-	-	+	-	-
<i>chinensis</i>	-	+	-	-	-
<i>comatus</i>	-	-	-	-	-
<i>halepensis</i>	-	+	+	-	-
<i>hindustanicus</i>	+	-	-	+	+
<i>kabulensis</i>	-	-	-	+	-
<i>longiductus</i>	+	+	-	+	+
<i>rupester</i>	-	-	-	+	-
<i>salangensis</i>	+	-	-	+	-
<i>sichuanensis</i>	-	+	-	-	-
<i>turanicus</i>	-	-	-	+	-
<i>zulfagarensis</i>	-	-	-	+	-
<b>2. <i>Anaphebotomus</i></b>					
<i>colabaensis</i>	+	-	-	-	+
<i>hoepplii</i>	-	+	-	-	+
<i>stantoni</i>	-	+	-	-	-
<b>3. <i>Euphlebotomus</i></b>					
<i>argentipes</i>	+	-	+	-	+
<i>autumnalis</i>	-	-	-	+	-
<i>caudatus</i>	-	-	-	+	-
<i>kiangsuensis</i>	-	+	-	-	-
<i>mesghalii</i>	-	-	+	-	-

<i>yunshengensis</i>	-	+	-	-	-
<b>4. Idiophlebotomus</b>					
<i>tubifer</i>	-	-	-	-	+
<i>longiforceps</i>	-	+	-	-	-
<b>5. Kasaulius</b>					
<i>newsteadi</i>	-	-	-	-	+
<b>6. Larrousius</b>					
<i>kandelakii</i>	-	-	+	+	-
<i>kandelakii burneyi</i>	+	-	-	?	-
<i>keshishiani</i>	+	-	+	+	-
<i>major</i>	+	-	+	-	+
<i>notus</i>	-	-	-	+	-
<i>perfiliewi-transcaucasicus</i>	-	-	+	-	-
<i>perniciosus</i>	-	-	-	-	-
<i>smirnovi</i>	-	+	-	-	-
<i>tobbi</i>	-	-	+	-	-
<i>wenyoni</i>	-	-	+	-	-
<b>7. Paraphlebotomus</b>					
<i>alexandri</i>	+	+	+	+	+
<i>andrejevi</i>	-	+	+	+	-
<i>mofidii</i>	-	-	+	-	-
<i>caucasicus</i>	-	+	+	+	-
<i>jascusieli</i>	-	-	+	-	-
<i>kazeruni</i>	-	-	+	+	-
<i>mongolensis</i>	-	+	+	+	-
<i>nuri</i>	+	-	+	+	-
<i>sergenti</i>	+	-	+	+	+

<b>8. Phlebotomus</b>					
<b>bergeroti</b>	-	-	+	-	-
<b>papatasi</b>	+	-	+	+	+
<b>salehi</b>	+	-	+	-	+
<b>9. Synphlebotomus</b>					
<b>ansarii</b>	-	-	+	-	-
<b>eleanorae</b>	-	-	+	-	+

phlebotomine sandflies in almost all major provinces since the early part of the century. R.A. Bolt was the first to report the presence of a sandfly from the vicinity of Beijing, later named as *Phlebotomus major chinensis* by Newstead (1916). Important reviews on the phlebotomine sandflies of China are those of Leng and Yin, (1983); Leng, (1987); Leng, (1988) and Leng and Zhang, (1993). A total of 42 species and sub-species belonging to 5 genera namely *Chinius*, *Grassomyia*, *Idiophlebotomus*, *Phlebotomus* (including 5 sub-genera) and *Sergentomyia* have been reported from China (Leng and Zhang, 1993; see Table 5.1). Among them five species are considered to be confirmed vectors of visceral leishmaniasis, namely: *Phlebotomus (Adlerius) chinensis*, *P.(Adlerius) longiductus* (in Xinjiang), *P.(Adlerius) sichuanensis* (in north-west Sichuan above 900m. sea level), *P.(Larroussius) smirnovi* (in Xinjiang, Gansu and Inner Mongolia as *P.wui*) and *P.(Paraphlebotomus) alexandri*.

## ii) Iran.

There is a wealth of published information on the sandfly fauna of Iran (Table 5.1) including their geographical distribution and known and suspected vectors of cutaneous and visceral leishmaniasis ( Adler *et al.*, 1930; Pervomaiski, 1948; Lewis, 1957; Lewis *et al.*, 1961; Mesghali 1961; Theodor and Mesghali 1964; Javadian and Mesghali, 1975). Among these species, *Phlebotomus major*, *P.chinensis halepensis*, *P.chinensis brevis*, *P.kandelakii*, *P.keshishiani*, *P.tobbi* and *P.wenyoni* were recorded in areas where cases of visceral leishmaniasis were reported but *P.major* and *P. alexandri* were suspected on epidemiological grounds as the most likely vectors of visceral leishmaniasis (Nadim *et al.*, 1978; Nadim, 1988).

### iii) Afghanistan.

The most complete work on the sandfly fauna of Afghanistan has been published in the series of papers by Artemiev (1974a), (1974b), (1976a), and (1976b). The first deals with the genus *Phlebotomus* and the remaining papers deal with the genus *Sergentomyia*. Later, Artemiev, (1978) prepared a comprehensive list of 42 sandfly species recorded in Afghanistan (Table 5.1) with 11 others occurring in neighbouring countries with brief notes on their geographical distribution, known general ecology and medical importance. All the recorded species belong to the fauna of the Palaearctic region, except *S. babu* of the Indo-Malayan fauna which occurs in the monsoon areas of the east.

### iv) India.

The phlebotomine fauna of India (Table 5.1) includes 44 species of which 11 are *Phlebotomus* and 33 *Sergentomyia* (Lewis, 1978). *Phlebotomus (Euphlebotomus) argentipes* has been incriminated as the principal vector of visceral leishmaniasis in India but it has never been recorded from the visceral leishmaniasis foci of Pakistan.

The Palaearctic species occur mainly in north west India and therefore the sandfly fauna of the western Himalayan region and Indian held Jammu and Kashmir is of particular interest to us due to the close proximity of these regions to Northern Areas of Pakistan and Azad Jammu & Kashmir (the active foci of visceral leishmaniasis) and similarly of vegetation, landscape and climate. The only report on the sandflies of the western Himalayan region of India is in a general survey of haematophagous arthropods conducted during 1966-1970 in the western Himalayan

region and the Himalayan districts of West Bengal (Kulkarni *et al.*, 1978). Sandflies were collected by hand capture in houses and cattle sheds as well as from outside resting sites. Out of the 13 species collected only three belonged to the genus *Phlebotomus* and these were: *P. longiductus*, *P. argentipes* and *P. major*.

In Himachel Pradesh, *Phlebotomus longiductus* (as *P. chinensis longiductus*), was collected from indoor resting sites at an altitude of 1,570-2,700 m, and from Uttar Pradesh at an altitude of 780 and 2300-2,500 meters. *P. major* was recorded only from indoor sites at an altitude of 2,000-2,300 m. and was associated with hilly areas, along the Himalayan foot hills at an altitude of 1,500-2,100 metres.

Information on the phlebotomine sandflies of the Kashmir valley, in India is limited. A total of 9 species have been reported including 4 species of *Phlebotomus* (Jacob and Kalra, 1951). These include *Phlebotomus papatasi*, *P. sergenti*, *P. major*, *P. longiductus*. In the subsequent survey carried out in three districts of Jammu region (Kaul and Shetty, 1983) only 191 sandflies were captured, all belonging to the genus *Sergentomyia*.

### 5.3 MATERIALS AND METHODS

Specimen for the taxonomic studies are from two sources- The general survey and the longitudinal studies (reported later in this thesis, Chapter 6). Longitudinal entomological studies were carried out in the visceral leishmaniasis foci of Azad Kashmir (Bagh District) and Northern Pakistan (Chilas District) during the period March to November 1991 and in the same months in 1992. The general survey was conducted only once during the peak sandfly season in Perkuta, Keris, Kuru



(Northern Areas), Malot, Banjosa, and Beesbagla (Azad Jammu & Kashmir) as well as in Margella hills surrounding Islamabad (the capital). Sandflies were collected in different habitats (indoors, outdoors and wild sites) using mouth aspirators, sticky-paper traps (coated with deodorized castor oil) and CDC light-traps (from John Hock Co., Gainesville, Florida, USA). All sandflies belonging to the genus *Phlebotomus* were mounted and identified to species . Detailed methodology for the general survey and longitudinal studies have been discussed in the previous chapter (chapter 4) on Material and Methods.

### **5.3.1. Laboratory methods for morphometric measurements.**

For morphometric measurements flies were mounted in Berlese medium. This medium has many advantages over Canada balsam which is a comparatively slow technique requiring dehydration of the specimen and the risk of inhalation of xylene. Apart from enabling slides to be made faster it has a good refractive index, revealing transparent structures like the spermathecae (Murray, 1993).

### **5.3.2. Morphometric Characters studied.**

The taxonomic characters used for the differentiation and classification of sandflies were selected on the base of their taxonomic use with other sandflies, mainly following Lewis (1967).

The following characters (and their abbreviations as used in the subsequent tables) were measured (mm.) at appropriate magnifications using the Video Plan computerised system at the Department of Entomology, British Museum (Natural

History). Ratios were also calculated to assess their diagnostic use.

- Head Length (HDL):- measured from the anterior margin of the clypeus to posterior margin of head.
- Head Width (HDW):- measured between inner margins of eyes i.e. occiput.
- Head length/Head width ratio (HDL/HDW).
- Antenna 3, 4 & 5 (A3, A4 & A5):- Length of antennal segments 3, 4 & 5 respectively, each segment measured individually including the base inserted in the preceding segment.
- Ascoid 3,4 & 5 (Asc3, Asc4 & Asc5):- Length of Ascoid on A3, A4 and A5 respectively.
- A4 + A5:- Summed length of segments A4 and A5.
- A3/A4+A5 Ratio.
- Labrum length (LBL):-Length of labrum measured from the anterior margin of clypeus to the extreme tip of labral sensilla.
- A3/Labrum length Ratio.
- Palp 1,2,3,4 & 5 (P1,P2,P3,P4 & P5):- Respective lengths of the palpal segments 1,2,3,4 & 5 measured individually.
- Pharynx Length (PHXL):-Maximum length of the pharynx.
- Pharynx Width (PHXW):-Maximum width of the pharynx.
- Pharynx Length/Pharynx Width ratio (PHXL/PHXW).
- Wing length (WL):- Straight length of the wing from the basal end of the hairy node at the base of the costa to the wing tip.
- Wing Width (WW):- Width of the wing at its widest point.

- Wing length/Wing width (WL/WW) ratio.
- Antennal segment 3/Wing length ratio (A3/WL).
- Labrum length/Wing length ratio (LBL/WL).
- Common Duct Length (CDL):- Total length of the common duct from its base to the tip of bifurcation of the individual spermathecal ducts.
- Common Duct Width (CDW):- Width of the common duct at its widest point.
- Spermathecal Duct Length (SDL):- Entire length of the spermathecal ducts.
- Spermathecal Terminal Process Length (STPL):- Length of the terminal process of the spermatheca.
- Spermathecal Length (SPL):- Total length of the spermatheca from the tip of the terminal process to the junction of the spermatheca and the individual ducts.
- Number of Spermathecal Rings (SPR.No.): - Count of spermathecal rings/segments.
- Aedeagus Length (ADL):- Maximum straight length of the aedeagus from the base to the tip.
- ADL/WL ratio.
- ADL/A3 ratio.
- Paramere Length (PRL):- Total straight length of paramere.
- Coxite Length (CXL):- Total straight length of coxite from its base to the tip.
- Coxite Width (CXW):- Width of coxite measured at its widest point.
- ADL/CXL ratio.
- CXL/WL ratio.

- Style Length (STL):- Total straight length of the style.
- Style Width (STW):- Width of the style measured at its widest point.
- CXL/STL ratio.
- Distance of terminal spine from the proximal end of the style(DTSPE).
- Distance of middle spine from the proximal end of the style (DMSPE).
- Distance of posterior spine from the proximal end of the style (DPSPE).
- Pump Length (PML):- Length of the sperm pump
- Genital Filament Length (GFL): length from base of pump to tip of filaments.
- GFL/WL ratio.
- Number of Hairs on Coxite hair patch (HRSNo.).
- Position of hair patch on the coxite (CXHRDST). distance from base of coxite to distal margin of hair patch.

## 5.4 RESULTS AND DISCUSSIONS.

A total of 9656 sandflies belonging to the genus *Phlebotomus* were collected from visceral leishmaniasis foci of Northern Areas and azad Jammu & Kashmir (AJK). Out of these 8797 sandflies were captured during the longitudinal studies (discussed in detail in the subsequent chapter 6 of this thesis) and 859 during the general survey as discussed below:

### 5.4.1. General survey.

A total of 859 sandflies were captured. Out of these 565 were from Northern Areas, 237 from Azad Jammu & Kashmir and 57 from the Margella hills. Table 5 2

Table 5.2. *Phlebotomus* species captured from different localities during the general survey in Northern Areas, Azad Jammu & Kashmir and the Margella hills (Islamabad).

Species	AREAS						
	Northern Areas (n=565)			Azad Jammu & Kashmir (n=237)			Margella Hills
	Kuru (2600m) n=189	Keris (2500m) n=102	Mehdiabad (2400 m) n=274	GaliMalot (1700m) n=95	Beesbagla (1890 m) n=27	Banjosa (1800 m) n=115	(1700 m) n=57
<i>P.keshishiani</i>	12 (6.3)	7 (6.86)	101 (36.86)	-	-	-	4 (7.01)
<i>P.kandelakii burneyi</i>	36 (19.4)	28 (27.5)	10 (3.64)	-	-	-	-
<i>P.major</i>	-	-	-	-	-	-	17 (29.8)
<i>P.Sp.A</i>	-	-	-	67 (70.52)	18 (66.6)	58 (50.4)	-
<i>P.salangensis</i>	111 (58.73)	43 (42.35)	141 (51.45)	-	-	-	-
<i>P.hindustanicus</i>	-	-	-	16 (16.84)	9 (33.3)	47 (40.86)	8 (14.0)
<i>P.alexandri</i>	9 (4.7)	7 (6.86)	3 (1.09)	-	-	-	-
<i>P.sergenti</i>	21 (11.1)	17 (16.6)	19 (6.93)	12 (12.63)	-	10 (8.69)	22 (38.5)
<i>P.papatasi</i>	-	-	-	-	-	-	6 (10.52)
<b>Total</b>	189	102	274	95	27	115	57

Note: Within parenthesis are the percentages calculated from the total number of flies captured from each locality.

shows the species composition within each locality.

A total of 5 *Phlebotomus* species (*P.salangensis*, *P.kandelakii burneyi*, *P.keshishiani*, *P.alexandri* and *P. sergenti*) were recorded from Northern Areas and four species (*P.Sp.A*, *P.major*, *P.hindustanicus* and *P.sergenti*) from Azad Jammu & Kashmir (AJK). The species belonged to three sub genera: *Phlebotomus (Larroussius)*, *P. Adlerius* and *P. Paraphlebotomus*.

In Northern Areas at higher altitudes *Phlebotomus salangensis* was the predominant species in all the three villages sampled. Species composition however varied between the localities even at the same approximate altitude. *P.kandelakii burneyi* was the second dominant sandfly followed by *P.sergenti* in Kuru and Keris village, whereas it was *P.keshishiani* followed by *P.sergenti* in Mehdiabad village). In AJK *Phlebotomus (L) Sp.A* was the most abundant followed by *P.hindustanicus* and *P. sergenti* in all the three localities (Table 5.2).

From the Margella hills four *Phlebotomus* species belonging to 4 subgenera: *Phlebotomus (Larroussius)*, *P. (Adlerius)*, *P. (Paraphlebotomus)* and *P. (Phlebotomus)* were captured. The species in order of decreasing abundance were *Phlebotomus sergenti*, *P.major*, *P.hindustanicus* and *P.papatasi* (Table 5.2).

#### 5.4.2. SYSTEMATICS.

The main objective of this chapter was to identify *Phlebotomus* species captured during the present study from the active foci of visceral leishmaniasis in Northern Pakistan. Although most of the species have been recognised by Lewis (1967) there is still confusion regarding the identity of some species belonging to

subgenera *Phlebotomus Adlerius* and *Phlebotomus (Larroussius)*. This chapter thus provide detailed information on the morphometrics of *Phlebotomus salangensis*, *P.hindustanicus*, *P.keshishiani*, *P.major*, *P.Sp.A*, and *P. kandelakii burneyi*. Comparisons were made with the Type specimens when needed.

**GENUS *Phlebotomus* Rondani & Berté, 1840**

**Sub-genus *PHLEBOTOMUS* s.str.**

***Phlebotomus papatasi* Scopoli, 1786.**

*Bibio papatasi* Scopoli, 1786: 55 Type(s) Italy (Depository unknown).

*Phlebotomus papatasi*; Grassi, 1907; Newstead and Sinton, 1921: 104[surstyles].

*Phlebotomus (Phlebotomus) papatasi*; Lewis 1967: 14; 1978: 223; 1980: 138; Perfil'ev, 1968: 228; Bhat and Modi, 1976: 265, 266; Lane, 1986:15; Lane & Fritz (1986).

This species has been a subject of numerous morphological studies because of its role as a disease vector. The complex nomenclatural history of this species has been reviewed in depth by Lewis (1982). Briefly, the early anatomical descriptions are those of Grassi (1907), Newstead (1911-1912), Adler and Theodor (1926), Perfil'ev (1928), Parrot (1937, 1940, 1946) and Sinton (1927, 1928). The comprehensive work on the taxonomy of this fly include those of Sinton (1932, 1933); Kirk and Lewis (1951) and Theodor (1958).

The recent full or partial descriptions of *P.papatasi* are given by Perfil'ev (1968), Quate (1964), Lewis (1967), Abonnenc (1972), Bhat and Modi (1976), Lewis (1978, 1982), Lewis and Buttiker (1982) and Lane (1986). The morphological differences to distinguish between the females of *P.papatasi* and the closely related

species *P. bergeroti* has been shown by Lane & Fritz (1986).

This insect has been described so frequently and is now so well known that description, synonymy and distribution is well established (see Lewis, 1967, 1978, 1982).

The males and females were easily identified on the following taxonomic characters:

**Female.**

Cibarium with few minute ventral teeth and some lateral spicules; Pharynx stout narrowing after posterior bulge; A3 shorter than labrum. Spermatheca with 8-10 segments approximately equal in size and straight sided with separate ducts.

**Male.**

Cibarium very much like that in the females. Terminalia very long. Paramere with three lobes, dorsal lobe very much longer than the broad median lobe. Coxites with small tufts of hairs on basal plate, and another clump of long hairs distally. Style, long slender, with five short pointed spines, three terminal, one median and one subapical. Distance between median and subapical spine less than between subapical and terminal spine.

*P.papatasi* is commonest species in most parts of the country, but has never been found in the northern areas above 5000ft. Lewis (1967) compared the Pakistani specimens with the description of Egyptian *P.papatasi* by Schmidt and Schmidt (1963) but did not find any marked regional variations.

**Distribution.**

*Phlebotomus papatasi* is the most widespread of any sandfly species. It has



been recorded from Portugal in the west to Bangladesh in the east and from southern U.S.S.R. in the north to Sudan in the south.

This species has been reported from Pakistan by various workers (Figure 5.1). Newstead & Sinton (1921): Bannu, Dera Ismail Khan, Idak, Tank. Sinton (1924): Kohat, Lahore, Miramshah, Nowshera, Quetta, Rawalpindi. Sinton (1927): Jandola, Khirgi, Landikotal, Peshawar. Sinton (1932): scattered all over the plains of Indo-Pakistan subcontinent. Munir (1963): Chilas, Gilgit; Lewis (1967): Digri, Jhelum, Kandhkot, Kashmore, Larkana, Mirpur Khas, Shikarpur, Chaman, Hyderabad, Jamesabad, Jamrud, Karachi, Khairpur, Pano Aqil, Tando Bago, Abbottabad, Mir Muhammad, Saidpur, Taxila.

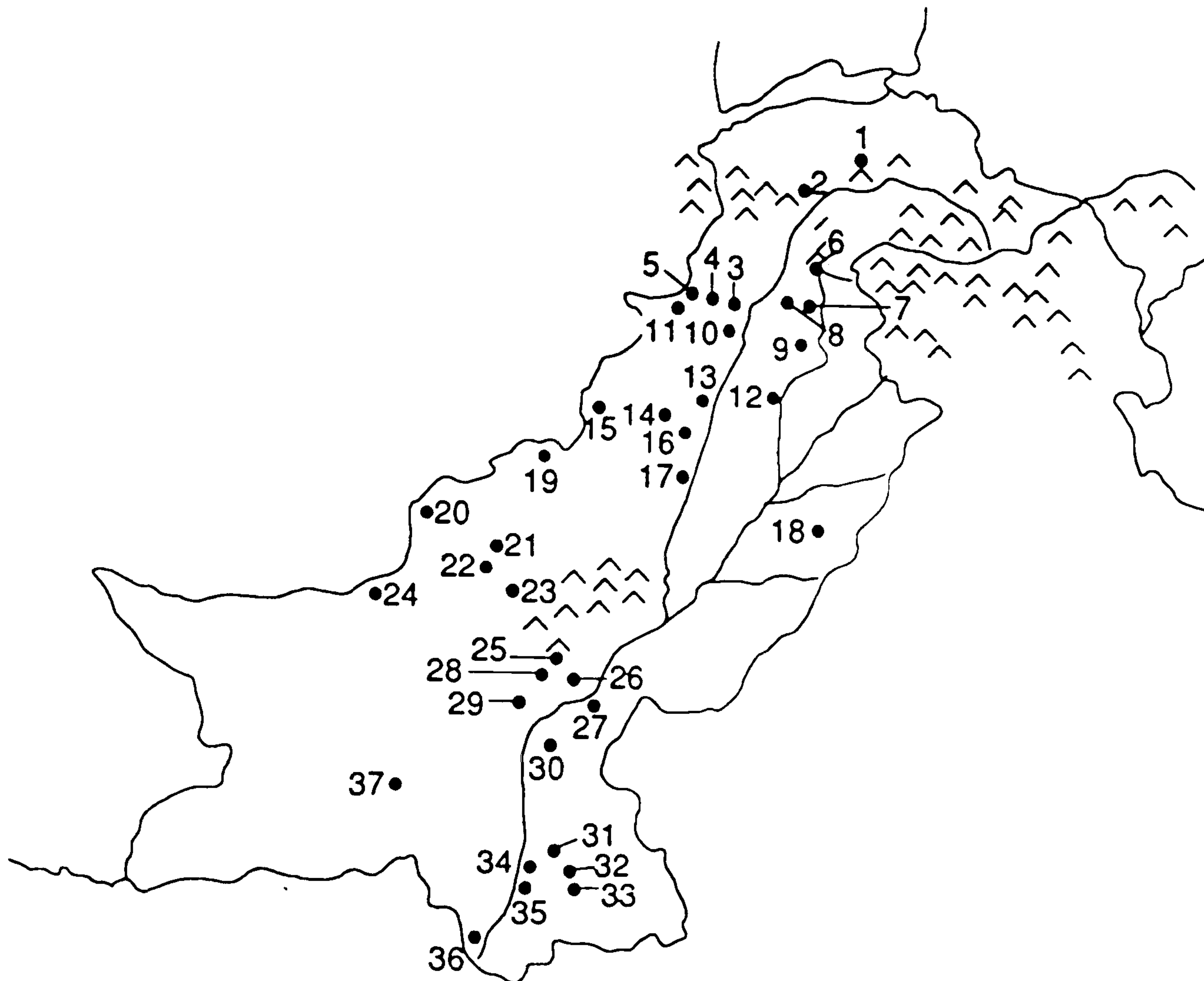
During the present survey this species was recorded from Chilas, Gilgit and Islamabad (Margella Hills).

#### **Medical importance.**

This is probably the most studied of all sandflies because of its abundance and widespread distribution, anthropophily and peridomestic habits. It is the main vector of cutaneous leishmaniasis (*Leishmania major*) to man in many parts of its range (Lewis 1974; Theodor, 1964; Williams & Coelho, 1978; Lane, 1986). In the Mediterranean basin and the Middle East, *P. papatasi* has been proved a vector in several countries and strong circumstantial evidence exists for its vectorial status in many other countries. It is a proven vector of *Leishmania major* in eastern Saudi Arabia, southern Morocco and eastern Tunisia (Killick-Kendrick, 1990) Jordan valley (Schlein, et al., 1982), Israel, Arva (Schlein *et al.*, 1984), and highly suspected vector in several other countries such as Iran (Nadim, 1988); Egypt (Lane, 1986 and

**Figure 5.1**

**Geographical distribution of *Phlebotomus papatasi* in Pakistan**



- 1 = GILGIT; 2 = CHILAS; 3 = NOWSHERA; 4 = LANDIKOTAL;  
5 = MIR MOHAMAND; 6 = ABBOTTABAD; 7 = TAXILA; 8 = ISLAMABAD;  
9 = RAWALPINDI; 10 = PESHAWAR; 11 = JAMRUD; 12 = JHELUM; 13 = KOHAT;  
14 = BANNU; 15 = MIRAMSHAH; 16 = TANK; 17 = D.I.KHAN; 18 = LAHORE; 19 = JANDOLA;  
20 = CHAMAN; 21 = ZHOB; 22 = QUETTA; 23 = SIBI; 24 = BOSTAN; 27 = KHANDKOT;  
28 = SHIKARPUR; 29 = LARKANA; 30 = KHAIRPUR; 31 = DIGRI; 32 = MIRPURKHAS  
33 = JAMSHEDABAD; 34 = TANDO BAGO; 36 = KARACHI; 37 = KHUZDAR;

unpublished), Afghanistan (Nadim *et al.*, 1979) and North Africa (Review: Dedet, 1979). In Pakistan strong circumstantial evidence exists regarding this species being the vector of zoonotic cutaneous leishmaniasis in the southern parts mainly Baluchistan (Munir, *et al.*, 1989).

*P. papatasi* has been considered to be involved in the transmission of visceral leishmaniasis in some of the countries where no other likely vector has been found e.g. Saudi Arabia (Lewis & Buttiker, 1980) and Iraq (Abul-Hab & Azawia, 1978; Adler & Theodor, 1957; Sukkar, 1972). However it is not an efficient vector of visceral leishmaniasis and is extremely unlikely to transmit the parasite in nature.

### Subgenus *PARAPHLEBOTOMUS* Theodor, 1948.

#### *Phlebotomus sergenti* Parrot, 1917.

*Phlebotomus(Phlebotomus) sergenti* Parrot; Syntypes [♂], Algeria [examined]. Abonnenc & Laviere, 1957; Abonnenc, 1957 (table of differences of ♂s and ♀s from *P. alexandri* and *P. saevus*).

*Phlebotomus(Paraphlebotomus) sergenti* Parrot; Perfil'ev 1968: 8, 9, 49 [larva], 50, 60 [egg], 67, 72-74, 81, 236; Lewis, 1967: 17 [recorded from Pakistan]; Lewis, 1978 [synonymy]; Artemiev 1978 [Afghanistan distribution, medical importance, ecology]; Lewis and Buttiker, 1980: 263[synonymy]; Lewis, 1982 [Synonymy, distribution]; Lane, 1986: 17 [Egypt, Synonymy].

Males and females of *P. sergenti* collected during the present study were identified according to the following taxonomic characters:

#### **Males:**

Pharyngeal armature with series of scales with minute teeth along anterior margin. A3 slender, slightly longer or equal to the length of labrum. Terminalia short. Basal process of coxite thin with narrow head directed obliquely down. Style very short, globular with four spines (2 terminal and 2 at the middle of the style). Surstyless

longer than coxite.

**Female:**

Pharynx with large scales anteriorly, some produced into long broad spines, scales becoming broader and flatter posteriorly. A few rows of finely punctate lines or fine teeth at the base of the pharynx. Length of the A3 almost equal or slightly greater than length of the labrum. Spermatheca with five segments with the characteristic large apical segment. The other segments smaller and narrower towards the spermathecal ducts.

**Distribution:**

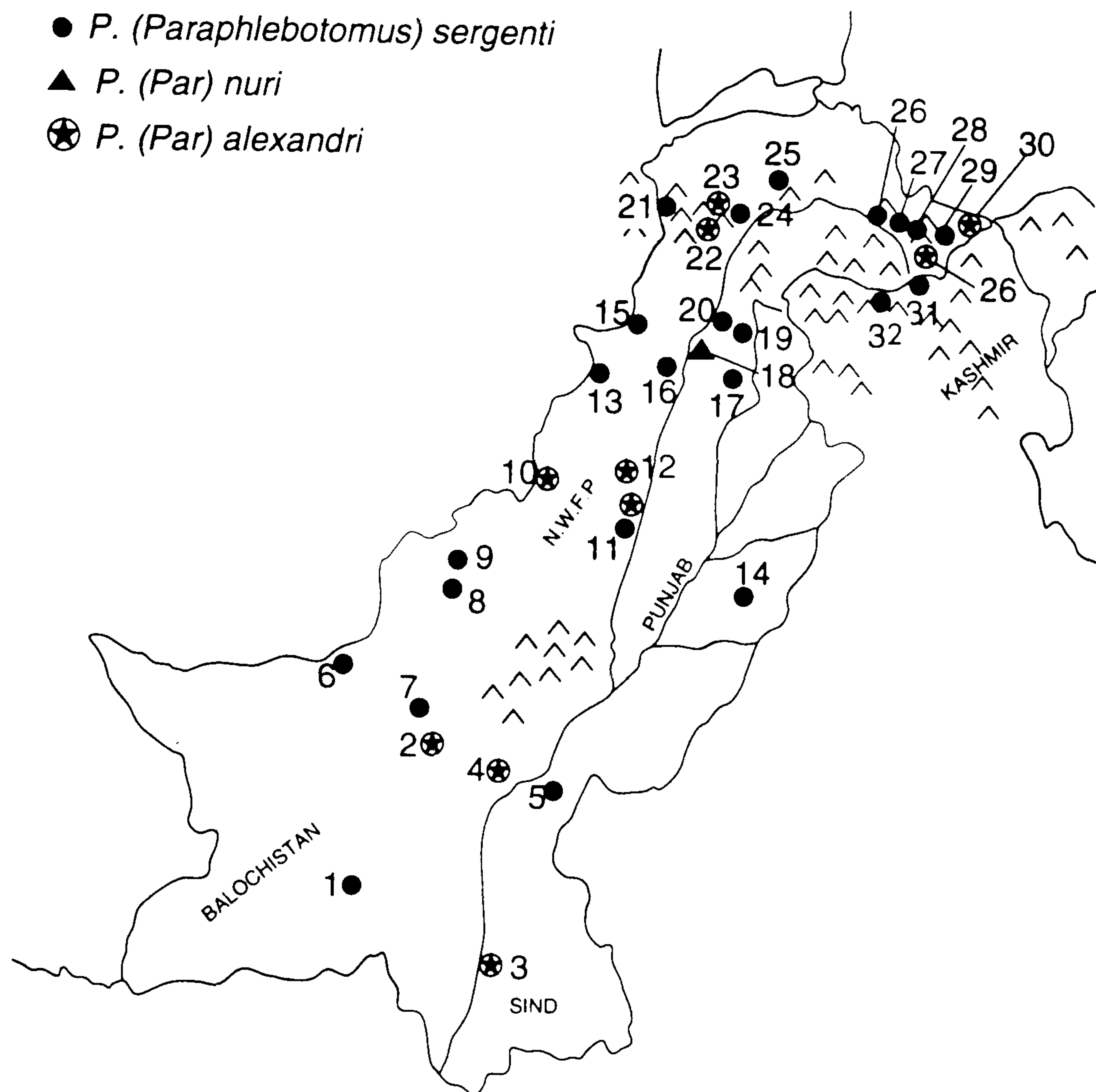
Mediterranean basin eastward through Israel, Egypt, Iraq, Iran, Afghanistan, India, Pakistan and southwards to Saudi Arabia, Yemen, and Ethiopian Highlands. Also recorded from USSR. *P. sergenti* has been recorded from almost all the provinces of Pakistan including Punjab: Lahore (Nasir 1958), Rawalpindi, Saidpur and Texala (Lewis 1967); NWFP: Dera Ismail Khan (Newstead and Sinton 1921), Chitral and Landikotal (Sinton 1927), Peshawar (Lewis 1967); Northern Areas: Chilas Gilgit, Gol, Gowadi, Perkuta, Keris, Mir Mohammad (Lewis 1967); Baluchistan: Quetta (Sinton 1924); Sind: Sukkur (Sinton 1929). During the present survey this species was found in Chilas, Gilgit, Mehdiabad (previously known as Perkuta), Kuru, Keris, (Northern Areas), Margella hills (Islamabad), Rawalpindi and Azad Jammu and Kashmir (Bagh, Banjaso, Gali Malot) (Figure 5.2).

**Medical importance.**

*P. sergenti* is the probable vector of *Leishmania tropica* in almost all foci (Killick-Kendrick 1990), except Kenya where the parasite is most likely transmitted

## Figure 5.2

### Geographical distribution of *Phlebotomus sergenti*, *P. alexandri* and *P. nuri* in Pakistan and AJK



1 = KHUZDAR; 2 = SIBI; 3 = HYDERABAD; 4 = LARKANA; 5 = SUKKUR; 6 = BOSTAN; 7 = SIBI; 8 = QUETTA; 9 = ZHOB; 10 = WAZIRISTAN; 11 = D.I.KHAN  
 12 = TANK; 13 = LANDIKOTAL; 14 = LAHORE; 15 = MIR MOMAND; 16 = PESHAWAR;  
 17 = RAWALPINDI; 18 = SAIDPUR (MARGELLA HILLS); 19 = ISLAMABAD;  
 20 = TAXILA; 21 = CHITRAL; 22 = KHAMBAR; 23,24 = CHILAS; 25 = GILGIT;  
 26 = MEHDIABAD (PERKUTA); 27 = GOL; 28 = GOWADI; 29,30 = KERIS;  
 31 = GALIMALOT; 32 = BAGH.

by *P.saevus* (Lawyer *et al.*, 1989; Mebrathu *et al.*, 1988). The first proven record of the vectorial role of *P. sergenti* in the transmission of typed *Leishmania tropica* is from Saudi Arabia (Al-Zahrani *et al.*, 1988). In Pakistan this species is abundant through out the country, and is most probably involved in the transmission of *Leishmania tropica* in the endemic foci.

***Phlebotomus (Paraphlebotomus) alexandri* Sinton, 1928.**

*Phlebotomus sergenti* Var.; Newstead, 1920: 309[♂].

*Phlebotomus sergenti* Var. *alexandri* Sinton, 1928: 308 [♂] Adler Theodor and Lourie, 1930: 533[♀].

*Phlebotomus alexandri*, Sinton 1932: 58, 1933: 418. Lectotype [♂], Iraq(BMNH), designated by Lewis (1982:143) [examined].

*Phlebotomus (Paraphlebotomus) alexandri* Sinton; Theodor, 1958: 19, Theodor and Mesghali, 1964: 290; Perfil'ev, 1966:72-74 [synonymy]; Lewis, 1967 [synonymy, ♂ Pakistan]; Perfil'ev, 1968: 241 [variation]; Lewis, 1978: 235; Artemiev, 1978 [Afghanistan redescription]; Lewis and Buttiker, 1980 [Synonymy]; Lewis, 1982 [Extra facts Saudi Arabia, distribution]; Lane, 1986 [redescription Egypt].

**Males.**

Small rather dark sandfly easily mistaken for *Sergentomyia*. Genitalia very short. Coxite with short, broad basal process with fan shaped tuft of hairs. Style more slender than that of *P.sergenti*. A3 much shorter than labrum.

**Female.**

Pharynx with almost rectangular armature, consisting of numerous long fine teeth, posterior margin of pharynx straight or slightly concave. Asc3 short and blunt. Spermatheca with 6-7 segments.

**Distribution.**

*Phlebotomus alexandri* is usually considered as a highland species but it has also been recorded in suitable lowlands by Lane & Al Taqi (1983). The species is

widely distributed and "Mediterranean" in the widest sense (Theodor and Mesghali, 1964): Greece, Cyprus, Spain, North Africa, Turkey, southern parts of former Soviet Union, Israel, Ethiopia, Yemen, Saudi Arabia, Iraq, Iran, Kuwait, United Arab Emirates, Afghanistan, Pakistan, China. A single specimen was found in Sudan.

*P. alexandri* has been recorded in Pakistan from Waziristan (Sinton, 1928b), Qambar (Sinton 1932), Perkuta (Lewis 1967). During the present studies, *P. alexandri* was recorded from Chilas, Mehidabad, Kuru and Keris (Figure 5.2).

Theodor and Mesghali (1964) commented that this species is widely distributed around the Mediterranean and is apparently always rare. We also captured this species in rare numbers from Northern Areas of Pakistan.

#### **Medical importance.**

*P. alexandri* is the vector of visceral leishmaniasis in Tufan county, Xinjiang Uygur autonomous region, China (Leng and Zhang, 1993). The promastigotes isolated were identified by isoenzyme analysis as *Leishmania donovani* (Desjeux, 1991, Killick-Kendrick, 1990). It might well be a vector of *Leishmania infantum* in areas of the Middle East such as Iraq and Oman (Lane unpublished) where no other potential vector has been found.

#### ***Phlebotomus (Paraphlebotomus) nuri* Lewis, 1967.**

*Phlebotomus (Paraphlebotomus) nuri* Lewis, 1967: 15 [Described ♂ from Pakistan]; 1978b: 236; Artemiev, 1974a: 160, 161; 1978: 17 [Described ♀ from Afghanistan]. Holotype ♂ Pakistan (BMNH) [examined].

Lewis (1967) described the male of *P. nuri* from Rawalpindi and Saidpur

(Islamabad). Later, Artemiev, (1978) described females of *P.nuri* from Afghanistan. In the present survey I did not find this species in the type locality (Rawalpindi, Saidpur) nor from other areas despite sampling there..

**Males:**

This species differs from other *Paraphlebotomus* species in having long terminalia, a long, thick basal process with long curved hairs, long style (longer than *P. caucasicus*), and a conical straight, aedeagus.

**Females:**

Morphometric measurements of females are given by Artemiev (1978). Spermatheca consists of 7-8 segments, with narrow apical segment and deep furrows between the segments.

**Distribution:**

Pakistan (Rawalpindi, Saidpur) (Figure 5.2), Afghanistan and perhaps southern Iran.

*P.nuri* according to Artemiev, (1978), is a thermophilic and moderately hydrophilic mountain species, mainly found in the rocky mountains of southern Afghanistan.

**Medical importance.** Role of *P.nuri* in the transmission of leishmaniasis is not known.



**Subgenus *LARROUSSIUS* Nitzulescu, 1931.**

***Phlebotomus keshishiani* Schurenkova, 1936.**

*Phlebotomus keshishiani* Schurenkova, 1936: 922 [♂ ♀]. Syntypes ♂ ♀, Russia.(Tropical Institute, Tadzhikistan (SSR) Dushanbe). Syntypes male (BMNH) [examined].

*Phlebotomus (Larroussius) keshishiani* Schurenkova; Theodor, 1958: 24; Theodor and Mesghali, 1964: 291 (Description, Iran); Lewis, 1967: 19 (Description, Pakistan); Perfil'ev, 1968: 274 (Description); Artemiev, 1978 :19 (distribution, Afghanistan); Lewis, 1982: 155 (Taxonomic review, distribution)

This species was described from the Pamirs of Tadzhikistan, and reported from Pakistan by Lewis, (1967). Theodor (1958) listed the essential features of this species from the original description and Perfiliev (1968) mainly referred to the description of Theodor (1958).

Lewis (1967) compared specimens with the description of *P. keshishiani* listed by Theodor (1958) and found that specimens from Pakistan were relatively large and males showed a different ascoid distribution (2/3-8, 1/ 9 -15) than those of the Russian specimens (2/3-7; 1/8-15). He also noted variation in the length of palpal segments of Pakistani specimens compared to Russian. During the present study we found *P. keshishiani* males from Northern Areas of Pakistan which showed both types of ascoid distributions as mentioned above. These specimens were therefore measured again to find any other morphological differences between the specimens with two different antennal formulae. This was an important point to resolve because during the present study, it became increasingly obvious that this species could be the vector of visceral leishmaniasis in Northern Areas.

Table 5.3 shows the measurements of wings, head and associated structures of male *P. keshishiani* collected from Chilas (Thor) and Perkuta as well as females. The females from the two localities were measured separately because of the difference of

**Table 5.3. Mean morphological measurements of of male and female *P. keshishiani* from Northern Pakistan.**

CHARACTERS	SYNTYPE	MALES		FEMALES	
	MALE (n=1)	THOR/PKT (n=3)	THOR/PKT (n=10)	THOR (n=10)	PERKUTA (n=10)
ANTENNAL FORMULA	2/3-7; 1/8-15	2/3-7; 1/8-15	2/3-8; 1/9-15	2/3-15	2/3-15
WL	2.68	2.73 ± 0.9 (2.6-2.8)	2.52 ± 0.2 (2.26-2.73)	2.74 ± 0.3 (2.3-3.2)	2.76 ± 0.2 (2.3-2.9)
WW	0.77	0.77 ± 0.03 (0.74-0.82)	0.71 ± 0.04 90.66-0.80)	0.76±0.08 (0.64-0.94)	0.78 ± 0.06 (0.62-0.84)
WL/WW	3.50	3.53 ± 0.11 (3.41-3.69)	3.55 ± 0.2 (3.31-3.55)	3.63 ± 0.1 (3.4- 3.8)	3.52 ± 0.15 (0.45-0.55)
HDL	0.511	0.52 ± 0.02 (0.50-0.54)	0.48 ± 0.04 (0.42-0.53)	0.50 ± 0.04 (0.44-0.57)	0.52 ± 0.03 (0.45-0.55)
HDW	0.465	0.59 ± 0.03 (0.57-0.64)	0.54 ± 0.04 (0.48-0.59)	0.50 ± 0.07 (0.36-0.63)	0.53 ± 0.05 (0.45-0.61)
HDL/HDW	1.098	0.862 ± 0.01 (0.85-0.88)	0.90 ± 0.07 (0.76-1.04)	1.030 ± 0.1 (0.85-1.30)	0.97 ± 0.06 (0.87-1.03)
PHXL	0.218	0.23 ± 0.02 (0.21-0.25)	0.21 ± 0.02 (0.18-0.22)	0.23 ± 0.02 (0.19-0.27)	0.23 ± 0.02 (0.19-0.25)
PHXW	0.083	0.08 ± 0.008 (0.06-0.08)	0.06 ± 0.005 (0.06-0.09)	0.08 ± 0.007 (0.07-0.09)	0.07 ± 0.005 (0.06-0.08)
PHXL/PHXW	2.61	3.1 ± 0.6 (2.62-3.9)	3.44 ± 0.3 (3.04-3.87)	2.95 ± 0.3 (2.5-3.51)	3.35 ± 0.2 (0.3-0.4)
LBL	0.308	0.31 ± 0.02 (0.29-0.33)	0.29 ± 0.02 (0.23- 0.32)	0.38 ± 0.05 (0.31-0.49)	0.37 ± 0.03 (0.30-0.40)
LB/WL	0.115	0.114 ± 0.002 (0.11-0.12)	0.11 ± 0.007 (0.102-0.13)	0.14 ± 0.01 (0.13-0.17)	0.13 ± 0.005 (0.13-0.14)
A3	0.42	0.35 ± 0.008 (0.34-0.36)	0.34 ± 0.03 (0.3-0.38)	0.33 ± 0.04 (0.27-0.42)	0.31 ± 0.02 (0.26-0.34)
A4	0.163	0.141 ± 0.007 (0.131-0.147)	0.144 ± 0.009 (0.133-0.155)	0.128 ± 0.02 (0.110-0.162)	0.123 ± 0.006 (0.113-0.130)
A5	0.161	0.142 ± 0.004 (0.136-0.146)	0.141 ± 0.009 (0.131-0.154)	0.126 ± 0.02 (0.108-0.158)	0.120 ± 0.01 (0.101-0.131)
A3/L	1.359	1.108 ± 0.06 (1.028-1.167)	1.182 ± 0.06 (1.119-1.270)	0.865 ± 0.03 (0.813-0.933)	0.866 ± 0.04 (0.828-0.963)

<b>A3/A4+A5</b>	0.486	0.682 ± 0.4 (0.403-1.215)	1.197 ± 0.04 (1.135-1.243)	1.285 ± 0.04 (1.236-1.363)	1.289 ± 0.07 (1.200-1.392)
<b>A3/WL</b>	0.156	0.156 ± 0.004 (0.120-0.131)	0.135 ± 0.007 (0.128-0.148)	0.119 ± 0.007 (0.113-0.139)	0.114 ± 0.004 (0.107-0.121)
<b>P1</b>	0.057	0.050 ± 0.001 (0.048-0.0522)	0.043 ± 0.004 (0.038-0.049)	0.051 ± 0.005 (0.039-0.061)	0.058 ± 0.009 (0.045-0.076)
<b>P2</b>	0.195	0.188 ± 0.01 (0.169-0.205)	0.172 ± 0.008 (0.162-0.189)	0.205 ± 0.03 (0.166-0.263)	0.203 ± 0.02 (0.165-0.227)
<b>P3</b>	0.149	0.199 ± 0.006 (0.191-0.205)	0.194 ± 0.01 (0.173-0.204)	0.215 ± 0.2 (0.185-0.259)	0.207 ± 0.02 (-.173-0.229)
<b>P4</b>	0.181	0.155 ± 0.006 (0.147-0.162)	0.153 ± 0.004 (0.151-0.159)	0.161 ± 0.02 (0.126-0.207)	0.156 ± 0.018 (0.124-0.191)
<b>P5</b>	0	0.362 ± 0.005 (0.354-0.366)	0.306 ± 0.07 (0.187-0.377)	0.288 ± 0.07 (0.181-0.397)	0.324 ± 0.04 (0.271-0.403)
<b>Asc3</b>	0.043	0.045 ± 0.0002 (0.045-0.046)	0.043 ± 0.004 (0.039-0.049)	0.053 ± 0.005 (0.044-0.063)	0.049 ± 0.006 (0.0356-0.0546)
<b>Asc4</b>	0.044	0.044 ± 0.003 (0.039-0.046)	0.045 ± 0.001 (0.042-0.047)	0.055 ± 0.007 (0.043-0.071)	0.050 ± 0.006 (0.040-0.059)
<b>Asc5</b>	-	0.048 ± 0.001 (0.046-0.050)	0.047 ± 0.002 (0.043-0.052)	0.055 ± 0.009 (0.044-0.078)	0.050 ± 0.006 (0.041-0.060)
<b>Asc4/A4</b>				0.427 ± 0.02 (0.392-0.456)	
<b>SPRNo</b>				17 ± 0.8 (16-18)	
<b>SPL</b>				0.037 ± 0.007 (0.026-0.048)	
<b>TPL</b>				0.015 ± 0.002 (0.011-0.018)	-

Table gives means, standard deviation (±) and ranges (in parentheses).

antennal formula in the male specimens from Chilas and Perkuta. Three males from Chilas showed the antennal formula (2/3-7, 1/8-15) corresponding to the original description (and syntype), whereas the rest of the males both from Chilas and Perkuta had a different antennal formula (i.e. 2/3-8, 1/9-15).

#### **Male.**

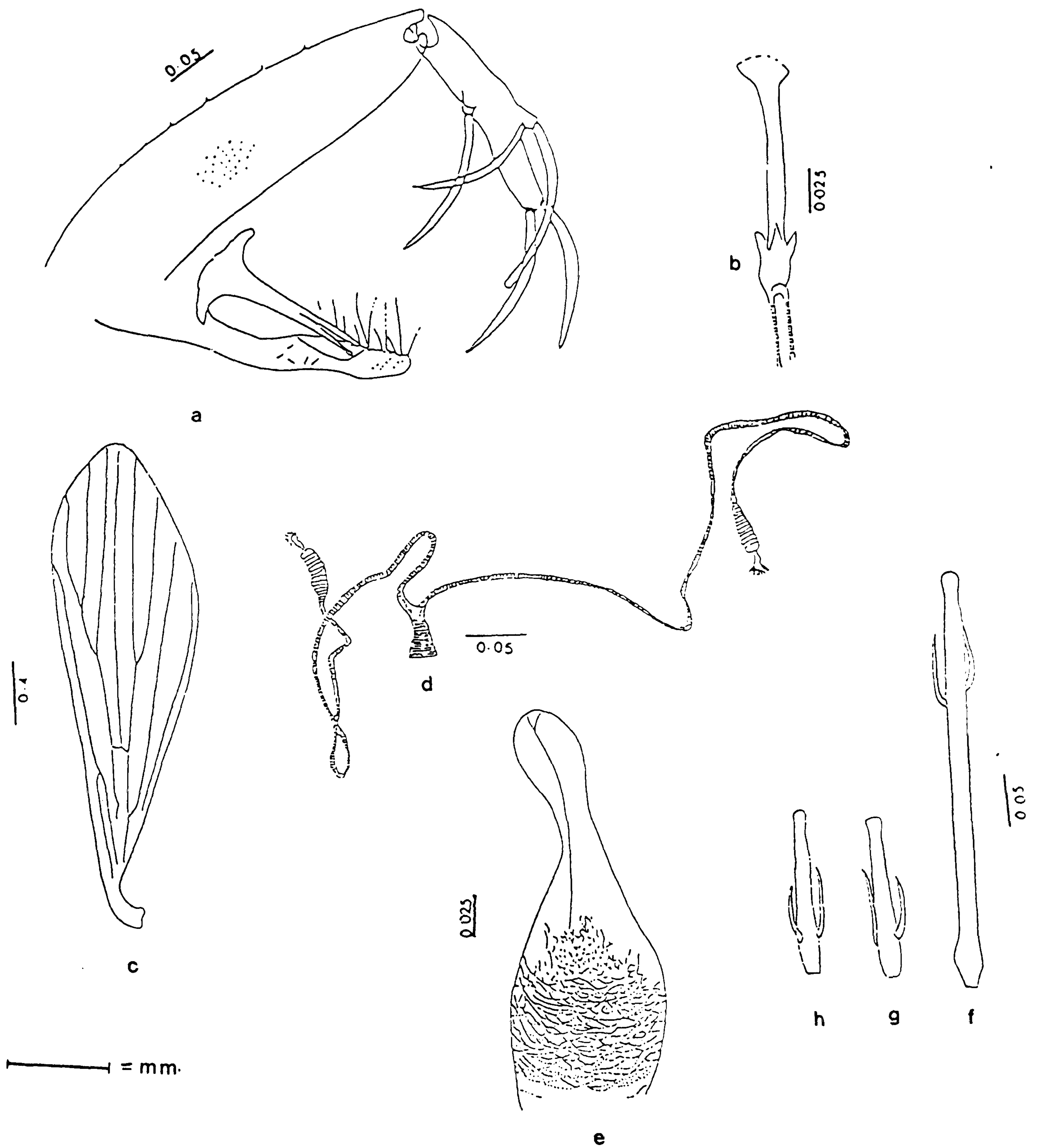
Pharynx armed with punctiform teeth and a few scales anteriorly. A3 greater than A4 + A5. Only three specimens from Chilas (Thor) showed two ascoids on antennal segments 3-7 and one on antennal segments 8-15 (as in the syntype), the remainder of the specimens from Chilas and those from Perkuta showed two ascoids on antennal segments 3-8 and one on antennal segment 9-15. Palpal formula 1,4,2,3,5 as in females.

Style with five spines, two terminal, two sub-terminal and one proximal; tips of spines usually spatulate. Patch of 29 (28-30) hairs present on basal half of coxite. Base of aedeagus comparatively broader than other species of *Larroussius*; aedeagus long, conical with extremely narrow, rounded tips. Parameres shorter than coxites (Figure 5.3a). Genital filaments 3.5 times length of sperm pump.

Measurements of the wings, head and associated structures are given in table 5.3 and the genitalia in table 5.4. Damaged parts of specimens were not measured.

#### **Female.**

The pharyngeal armature occupies almost all the broad part of the pharynx; anterior armature consists of scales with finely serrated margins and short, slightly curved, longitudinal lines laterally, posterior armature consists mainly of concave rows of coarsely dotted broken lines giving most of the pharyngeal armature a beaded



*Phlebotomus keshishiani*, a-b, ♂: a, terminalia; b, sperm pump. c-h, ♀: c, wing; d, spermatheca; e, pharynx; f, g, h, antennal segments 3,4,5

**Table 5.4. Mean morphological measurements of the male genitalia of *P. keshishiani* (syntype and Northern Pakistan samples having different antennal formula).**

CHARACTERS	SYNTYPE	MALES	
	MALE (n=1)	THOR/PKT (n=3)	THOR/PKT (n=10)
ANTENNAL FORMULA	1/3-7; 2/8-15	1/3-7; 2/8-15	1/3-8; 2/8-15
AD/A3	-	-	0.506 ± 0.03 (0.470-0.538)
AD/CXL	0.394	0.437 ± 0.04 (0.385-0.0484)	0.422 ± 0.035 (0.356-0.441)
PARAMERE	0.257	0.034 ± 0.03 (0.216-0.296)	0.246 ± 0.021 (0.224-0.292)
CXL	0.395	0.400 ± 0.02 (0.367-0.420)	0.410 ± 0.03 (0.380-0.466)
CXW	0.0742	0.097 ± 0.004 (0.093-0.103)	0.095 ± 0.008 (0.082-0.099)
CXL/CXW	5.329	4.141 ± 0.2 (3.924-4.426)	4.325 ± 0.4 (3.581-4.662)
CXL/WL	0.028	0.035 ± 0.002 (0.033-0.037)	0.163 ± 0.01 (0.151-0.184)
STL	0.195	0.2009 ± 0.003 (0.198-0.206)	0.197 ± 0.01 (0.180-0.212)
STW	0.025	0.037 ± 0.003 (0.032-0.040)	0.040 ± 0.004 (0.035-0.049)
STL/STW	7.594	5.505 ± 0.5 (4.956-6.247)	4.901 ± 0.444 (3.945-5.294)
STL/WL	0.073	0.074 ± 0.002 (0.071-0.076)	1.214 ± 0.11 (1.040-1.241)
DTSPE	0.188	0.191 ± 0.01 (0.177-0.204)	0.194 ± 0.009 (0.176-0.208)
DSTSPE	0.1456	0.143 ± 0.01 (0.129-0.152)	0.140 ± 0.006 (0.134-0.149)
DMSPE	0.089	0.095 ± 0.009 (0.085-0.108)	0.090 ± 0.009 (0.079-0.104)
PUMP	0.159	0.143 ± 0.01 (0.128-0.154)	0.139 ± 0.007 (0.123-0.144)

<b>GFL</b>	0.824	0.521 ± 0.1 (0.348-0.656)	0.516 ± 0.15 (0.338-0.722)
<b>F/P</b>	5.166	3.654 ± 0.9 (2.372-4.328)	3.695 ± 1.036 (2.333-5.224)
<b>GF/WL</b>	0.308	0.191 ± 0.05 (0.124-0.235)	0.203 ± 0.049 (0.129-0.265)
<b>HRSNo</b>		29 ± 0.8 (28-30)	25 ± 1.8 (21-27)

Table gives means, standard deviation ( $\pm$ ) and ranges (in parentheses).

appearance (Figure 5.3e). Palpal formula 1, 4, 2, 3, 5; Antennal formula 2/3-15 (two ascoids from antennal segments 3 -15).  $A_3 > A_4 + A_5$ .

Spermatheca (Figure 5.3 d) with 16-18 segments and body  $0.037\text{mm.} \pm 0.007$  (0.026-0.048) mm; terminal process rather short length  $0.015 \pm 0.002$  (0.011-0.018) mm; spermathecal ducts very long much longer than other *Larroussius* species and open into a common duct the shape of which is roughly rectangular.

### Discussions.

The morphology and measurements (Table 5.3 and 5.4) of most of characters of male and female specimens collected from Chilas and Parkuta tally fairly well with the early descriptions of Theodor (1958) and Lewis (1967) and with the male syntype in the British Museum (Natural History), London.

One of the most striking morphological similarity between the males from Pakistan and USSR (including the syntype) is the shape of the aedeagus and pharynx. The tip of the aedeagus, being extremely narrow and rounded (Figure 5.3a), differentiates this species from other species of the subgenus *Larroussius*. Lewis (1967) and Theodor and Mesghali (1964) consider this the main character for differentiating males of *P. keshishiani* from those of *P. major* and *P. wenyoni*.

There are however noticeable differences between the males from Pakistan and the Syntype [examined], as well as the early descriptions given for the Russian specimen of *P. keshishiani* by Theodor (1958) and Perfil'ev (1968). The differences are mainly in the relative length of the palpal segments, thus the palpal formula of the specimens from Pakistan (1,4,2,3,5) differs from the syntype (1,3,4,2,5), indicating that



palpal segment 4 is characteristically shorter than the second, third and fifth in Pakistani specimens in contrast to the syntype in which the third palpal segment is shorter than the fourth, second and fifth. This is in agreement with that of the early description of *P. keshishiani* from Pakistan by Lewis (1967).

The relative lengths of the third, fourth and fifth antennal segments [  $A_3=0.344 \pm 0.008$  (0.337-0.356) mm,  $A_4=0.140 \pm 0.007$  (0.131-0.147) mm,  $A_5=0.142 \pm 0.004$  (0.136-0.146) mm] (Figure 5.3 f, g & h) of the specimens from Pakistan are shorter than the measured syntype ( $A_3=0.419$  mm,  $A_4=0.163$  mm,  $A_5=0.161$  mm). The length of the third antennal segment of the Pakistani specimens however fall within the range ( $A_3= 0.33-0.42$  mm) given for the Russian specimens (Theodor, 1958; Perfil'ev, 1968).  $A_3$  is longer than the combined length of  $A_4 + A_5$  as in the descriptions of Lewis (1967), Theodor (1958) and Perfil'ev (1968).

Measurements of the genitalia of males of *P. keshishiani* from Pakistan and the syntype also show differences to Russian descriptions:- the relative length of the genital filaments and the sperm pump (Figure 5.3b) is shorter in the specimens from Pakistan and mean number of hairs on the coxite (Figure 5.3a) in the specimens from Pakistan is greater (29).

In the present study we found males with two different ascoidal distributions on the antennal segments 3-15 (antennal formula). In some, the antennal formula was (2/3-7, 1/8-15) which is the same as in the measured syntypes, and the descriptions of Theodor (1958) and Perfil'ev (1968) for the 'Russian' specimens. In other specimens the antennal formula was (1/3-8, 2/9-15) which conforms to Lewis (1967). However, specimens of the two different antennal formula types did not show any

differences in the measurements of the other characters (table 5.3, 5.4). Variations in the antennal formula is known in different species of the subgenus *Larrousius*; e.g. Parrot (1936) examined 32 specimens of male *P.orientalis* out of which 30 had an antennal formula of 2/3-7, 1/8-15 and 2 had the formula 2/3-8, 1/9-15.

Since no female type specimens of *P.keshishiani* are available in the British Museum (Natural History), London, the measurements of the morphological characters of females *P.keshishiani* from Pakistan were compared with the early descriptions of Theodor (1958), Lewis (1967) and Perfil'ev (1968). The females from Pakistan show no statistical differences in most of the characters measured. The arrangement of the pharyngeal armature and the length of the spermathecal ducts remain the most important character to differentiate this species from *P.major*, *P.major major*, *P.major kriamensis*, *P.neglectus*, *P.syriacus* and *P.wenyoni*. In my opinion, the length of the third antennal segment in relation to the labrum is an important character distinguishing this species from *P.major*: the length of the labrum in *P.keshishiani* is shorter than the length of A3, whereas in *P.major* this ratio is the reverse i.e the labrum is longer than A3.

Like males, the females *P.keshishiani* from Pakistan also showed certain variations compared to previous descriptions of this species. The length of the third, fourth and fifth antennal segments (Figure 5.3 f, g & h) are relatively shorter than 'Russian' specimens. The palpal segments also show variations in their relative lengths, thus the females from Pakistan have the same palpal formula as the males (1,4,2,3,5) in contrary to the formula [1, (2,4), 3, 5] given for the 'Russian' specimens by Perfil'ev (1968) and Theodor (1958). The morphological measurements of female *P.keshishiani*

from Pakistan are however in complete agreement with the descriptions of Lewis (1967).

From the above observations it seems that variations in the length of palpal and antennal segments between specimens from two different regions are clinal, varying in response to climatic and other environmental conditions. However sympatric males with antennal formulæ different from the nominate form need further study; by e.g. enzyme analysis to determine how different they are genetically. Studying isofemale broods may also further clarify the situation. It should be noted that the presence of males with two different antennal formulae in the same locality excludes the possibility of the existence of a subspecies on the grounds that the two populations are not geographically isolated.

#### **Distribution.**

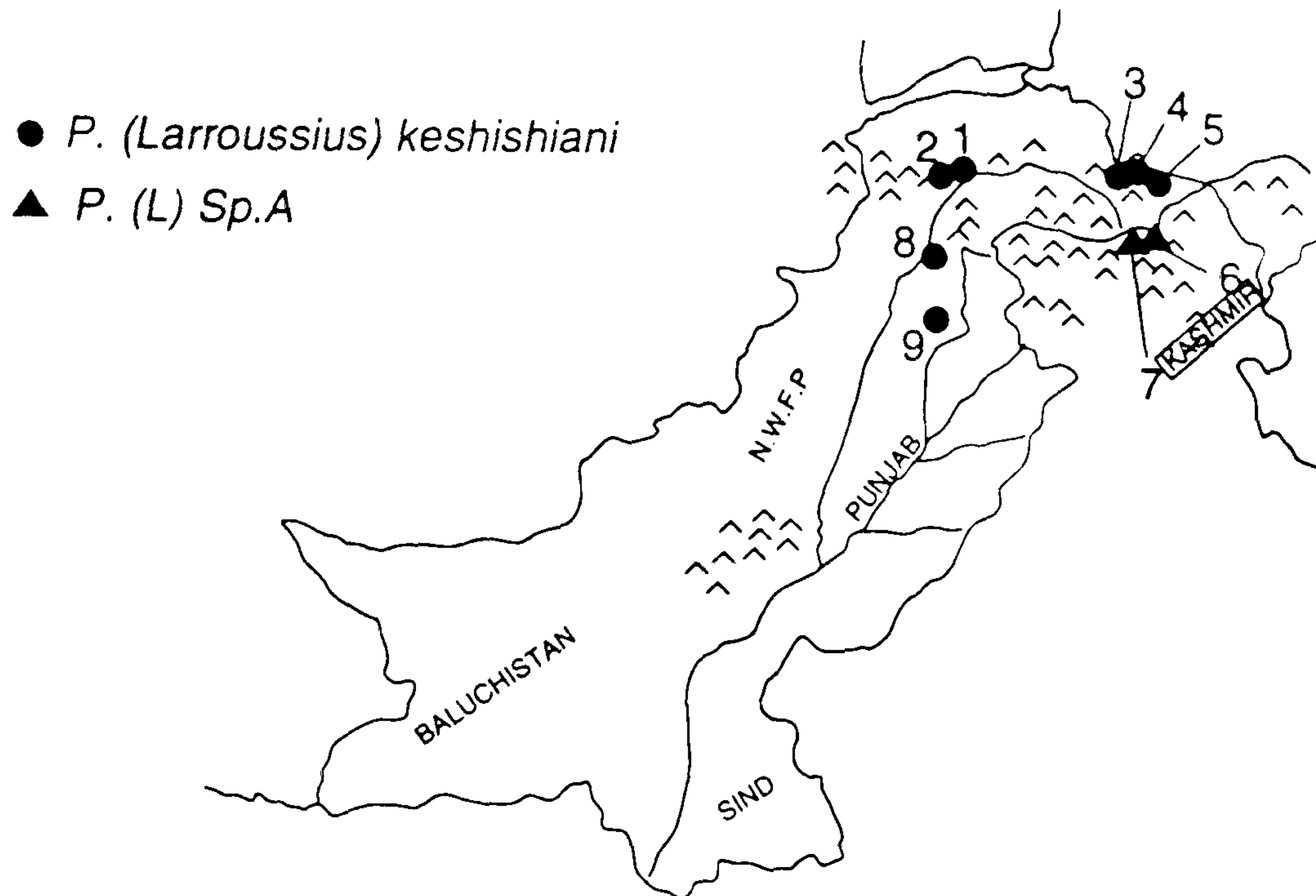
Tajikistan, Afghanistan, Pakistan and Iran. *P. keshishiani* has previously been recorded in Pakistan by Lewis (1967) from Gilgit, Perkuta, Rawalpindi and Saidpur. During the present survey this species was collected from Chilas, Keris, Kuru & Mehdiabad (Perkuta) and the Margella Hills (Islamabad) (Figure 5.4 A).

#### **Medical importance**

The role of *P. keshishiani* in the transmission of leishmaniasis is not yet proved, it is however considered a vector of visceral leishmaniasis by Artemiev (1978).

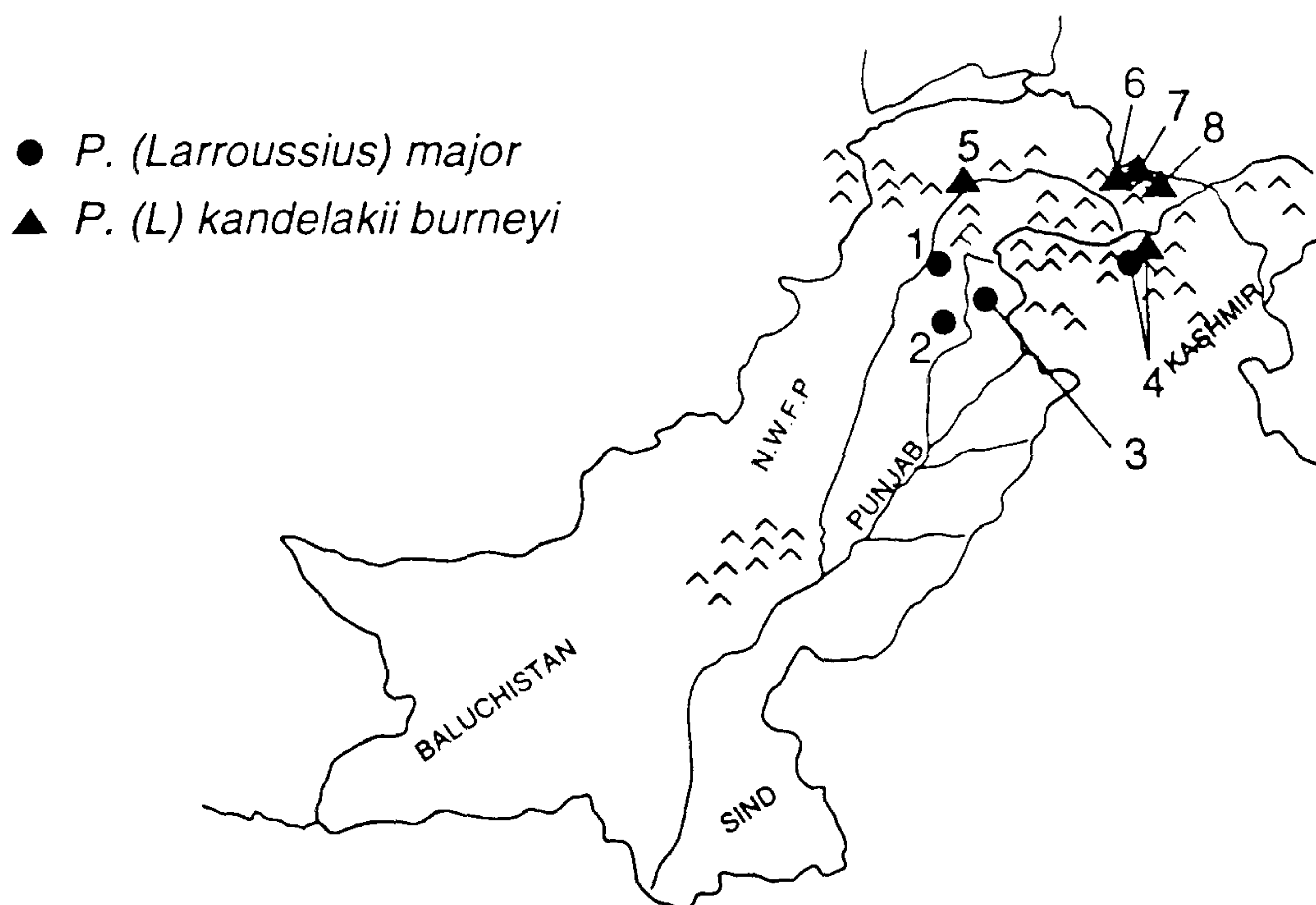
**Figure 5.4**

**A. Geographical distribution of *Phlebotomus keshishiani* & *P. Sp.A* in Pakistan and AJK**



1 = GILGIT; 2 = CHILAS; 3 = MEHDIABAD (PERKUTA); 4 = KERIS;  
5 = KURU; 6 = BANJOSA; 7 = MALOT; 8 = ISLAMABAD; 9 = RAWALPINDI

**B. Geographical distribution of *Phlebotomus major* & *P.kandelakii burneyi* in Pakistan and AJK**



1 = ABBOTABAD; 2 = RAWALPINDI; 3 = ISLAMABAD (MARGELLA HILLS)  
4 = BAGH; 5 = CHILAS; 6 = KERIS; 7 = MEHDIABAD (PERKUTA); 8 = KURU

***Phlebotomus (Larrousius) major* Annandale, 1910.**

*Phlebotomus major* Annandale, 1910:46 [♂ description and distribution]; Summers, 1911:110; 1925:107 [♀]; Sinton, 1928:303 [synonymy]; 1932:59 [key]; 1933:418; Sinton and Barraud, 1928:329 [spermatheca]; Lectotype [♀], India designated by Quate, 1962:157 [examined].

*Phlebotomus major* var. *perniciosus* Newstead [in part]; Brunetti, 1912:201. Synonymized by Perfil'ev, 1968:253.

*Phlebotomus (Larrousius) major* Annandale; Theodor, 1958; Theodor and Mesghali, 1964:291 (Iran); Perfil'ev, 1968:253, 260 (taxonomic notes, distribution, biology and medical importance); Lewis, 1967:21 [Pakistan]; Perfil'ev, 1968:253 [description]; Artemiev, 1978 [description Afghanistan]; Lewis, 1982:157 (taxonomic review and distribution); Lane, 1986 [description Egypt].

*Phlebotomus major* was first described from north-west India by Annandale in 1910, who initially considered it as the Himalayan form of *P. argentipes*, but later described it as a separate species. Franca and Parrot (1921) considered *P. perniciosus* to be a variety of *P. major* and named it as *P. major* var. *perniciosus*. After the introduction of new methods for the identification of sandflies (1926), *P. major* was confirmed as a well defined and valid species. Contrary to the early belief that the distribution of *P. major* is restricted to India only, faunistic studies in the 1920's in southern Europe and western Asia showed that this species has a very wide distribution (Perfil'ev 1968). *P. major* was therefore recorded from India, southwest Asia, and the eastern Mediterranean (Dalmatia, Italy, Crete etc). The forms recorded from areas other than India were later considered as varieties. Thus *P. major* described from western Asia in 1929 was named as *P. major* var. *syriacus* (Adler & Theodor 1931). Subsequently, Theodor (1958) divided *P. major* from western Asia and southern Europe into two sub species: *P. major syriacus* in the eastern Mediterranean and *P. major neglectus* in the central Mediterranean (Yugoslavia and Italy). Perfil'ev (1968) added the third sub species of *P. major* from Crimea in Asia minor as *P. major*

*krimensis* with the nominate subspecies as *P. major major* from Pakistan and northern India (Lewis, 1982). *P. major wui*, described from northern China, is not a member of the *P. major* species group but a synonym of a closely related species (Leng *et al.*, 1987). Due to the substantial geographical variation within these species and the geographical overlap between them, Lane (1986, 1988) suggested that these may not be true subspecies, and should be treated as a group of closely related species.

The first report of *P. major* from Pakistan was that of Lewis (1967) who measured only male specimens collected from Saidpur (Margella hills). On the basis of the relative length of palpal segment 2 and 3, he suggested that the Pakistani sample had some affinity to *P. major syriacus*, but in view of the degree of variation he treated it as *P. major major*.

Female specimens of *P. major* collected in Pakistan were compared with the paralectotype of *P. major* from Naini Tal, India (Indian Museum No. 7118/16) obtained on request from the Zoological survey of India. Due to the inaccessibility of the lectotype type male, specimens from Pakistan were compared with the early descriptions of Theodor (1958) and Lewis, (1967; 1982).

#### **Males.**

Measurements of the wings, head and associated structures are given in table 5.5 and those of the genitalia in Table 5.6. Antennal formula 2/3-8, 1/9-15. Antennal segment 3 greater than combined length of segments A4 + A5 but shorter than labrum. Palpal formula 1, 4, 2, 3, 5.

Style (Figure 5.5a) little less than half coxite length, bearing five spines with spatulate tips, two terminal, two sub terminal and one medial. Aedeagus long, almost

Table 5.5. Morphological measurements of male and female *P. major* and *P.Sp.A*.

CHARACTERS	PARALECTOTYPE	FEMALES		MALES	
	<i>P.major</i> (n=1)	<i>P.major</i> (n=7)	<i>P.Sp.A</i> (n=47)	<i>P.major</i> (n=7)	<i>P.Sp.A</i> (n=28)
WL	2.74	2.8±0.05 (2.7-2.9)	3.3 ± 0.2 (2.16-3.68)	2.4 ± 0.2 (2.2-2.6)	2.9 ± 0.2 (2.4-3.2)
WW	0.8	0.83 ± 0.02 (0.80-0.86)	0.98 ± 0.09 (0.68-1.17)	0.7 ± 0.04 (0.65-0.77)	0.82 ± 0.09 (0.54-0.97)
HDL	0.51	0.50 ± 0.02 (0.47-0.54)	0.55 ± 0.03 (0.47-0.61)	0.44 ± 0.02 (0.41-0.47)	0.50 ± 0.02 (0.44-0.53)
HDW	0.34	0.54 ± 0.04 (0.50-0.61)	0.54 ± 0.04 (0.46-0.63)	0.5 ± 0.04 (0.44-0.47)	0.5 ± 0.04 (0.44-0.62)
PHX	0.24	0.22 ± 0.009 (0.21-0.24)	0.24 ± 0.02 (0.21-0.27)	0.17 ± 0.02 (0.16-0.20)	0.21 ± 0.01 (0.18-0.24)
PHXW	0.09	0.08 ± 0.01 (0.05-0.09)	0.084 ± 0.01 (0.04-0.1)	0.06 ± 0.002 (0.055-0.062)	0.06 ± 0.08 (0.50-0.09)
LBL	0.41	0.39 ± 0.007 (0.38-0.40)	0.46 ± 0.03 (0.39-0.52)	0.27 ± 0.02 (0.25-0.30)	0.33 ± 0.02 (0.30-0.38)
A3	0.39	0.38 ± 0.01 (0.36-0.40)	0.45 ± 0.03 (0.38-0.57) n=45	0.43 ± 0.03 (0.38-0.47)	0.53 ± 0.03 (0.46-0.60) n=26
A4	-	0.15 ± 0.004 (0.15-0.16)	0.17 ± 0.01 (0.15-0.19)	0.17 ± 0.01 (0.15-0.19)	0.22 ± 0.02 (0.16-0.24) n=26
A5	-	0.15 ± 0.003 (0.14-0.15)	0.18 ± 0.11 (0.15-0.20)	0.19 ± 0.01 (0.14-0.18)	0.22 ± 0.02 (0.15-0.24) n=26
P1	0.044	0.06 ± 0.006 (0.05-0.07)	0.061 ± 0.01 (0.035-0.090)	0.04 ± 0.005 (0.036-0.050)	0.05 ± 0.01 (0.03-0.07)
P2	0.253	0.23 ± 0.02 (0.21 - 0.28)	0.25 ± 0.02 (0.18-0.30)	0.18 ± 0.008 (0.16-0.19)	0.20 ± 0.01 (0.18-0.24)
P3	0.214	0.22 ± 0.01 (0.21-0.24)	0.25 ± 0.2 (0.18-0.32)	0.18 ± 0.01 (0.16-0.20)	0.22 ± 0.02 (0.17-0.24)
P4	0.157	0.15 ± 0.01 (0.13-0.17)	0.18 ± 0.01 (0.13-0.21)	0.14 ± 0.03 (0.11-0.21)	0.16 ± 0.02 (0.13-0.415) n=26
P5	0.270	0.35 ± 0.6 * (0.29-0.47)	0.35 ± 0.08 (0.15-0.58)	0.27 ± 0.07 (0.20-0.40)	0.32 ± 0.06 (0.2-0.43)
Asc3	0.055	0.05 ± 0.006 (0.05-0.06)	0.06 ± 0.01 (0.03-0.08)	0.05 ± 0.005 (0.04-0.06)	0.05 ± 0.009 (0.04-0.07) n=26
Asc4	N/A	0.06 ± 0.004 (0.06-0.07)	0.064 ± 0.008 (0.037-0.076)	0.05 ± 0.005 (0.04-0.06)	0.06 ± 0.01 (0.05-0.09)

<b>Asc5</b>	N/A	0.06 ± 0.006 (0.05-0.07)	0.064 ± 0.007 (0.05-0.08)	0.05 ± 0.005 (0.04-0.06)	0.05 ± 0.008 (0.03-0.06) n=26
<b>WL/WW</b>	3.46	3.37 ± 0.050 (3.32-3.45)	3.33 ± 0.16 (2.96 -3.8)	3.50 ± 0.2 (3.25-4.0)	3.7 ± 0.4 (3.2-5.3)
<b>HDL/HDW</b>	1.51	0.9 ± 0.03 (0.87-0.96)	1.02 ± 0.07 (0.87-1.20)	0.88 ± 0.05 (0.8-0.93)	1.00 ± 0.06 (0.82-1.10)
<b>PHXL/PH XW</b>	2.64	3.0 ± 0.3 (2.5-4.6)	2.95 ± 0.30 (2.36-4.6)	3.00 ± 0.2 (2.7-3.3)	3.4 ± 0.4 (2.4-4.0)
<b>LB/A3</b>	1.07	1.02 ± 0.04 (0.98-1.08)	1.02 ± 0.08 (0.76-1.19)	0.64 ± 0.08 (0.54-0.78)	0.63 ± 0.04 (0.56-0.72) n=26
<b>A3/A4 + A5</b>	N/A	1.28 ± 0.04 (1.21-1.32)	1.29 ± 0.1 (1.06-1.57)	1.26 ± 0.05 (1.17-1.33)	1.23 ± 0.06 (1.14-1.47) n=26
<b>LB/WL</b>	0.151	0.14 ± 0.004 (0.13-0.15)	0.47 ± 0.05 (0.34-0.69)	0.40 ± 0.02 (0.37-0.44)	0.11 ± 0.004 (0.10-0.13)
<b>SPBL</b>	N/A	0.058 ± 0.03 (0.04-0.13)	0.052 ± 0.012 (0.03-0.14)	-	-
<b>SPBW</b>	N/A	0.021 ± 0.002 (0.018-0.025)	0.012 ± 0.003 (0.013-0.027)	-	-
<b>SPDL</b>	N/A	0.185 ± 0.04 (0.1-0.23)	0.203 ± 0.07 (0.094-0.36)	-	-
<b>SPL</b>	N/A	0.042 ± 0.006 (0.033-0.048)	0.055 ± 0.01 (0.026-0.068)	-	-
<b>TPL</b>	N/A	0.019 ± 0.001 (0.018-0.022)	0.033 ± 0.005 (0.02-0.0425)	-	-
<b>No.SPR</b>	N/A	13.2 ± 1.03(12-15)	17.4 ± 1.8(16-21)	-	-

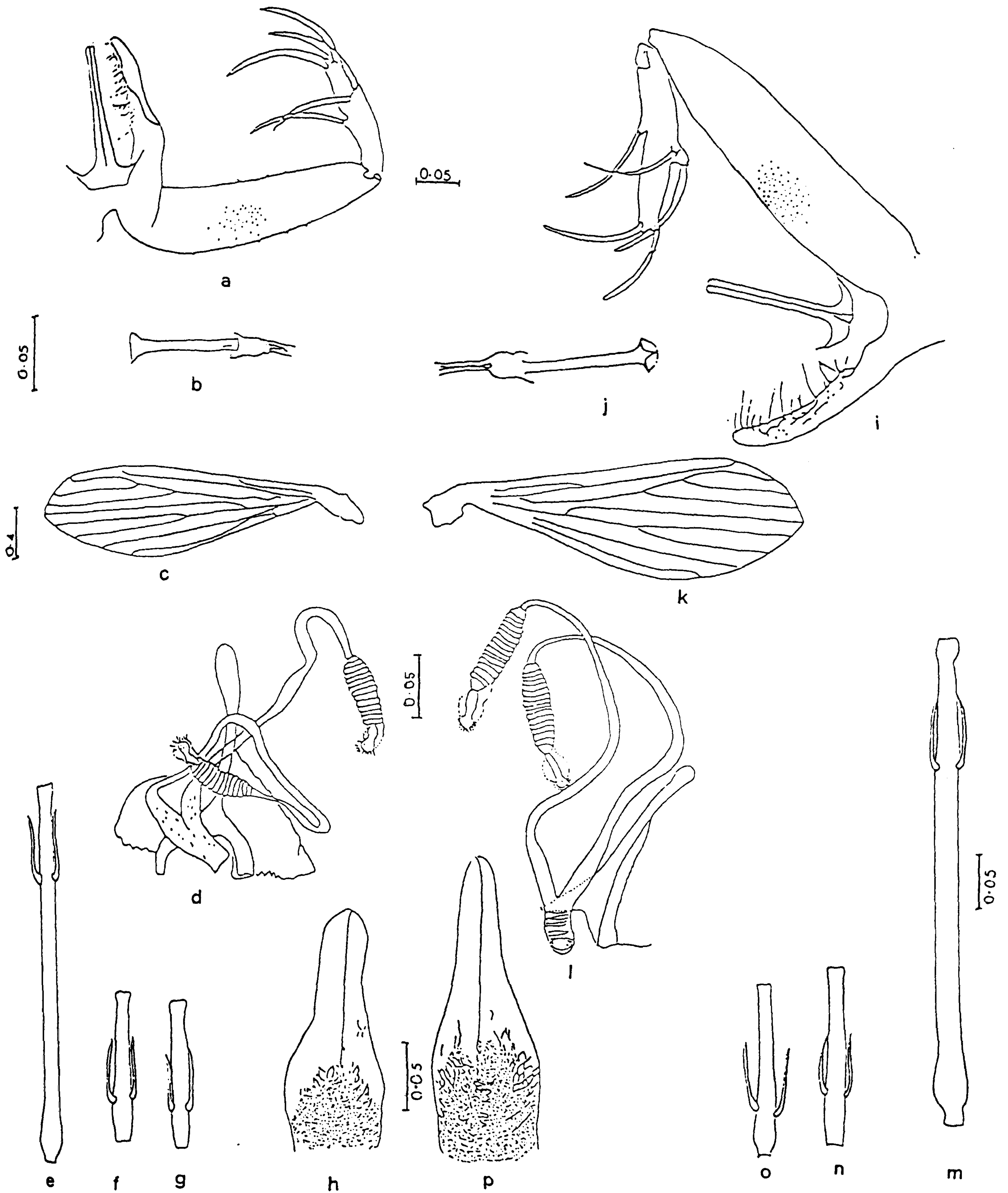
**Table gives means, standard deviation (±) and ranges (in parentheses).**



**Table. 5.6. Morphometric comparison of male genitalia of *P.major* and *Sp.A.* collected from AJK.**

CHARACTERS	<i>P.major</i> n=7	<i>P.sp.A.</i> n=28
ADL	0.214 ± 0.03 (0.196-0.276)	0.226 ± 0.02 (0.168-0.256)
PRL	0.247 ± 0.02 (0.223-0.284)	0.285 ± 0.03 (0.218-0.351)
CXL	0.358 ± 0.01 (0.344-0.382)	0.502 ± 0.06 (0.32-0.57)
CXW	0.085 ± 0.004 (0.08-0.09)	0.102 ± 0.02 (0.044-0.128)
STL	0.183 ± 0.002 (0.180-0.190)	0.27 ± 0.03 (0.196-0.313)
STW	0.041 ± 0.004 (0.033-0.461)	0.041 ± 0.007 (0.026-0.054)
PML	0.145 ± 0.007 (0.136-0.160)	0.178 ± 0.02 (0.144-0.288)
GFL	0.326 ± 0.15 (0.103-0.506)	0.551 ± 0.2 (0.274-1.15)
DTSPE	0.175 ± 0.009 (0.157-0.185)	0.262 ± 0.02 (0.181-0.295)
D SP E	0.126 ± 0.005 (0.118-0.134)	0.168 ± 0.01 (0.124-0.192)
D PSPE	0.073 ± 0.005 (0.063-0.080)	0.114 ± 0.01 (0.074-0.137)
HRS No.	31.2 ± 2.4 (29 - 35)	60.11 ± 11.7 (37 - 90)
RATIO GF/WL	0.465 ± 0.2 (0.145-0.782)	0.191 ± 0.02 (0.95-0.38)
RATIO AD/CX	0.595 ± 0.05 (.542-0.721)	0.451 ± 0.03 (0.396-0.526) n=26
RATIO CX/A3 LENGTH	0.857 ± 0.09 (0.743-0.994)	0.980 ± 0.1 (0.717-1.19) n=26
RATIO CXL/WL	0.514 ± 0.04 (0.45-0.56)	0.173 ± 0.02 (0.125-0.213)
RATIO ADL/A3L	0.512 ± 0.1 (0.416-0.717)	0.434 ± 0.04 (0.36-0.53)
RATIO CX/ST L	1.953 ± 0.05 (1.90-2.06)	0.384 ± 0.07 (0.196-0.313)

Table gives means, standard deviation (±) and ranges (in parentheses).



*Phlebotomus major*, a-b, ♂; c-h, ♀. a: terminalia, b: sperm pump, c: wing, d: spermatheca, e: antennal segment 3, f: antennal segment 4, g: antennal segment 5; h: pharynx. *Phlebotomus* spp. A, I-J, ♂; k-p, ♀. i: terminalia, j: sperm pump, k: wings, l: spermatheca,, m: antennal segment 3, n: antennal segment 4, o: antennal segment 5; p: pharynx.

equal to the length of parameres, with smooth parallel sides and blunt rounded ends. Coxite long, with group of 29-35 hairs on its inner surface.

### **Females.**

Table 5.5 show the morphological measurements of the lectotype female from northern India, and the specimens collected from Pakistan during the present study.

The pharyngeal armature occupies little more than half of the broad part of the pharynx and consists of convex rows of small denticles and short toothed lines, and a few scales with finely serrated margins anteriorly (Fig. 5.5e). Two ascoids on antennal segments three to fifteen. A3 almost equal to length of labrum and longer than combined length of A4 + A5. Palpal formula 1, 4, 2, 3, 5.

Spermatheca (Fig. 5.5d) with 12-15 rings and  $0.042 \pm 0.006$  (0.033-0.048) mm long. Length of terminal process  $0.019 \pm 0.001$  (0.018-0.022) mm. Spermathecal ducts  $0.185 \pm 0.04$  (0.1-0.23) mm long, uniting before common duct. Expanded base  $0.058 \pm 0.03$  (0.038-0.126) mm long and  $0.021 \pm 0.002$  (0.018-0.025) mm wide.

### **Discussion.**

Species of the *P.major* group have conventionally been distinguished by differences in their absolute size, proportion of the pharynx occupied by pharyngeal armature, and the number of hairs on male coxites. Although males can be identified by the conventional morphological methods, it is sometimes almost impossible to distinguish sympatric females. A more practical and reliable way of distinguishing sympatric female *Larrousius* is by examining the morphology of the base of the spermathecal ducts. This method originally introduced by Leger *et al.*, (1983), has been successfully used to identify some sympatric females of the subgenus *Larrousius*

(Killick-Kendrick, <sup>et al.</sup> 1991). We used both morphometrics and the base of the spermathecal ducts to identify specimens from Pakistan.

The morphological measurements (Table 5.5 & 5.6 ) of female *P. major* specimens from Pakistan and of the lectotype do not show any significant differences in almost all the characters measured. The pharynx of Pakistani specimens has an armature typical of *P. major* from India, except that the anterior scales look slightly larger in the Pakistani forms. It differs from *P. neglectus* in lacking lateral scales anteriorly, and from *P. syriacus* in occupying little more than half the length of the pharynx (Fig. 5.5e). However, the ratio of armature: pharynx length is not a reliable character as it is hard to determine the limits of the armature with any degree of accuracy, as observed by Lane (1988).

The length of the spermathecal ducts as well as the length and width of the expanded base (following Leger *et al.*, 1983) could not be measured for the lectotype which is mounted in Canada balsam. However, the shape of the dissected-out base of Pakistani specimens was compared with the drawings of the spermathecal base of *P. neglectus* (Leger *et al.*; 1983; Killick-Kendrick, <sup>et al.</sup> 1991) and *P. syriacus* (Killick-Kendrick, <sup>et al.</sup> 1991). Specimens from Pakistan differ from *P. neglectus* in the length of the spermathecal base (shorter in Pakistani specimens). However, I did not find much difference in the length and shape of the spermathecal base of *P. major* from Pakistan and of *P. syriacus* from Syria. Although the length of the base in the latter species looks a little longer and less broader than the former, this difference does not seem to be significant. Whether the relative length and width of the base of spermathecal ducts is a reliable character to differentiate between species in this species group or

is simply clinal variation needs to be investigated further.

The only difference noticed between the female of *P. major* from India and the specimens from Pakistan is in the length of the palpal segments. The former has the palpal formula of 1, 4, (2, 3), 5, and the Pakistani specimens 1, 4, 2, 3, 5. However, this is a small difference. The relative length of palpal segment 5 in the specimens from Pakistan is significantly longer than *P. major* from India. Interestingly the length of palpal segment 2 is also always shorter than palpal segment 3 in *P. neglectus* (Theodor, 1958; Perfil'ev, 1968), but then there is a clear difference in the length of the base of spermathecal ducts in both the species as mentioned above.

No notable differences were observed in almost all the measurements of males *P. major* from Pakistan and previous descriptions, except that the males also showed the same palpal variations as for the females. On the basis of palpal variation alone, Lewis (1967) suggested that the males from Pakistan show some affinity to *P. syriacus*, but in view of the degree of the variation he treated them as a separate subspecies of *P. major major*.

The number of hairs on the coxites (Figure 5.4a) of male *P. major* from Pakistan [31(29 - 35)] show a considerable overlap when compared with *P. syriacus* (about 30) and *P. neglectus* (28-38). Similarly Lane (1988) showed that the typical form (from the Indian subcontinent) could not be differentiated from *P. syriacus* and *P. neglectus* (from the Mediterranean). According to him this character shows a remarkable cline especially in *P. syriacus* and that the number of hairs increases in the samples from Greece, Yugoslavia and eastern Turkey, through Syria down to Israel.

In view of the overlap in various morphological characters in the species of

the *P. major* group and to understand if these differ genetically, recently established techniques of chemotaxonomy (enzyme characterization) and molecular taxonomy (DNA sequencing) may be helpful in solving this ambiguity.

### **Distribution.**

India (along the Himalayas), Nepal and Pakistan. In Pakistan this species has been recorded by Lewis (1967) from Abbottabad, Rawalpindi and Saidpur. During the present survey *P. major* was collected from Margella hills near Islamabad and Bagh district of Azad Jammu and Kashmir (Figure 5.4 A).

### **Medical Importance.**

*P. major* is the suspected vector of visceral leishmaniasis according to Artemiev, (1978) but Killick-Kendrick, (1990a) did not include this species in his list of suspected vectors of *Leishmania infantum*. The closely related species *P. syriacus* is on the contrary considered to be the vector of visceral leishmaniasis in the eastern Mediterranean area (Lane, 1986) and *P. neglectus* as the suspected vector of *Leishmania infantum* in Greece (Killick Kendrick, 1990a)

### **Phlebotomus (Larrousius) sp. A new species.**

This species has been collected only from altitudes above 1500 metres in Azad Jammu & Kashmir (28 males, 47 females). The species is a member of the *Phlebotomus major* group by its general appearance. However, measurements of the various morphological characters and comparison of the means of both sexes show that all parts of *P. Sp.A* are bigger than those of *P. major* ( $P < 0.001$ ) from the same

general area (Tables 5.5 & 5.6). The characters which do not show a statistically significant difference between *P.sp.A* and *P.major* include, head width, A5 length, Pharynx width, and P3 length in the females and aedeagus length, paramere length, head width, pharynx width and P5 length in the males.

#### **Male.**

Measurements of wings, head and the associated structures are given in Table 5.5 and those of the genitalia in Table 5.6. Antennal formula 2/3-8, 1/9-15. A3  $0.53 \pm 0.03$  (0.50-0.60) mm. long, longer than A4 + A5. Labrum distinctly shorter than A3. Palpal formula 1, 4, (2, 3), 5 as in the females.

Five spines on style (Figure 5.5i) positioned as follows: 2 terminal spines at a distance of  $0.3 \pm 0.02$  (0.2-0.3) mm; middle spine  $0.17 \pm 0.01$  (0.12-0.19) mm and posterior spine is  $0.114 \pm 0.01$  (0.07-0.1) mm from the proximal end of style. Tips of spines usually spatulate. Patch of  $60 \pm 11.7$  (37-90) hairs near basal half of the coxite. Aedeagus as in *P. major* (Figure 5.5i).

#### **Females.**

Measurements of the wings, head and associated characters are given in Table 5.5. Pharynx with armature occupying posterior quarter, anterior edge forming a convex line; posteriorly, pharyngeal armature consisting of irregular rows of tightly-packed fine and coarse denticles extending forward little beyond the broad part of the pharynx; anterior part consists of the well developed scales laterally.

A3 (Figure 5.5 n)  $0.45 \pm 0.03$  (0.39-0.51) mm. long; longer than A4 + A5 but almost equal or slightly shorter than labrum. Two ascoids on antennal segments 3-15. Palpal formula 1, 4,(3,2), 5.

Spermatheca (Figure 5.5 l) with 16-21 rings; capsule  $0.055 \pm 0.01$  (0.026-0.043) mm long; terminal process  $0.033 \pm 0.005$  (0.02-0.043) mm; spermathecal duct

$0.2 \pm 0.07$  (0.094-0.36) mm long. Base of the spermathecal ducts roughly rectangular, individual spermathecal ducts unite posteriorly to form a common duct which in turn opens into the genital atrium.

### **Distribution.**

During the present survey this species was found widely distributed in Azad Jammu & Kashmir (active focus of visceral leishmaniasis caused by *Leishmania infantum*) at altitudes of more than 1500 metres above sea level (Figure 5.4B). Specimens were collected from three localities namely Gali Malot (1700 metres), Beesbagla (1890 metres) and Banjosa (1800 metres). Both males and females were found to be mainly exophilic and phototropic. A few specimens were also caught inside living rooms and animal shelters.

### **Discussion.**

Morphologically this species is very similar to *P. major* particularly in the shape of the male aedeagus, which typically has smooth parallel sides with a broad rounded end, and the presence of a common spermathecal duct in females.

The females of *P. major* and *P.sp.A* can be distinguished on several features (Table 5.5) specifically, the pharyngeal armature (Figure 5.5 e,m) and proportion of head length to its width. The Labrum ( $t=6.10$ ,  $P < 0.001$ ) and the Antennal segments 3, and 4 ( $t=5.2$ ,  $2.10$ ,  $P < 0.05$ ) are significantly longer in the new species than those of *P. major*. The number of spermathecal rings in Sp.A is 16-21 as compared to 12-15 in *P. major*. The spermathecal process is also significantly longer ( $t=7.33$   $P < 0.001$ ) in *P.Sp.A* than *P. major* (Table 5.4). There is however, no significant difference between the length of the spermathecal ducts and length of the base of <sup>the</sup>spermathecal



ducts.

Males of *Sp.A.* can be readily distinguished from those of *P.major*. Measurements of the genitalia of males of both species (Table 5.6) show that all parts except the aedeagus and paramere length, are significantly bigger ( $P < 0.001$ ) than those of *P.major*. The aedeagus is almost equal to the length of parameres in *P.major* whereas it is shorter than the parameres in *Sp.A.* Antennal segments 3, 4 and 5 ( $t=7.7, 6.2, 3.7; P < 0.001$ ) are longer than those of *P.major*, as are the lengths of the genital pump ( $t=4.1, P < 0.001$ ) and the genital filaments ( $t=2.8, P < 0.05$ ). *Phlebotomus Sp.A.* has  $60 \pm 11.7$  (37-90) hairs on the basal half of its coxite, *P.major* has  $31 \pm 2.4$  (29-35). The style ( $t=7.4, P < 0.001$ ) and coxite ( $t=6.1, P < 0.001$ ) are significantly longer in *P.Sp.A* and the distances between terminal, posterior and the middle spines ( $t=10.9, 10.5, 10.2; P < 0.001$ ) also vary significantly between the two species. A3, A4, A5 length ( $t=7.7, 6.2, 3.7; P < 0.001$ ); P2, P3, P4 length ( $t=4.8, 5.01, 2.08; P < 0.005$ ) and labrum length ( $t=7, P < 0.001$ ) also show a significant difference between male *P.sp.A* and *P.major*

***Phlebotomus (Larrousius) kandelakii burneyi* Lewis, 1967.**

*Phlebotomus kandelakii* Schourenkova 1929; Barnet and Suyemoto, 1961: 616; Nasir, 1964:26.

*Phlebotomus kandelakii kandelakii* Schourenkova, 1929: 693: [ $\sigma$  ♀]. Adler, Theodor & Lourie, 1930: 536. Syntypes, USSR, Georgia, Tbilisi (Tiflis) [examined in BMNH].

*Phlebotomus (Larrousius) kandelakii burneyi* Lewis, 1967: 17 [ $\sigma$  ♀]; 1978: 238, 1982: 154; Artemiev, 1974a:160. Holotype  $\sigma$ . Pakistan [British Museum Natural History] London. [Examined].

Material examined. 12  $\sigma$  and 4 ♀ (Paratypes); 12  $\sigma$  and 1 ♀ (Northern Areas), 8  $\sigma$  and 10 ♀ (Iran); 9  $\sigma$  and 10 ♀ (USSR)

*Phlebotomus kandelakii* was first described from Georgia in the former USSR (Tiflis) in 1929 (Schourenkova, 1929). Two subspecies exist *P.kandelakii kandelakii* (Afghanistan, Iran, Turkey, USSR) (Seccombe *et al.*, 1993) and *P.kandelakii burneyi* (Pakistan) (Lewis, 1967).

*Phlebotomus kandelakii burneyi* was first described from Northern Pakistan as a subspecies of *Phlebotomus kandelakii* by Lewis (1967). The distinguishing character on which this species was differentiated from *P. kandelakii kandelakii* was the width of the distal part of the paramere (wider in *P.k.burneyi*) of the male and the length of palpal segment 5 in females. Lewis suggested further studies to show if any other morphological differences occur between these two subspecies. During the present study specimens of *P.kandelakii burneyi* were collected from the type locality (Kuru 2500 metres) as well as from Keris (2400 metres) and Mehdiabad (2300 metres) in Northern Pakistan. To understand if this subspecies differs significantly from *P.k. kandelakii*, I compared specimens of *Phlebotomus kandelakii kandelakii* from Russia and Iran with type specimens (Paratypes) of *P. kandelakii burneyi* from the collections of BMNH, London and also with wild caught specimens from Pakistan (specimens caught during the present study).

*Phlebotomus kandelakii* differs from other *Larroussi* species in the number of spermathecal segments (30-35) of the females and the aedeagus (fine ventral teeth) and paramere (bears a short ventral process with 6-7 spines) of males. Following is the description of males and females of *P. kandelakii burneyi* from Pakistan.

#### **Males.**

Pharynx with smaller armature than females. Labrum, 0.2 (0.19-0.21) mm. long; Width of distal part of paramere 0.034 (0.029-0.036) mm. Coxite with 17 (11-21) hairs in a group. Antennal formula, 2/3-7, 1/8-15 (2/3-5, 1/6-15 in *P. kandelakii kandelakii*). Paramere with a short subbasal ventral process bearing seven spines.

Aedeagus conical with blunt, transparent tip and a row of very fine ventral teeth which are mainly on the basal half. Morphological measurements of male characters for *P. kandelakii burneyi* and *P. kandelakii kandelakii* from Iran and USSR are given in table 5.7.

### **Females.**

Table 5.8 show the morphological measurements of various female characters. Pharyngeal armature 0.05 the length of the pharynx (same as in *P. k.kandelakii* from USSR), anterior teeth scale-like and bearing spicules, rest small denticles. Labrum 0.27 (0.26-0.27)mm, P5 0.67mm longer than *P. kandelakii kandelakii* (0.34 mm); Antennal segment 3 = 0.25 (0.22-0.26) mm. in length. Two ascoids on antennal segments 3-15.

### **Comparison of *Phlebotomus kandelakii* sub species:**

Tables 5.7 and 5.8 summarize the means, standard deviations and ranges for male and female characters measured. Of the 11 characters observed in males, only two characters, the depth of the paramere and length of palpal segment showed significant differences between *P.kandelakii burneyi* from Pakistan, and *P. kandelakii kandelakii* from Iran ( $t=9.4$ ,  $P < 0.001$ ) and USSR ( $t=31.3$ ,  $P < 0.001$ ). In the 9 females characters observed, the mean length of palpal segment was the only character that showed a significant difference between *P. kandelakii burneyi* and *P.k*

Table 5.7. Mean morphometric measurements (mm). of male *P.kandelakii burneyi* from Pakistan and *P.kandelakii kandelakii* from Iran and USSR.

CHARACTERS	<i>P.k burneyi</i> (Pakistan) n=12	<i>P.K.burneyi</i> Wild caught specimen n=12	<i>P.k kandelakii</i> (Iran) n=8	<i>P. kandelakii</i> (USSR) n=9
A3	0.28 ± 0.193 (0.243-0.319)	0.296 ± 0.017 (0.26-0.33)	0.234 ± 0.078 (0.136-0.225)	0.260 ± 0.004 (0.254-0.270)
A4	0.130 ± 0.08 (0.111-0.145)	0.141 ± 0.005 (0.11-0.15)	0.106 ± 0.078 (0.115-0.126)	0.118 ± 0.003 (0.115-0.126)
LBL	0.197 ± 0.006 (0.185-0.206)	0.217 ± 0.012 (0.21-0.23)	0.187 ± 0.065 (0.189-0.231)	0.205 ± 0.006 (0.193-0.214)
HDL	0.378 ± 0.01 (0.353-0.391)	0.382 ± 0.02 (0.36-0.41)	0.342 ± 0.119 (0.338-0.407)	0.370 ± 0.01 (0.355-0.386)
P5	0.404 ± 0.02 (0.340-0.441)	0.242 ± 0.027 (0.19-0.28)	0.339 ± 0.1 (0.340-0.422)	0.295 ± 0.03 (0.262-0.342)
P4	0.132 ± 0.09 (0.115-0.151)	0.105 ± 0.009 (0.09-0.12)	0.113 ± 0.04 (0.096-0.138)	0.116 ± 0.2 (0.101-0.160)
P3	0.147 ± 0.1 (0.130-0.176)	0.152 ± 0.12 (0.13-0.17)	0.136 ± 0.045 (0.134-0.160)	0.144 ± 0.008 (0.126-0.155)
CXL	0.287 ± 0.005 (0.277-0.289)	0.291 ± 0.015 (0.27-0.30)	0.220 ± 0.077 (0.227-0.273)	0.250 ± 0.08 (0.235-0.260)
STL	0.147 ± 0.007 (0.130-0.160)	0.166 ± 0.10 (0.15-0.2)	0.104 ± 0.03 (0.109-0.115)	0.120 ± 0.006 (0.109-0.130)
NoHRS	17 ± 2.0 (11-21)	16.9 ± (16-17)	13 ± 5.0 (9-21)	17 ± 2.0 (14-20)
PMD	0.034 ± 0.001 (0.029-0.036)	0.035 ± 0.002 (0.032-0.037)	0.018 ± 0.006 (0.017-0.021)	0.021 ± 0.001 (0.019-0.021)

Table gives means, standard deviation (±) and ranges (in parentheses).

**TABLE 5.8. Mean morphometric measurements (mm). of females *Phlebotomus kandelakii burneyi* from Pakistan and *P.kandelakii kandelakii* from Iran and USSR.**

PARAMETERS	<i>P.k burneyi</i> (Pakistan) n=4	<i>P.k burneyi</i> (Wild caught specimen) n=1	<i>P.k kandelakii</i> (Iran) n=10	<i>P.k kandelakii</i> (USSR) n=10
<b>A3</b>	0.247 ± 0.2 (0.218-0.264)	0.257	0.213 ± 0.3 (0.172-0.241)	0.236 ± 0.004 (0.193-0.240)
<b>A4</b>	0.105 ± 0.005 (0.097-0.109)	0.108	0.092 ± 0.007 (0.080-0.105)	0.100 ± 0.007 (0.084-0.103)
<b>LBL</b>	0.265 ± 0.003 (0.260-0.269)		0.277 ± 0.2 (0.252-0.302)	0.305 ± 0.006 (0.277-0.317)
<b>HDL</b>	0.425 ± 0.2 (0.395-0.441)	0.458	0.403 ± 0.3 (0.365-0.435)	0.427 ± 0.008 (0.400-0.441)
<b>PHXL</b>	0.190 ± 0.002 (0.189-0.193)	0.187	0.184 ± 0.01 (0.151-0.202)	0.191 ± 0.004 (0.175-0.200)
<b>PHARM</b>	0.052 ± 0.004 (0.048-0.059)	0.057	0.043 ± 0.007 (0.034-0.048)	0.056 ± 0.004 (0.042-0.063)
<b>P5</b>	0.672 ± 0.07 (0.570-0.780)	-	0.338 ± 0.03 (0.300-0.390)	0.345 ± 0.02 (0.302-0.458)
<b>P4</b>	0.131 ± 0.009 (0.122-0.145)	0.137	0.137 ± 0.1 (0.118-0.160)	0.139 ± 0.008 (0.170-0.190)
<b>P3</b>	0.162 ± 0.002 (0.160-0.164)	0.168	0.170 ± 0.01 (0.150-0.185)	0.180 ± 0.008 (0.170-0.190)

Table gives means, standard deviation (±) and ranges (in parentheses).

*kandelakii* from Iran ( $t=12.9$ ,  $P < 0.001$ ) and USSR ( $t=26.1$ ,  $P < 0.001$ ). Comparison between the specimens of *P.k burneyi* caught during this study and the type specimens showed significant differences in the mean length of P5 ( $t=14.9$ ,  $P < 0.001$ ).

### **Discussions.**

Comparison of morphological measurements (Tables 5.7 & 5.8) of the two subspecies *P. kandelakii kandelakii* and *P. kandelakii burneyi* revealed that males can be confidently differentiated on the width of the distal part of the paramere. Although the length of P5 in males also showed significant differences, in view of the variation in this character between the type specimens and the newly caught *P.k burneyi*, this character cannot be relied upon. Females differed only in the length of palpal segment 5, but the numbers of *P.k burneyi* were too small for a statistically valid comparison.

Although the results indicate that the two taxa *P. kandelakii burneyi* and *P.kandelakii kandelakii* can be distinguished, the question remains whether these represent two subspecies or separate species. By definition, a subspecies is an aggregate of phenotypically similar populations of a species inhabiting a geographic subdivision of the range of species, and differing taxonomically from other populations of the species (Mayr, 1969). In allopatric or geographical speciation, the essential feature is that the populations become spatially separated by any one of a range of barriers such as advancing ice caps, rising water levels, interstitial areas of hostile environment. Such separated populations become genetically differentiated due to genetic drift and natural selection in the new environment (Lane & Marshall, 1981). The significant variation in the width of male parameres of the two taxa could be the

result of such geographical isolation.

It must be kept in mind that sometimes geographical discontinuities between the samples could simply be the result of non-availability of material from the intervening regions; as collections become more comprehensive variation between two previously designated subspecies is sometimes found to be clinal and the discontinuities not as clear-cut as originally thought. It is therefore stressed that long term sampling of *P.k burneyi* from Northern Areas of Pakistan over the entire season, and complete information on its biology and ecology will be helpful in solving this problem. Till the time this information is made available I agree to accept *P.kandelakii burneyi* (Lewis, 1967) as a subspecies .

#### **Distribution.**

Afghanistan, Iran, Armenia, Azerbaidzhan, Georgia, Transcaucasia  
(Figure 5.4B).

#### **Medical Importance.**

*Phlebotomus kandelakii kandelakii* is a suspected vector of visceral leishmaniasis on ecological grounds in 'Transcaucasia' (Dergacheva, 1979; Safjanova, 1979). It was found infected with *Leishmania* promastigotes in Armenia (Sergiev, 1979). It is a suspected vector of *Leishmania infantum* rather than *Leishmania donovani* in USSR (Killick-Kendrick, 1990).

## Subgenus ADLERIUS Nitzulescu, 1931.

### *Phlebotomus (Adlerius) hindustanicus* Theodor 1958.

*Phlebotomus (Adlerius) chinensis hindustanicus* Theodor, 1958:29, 30 [description male female]. Syntype male, North-West of India (BMNH) [examined].

*Phlebotomus (Adlerius) chinensis longiductus* Parrot; Lewis, 1967:21 [Misidentification].

*Phlebotomus (Adlerius) hindustanicus* Theodor 1958; Artemiev, 1978:23; 1980: 1191.

*P. (Adlerius) hindustanicus* was first described as a subspecies of *P. chinensis* from India and later upgraded to a species by Artemiev, (1978). The species was first recorded from Pakistan by Lewis, (1967) as *P. (Adlerius) chinensis longiductus*. In his review on the taxonomy of genus *Phlebotomus* (Lewis, 1982) he recognised that he had misidentified the specimens from Pakistan and that they were in fact *P. hindustanicus*.

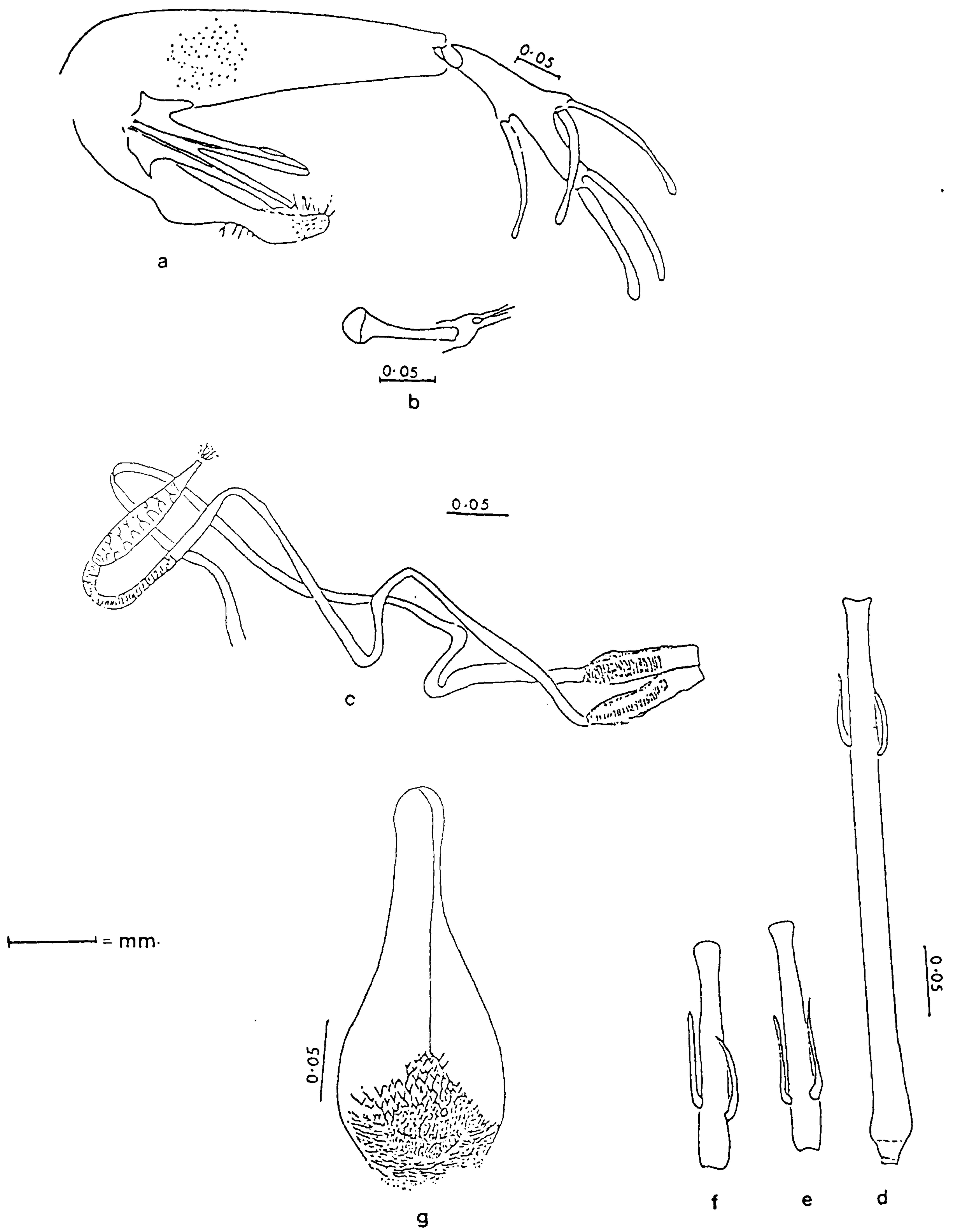
#### **Males.**

Large, moderately pigmented sandfly. Antennal formula 2/3-7,1/8-15. Some specimens with one long and one rudimentary ascoid on A7. Coxite with a group of 87 (45-96) hairs mainly on the basal half (Figure 5.6 a). Distal border of the hairy patch at 0.5 (0.45-0.58) length of coxite. Paramere with slightly widened distal part and short neck. Aedeagus with subterminal tooth at 22 (14-26)  $\mu\text{m}$  from the end (Figure 5.6a). Genital filaments 1067 (844-1286)  $\mu\text{m}$ . long; F/P ratio=9.8 (7.9-11.8). Table 5.9 show the morphological measurements of different male characters including genitalia.

**Females.** Table 5.10. gives the morphological measurements of female



FIGURE 5.6



*Phlebotomus hindustanicus*, a-b, ♂; c-g ♀. a, terminalia; b, sperm pump; c, spermatheca; d, pharynx; d, e, f, antennal segment 3, 4, 5; g: Pharynx

**Table 5.9. Mean morphological measurements of various characters of male *P.hindustanicus* and *P.salangensis*.**

<b>CHARACTERS</b>	<b><i>P.hindustanicus</i> (n=29)</b>	<b><i>P.salangensis</i> (n=10)</b>
<b>Antennal formula</b>	1/3-7; 2/8-15	1/3-5; 2/6-15
<b>WL</b>	2.514 ± 0.1 (2.23-2.67)	2.241 ± 0.1 (2.012-2.446)
<b>WW</b>	0.683 ± 0.041 (0.60-0.75)	0.622 ± 0.05 (0.552-0.734)
<b>WL/WW</b>	3.687 ± 0.2 (3.43-4.03)	3.61 ± 0.2 (3.33-3.83)
<b>HDL</b>	0.466 ± 0.02 (0.42-0.50)	0.442 ± 0.02 (0.408-0.478)
<b>HDW</b>	0.454 ± 0.04 (0.391-0.543)	0.440 ± 0.04 (0.382-0.494)
<b>HDL/HDW</b>	1.033 ± 0.07 (0.88-1.17)	1.009 ± 0.008 (0.88-1.13)
<b>PHXL</b>	0.207 ± 0.007 (0.18-0.23)	0.194 ± 0.01 (0.173-0.212)
<b>PHXW</b>	0.067 ± 0.007 (0.06-0.09)	0.057 ± 0.006 (0.049-0.07)
<b>PHXL/PHXW</b>	3.09 ± 0.3 (2.34-3.59)	3.404 ± 0.4 (2.74-3.85)
<b>LBL</b>	0.310 ± 0.02 (0.28-0.35)	0.261 ± 0.02 (0.232-0.300)
<b>LB/WL</b>	0.123 ± 0.005 (0.106-0.133)	0.117 ± 0.01 (0.105-0.141)
<b>A3</b>	0.441 ± 0.03 (0.4-0.5)	0.332 ± 0.03 (0.294-0.381)
<b>A4</b>	0.167 ± 0.1 (0.15-0.20)	0.130 ± 0.009 (0.116-0.145)
<b>A5</b>	0.164 ± 0.009 (0.15-0.20)	0.128 ± 0.01 (0.115-0.151)
<b>A3/LB</b>	1.417 ± 0.07 (1.208-1.559)	1.27 ± 0.08 (1.124-1.426)
<b>A3/A4 + A5</b>	1.325 ± 0.06 (1.160-1.465)	1.28 ± 0.04 (1.197-1.34)
<b>P1</b>	0.054 ± 0.008 (0.01-0.07)	0.046 ± 0.007 (0.038-0.0622)
<b>P2</b>	0.190 ± 0.02 (0.11-0.22)	0.164 ± 0.009 (0.148-0.181)
<b>P3</b>	0.192 ± 0.01 (0.17-0.21)	0.171 ± 0.01 (0.152-0.190)
<b>P4</b>	0.154 ± 0.01 (0.11-0.18)	0.134 ± 0.01 (0.105-0.154)
<b>P5</b>	0.352 ± 0.09 (0.13-0.47)	0.293 ± 0.08 (0.172-0.406)
<b>Asc3</b>	0.048 ± 0.004 (0.04-0.05)	0.035 ± 0.003 (0.030-0.040)
<b>Asc4</b>	0.048 ± 0.004 (0.03-0.06)	0.034 ± 0.002 (0.031-0.040)
<b>Asc5</b>	0.050 ± 0.004(0.04-0.06)	0.036 ± 0.004 (0.028-0.043)
<b>ADL</b>	0.190 ± 0.008 (0.17-0.21)	0.170 ± 0.01 (0.164-0.194)
<b>AD TOOTH FROM TIP</b>	0.022 ± 0.003 (0.014-0.026)	0.022 ± 0.003 (0.019-0.027)

<b>PML</b>	0.236 ± 0.02 (0.204-0.286)	0.220 ± 0.02 (0.197-0.249)
<b>CXL</b>	0.399 ± 0.02 (0.338-0.448)	0.381 ± 0.02 (0.347-0.407)
<b>CXW</b>	0.086 ± 0.02 (0.011-0.12)	0.101 ± 0.01 (0.079-0.12)
<b>CXL/CXW</b>	4.53 ± 0.6 (3.008-5.610)	3.84 ± 0.6 (3.07-5.02)
<b>CXL/AD</b>	2.09 ± 0.1 (1.759-2.358)	2.13 ± 0.009 (1.99-2.27)
<b>CXL/WL</b>	0.159 ± 0.01 (0.124-0.178)	0.171 ± 0.009 (0.156-0.193)
<b>STL</b>	0.196 ± 0.009 (0.183-0.222)	0.179 ± 0.008 (0.165-0.194)
<b>STW</b>	0.039 ± 0.006 (0.028-0.059)	0.039 ± 0.004 (0.033-0.044)
<b>STL/STW</b>	5.12 ± 0.7 (3.335-6.59)	4.62 ± 0.6 (3.80-5.78)
<b>DTSPE</b>	0.192 ± 0.01 (0.172-0.211)	0.176 ± 0.01 (0.159-0.199)
<b>DSTSPE</b>	0.137 ± 0.01 (0.114-0.197)	0.123 ± 0.009 (0.111-0.140)
<b>DMSPE</b>	0.082 ± 0.008 (0.060-0.10)	0.080 ± 0.007 (0.117-0.146)
<b>PUMP</b>	0.132 ± 0.01 (0.113-0.197)	0.129 ± 0.008 (0.117-0.146)
<b>GFL</b>	1.067 ± 0.1 (0.844-1.286)	0.814 ± 0.2 (0.5-1.03)
<b>F/P</b>	8.15 ± 1.2 (5.6-10.3)	7.2 ± 1.2 (4.156-7.746)
<b>GF/WL</b>	0.42 ± 0.05(0.336-0.530)	0.365 ± 0.09 (0.219-0.476)
<b>No.HRSCX</b>	88 ± 9.0 (45-96)	72 ± 5.7 (56-76)

**Table gives means, standard deviation (±) and ranges (in parentheses).**

**Table 5.10. Mean morphometric measurements (mm.) of female *P.hindustanicus* and *P. salangensis* from Northern Pakistan.**

<b>CHARACTERS</b>	<b><i>P.hindustanicus</i> n=25</b>	<b><i>P.salangensis</i> n=10</b>
<b>WL</b>	2.849 ± 0.1 (2.673-3.051)	2.842 ± 0.2 (2.218-2.754)
<b>WW</b>	0.824 ± 0.05 (0.743-0.949)	0.693 ± 0.05 (0.601-0.764)
<b>WL/WW</b>	3.463 ± 0.2 (3.10-3.80)	3.583 ± 0.12 (3.39-3.77)
<b>HDL</b>	0.512 ± 0.02 (0.476-0.537)	0.489 ± 0.002 (0.458-0.530)
<b>HDW</b>	0.520 ± 0.03 (0.476-0.599)	0.520 ± 0.05 (0.464-0.610)
<b>HDL/HDW</b>	0.985 ± 0.05 (0.85-1.07)	0.948 ± 0.07 (0.852-1.080)
<b>PHXL</b>	0.234 ± 0.01 (0.208-0.247)	0.222 ± 0.009 (0.200-0.238)
<b>PHXW</b>	0.093 ± 0.016 (0.080-0.106)	0.082 ± 0.002 (0.078-0.084)
<b>PHXL/PHXW</b>	2.496 ± 0.2 (2.10-2.88)	2.72 ± 0.1 (2.52-2.90)
<b>LBL</b>	0.408 ± 0.03 (0.325-0.447)	0.365 ± 0.03 (0.315-0.420)
<b>LB/WL</b>	0.143 ± 0.006 (0.122-0.153)	0.148 ± 0.005 (0.139-0.157)
<b>A3</b>	0.373 ± 0.04 (0.315-0.468)	0.294 ± 0.02 (0.257-0.340)
<b>A4</b>	0.146 ± 0.01 (0.125-0.174)	0.115 ± 0.006 (0.107-0.128)
<b>A5</b>	0.144 ± 0.1 (0.126-0.17)	0.114 ± 0.005 (0.107-0.125)
<b>A3/LB</b>	0.902 ± 0.09 (0.739-1.067)	0.804 ± 0.04 (0.727-0.854)
<b>A3/A4 + A5</b>	1.286 ± 0.06 (1.185-1.40)	1.284 ± 0.06 (1.18-1.36)
<b>P1</b>	0.054 ± 0.01 (0.03-0.08)	0.050 ± 0.005 (0.041-0.06)
<b>P2</b>	0.240 ± 0.02 (0.174-0.285)	0.207 ± 0.02 (0.177-0.233)
<b>P3</b>	0.216 ± 0.01 (0.186-0.243)	0.185 ± 0.02 (0.163-0.211)
<b>P4</b>	0.175 ± 0.01 (0.142-0.195)	0.141 ± 0.01 (0.125-0.165)
<b>P5</b>	0.370 ± 0.07 (0.228-0.533)	0.284 ± 0.05 (0.219-0.391)
<b>Asc3</b>	0.057 ± 0.009 (0.042-0.075)	0.044 ± 0.002 (0.038-0.08)
<b>Asc4</b>	0.059 ± 0.005 (0.052-0.072)	0.045 ± 0.003 (0.04-0.05)
<b>Asc5</b>	0.060 ± 0.007 (0.044-0.074)	0.047 ± 0.004 (0.038-0.052)
<b>Asc4/A4</b>	0.410 ± 0.03 (0.361-0.496)	0.396 ± 0.03(0.352-0.451)

Table gives means, standard deviation (±) and ranges (in parentheses).

*P.hindustanicus* collected from Pakistan. Posterior part of the pharyngeal armature consisting of very fine teeth and well developed concentric lines (Figure 5.7d). Anterior part of the armature consists of numerous small backward-pointed teeth. Teeth are larger and more developed at the extreme anterior end of the armature. Ascoid 4 = 48  $\mu\text{m}$  long. Spermatheca (Figure 5.7 c) with irregular segments and short neck.

### **Discussion.**

A comparison of measurements of specimens of *P.hindustanicus* with those from Afghanistan given in by Artemiev, (1980) (Table 5.11, 5.12) show only slight differences in some of the characters.

In males, the genital filaments are generally shorter in the specimens from Pakistan 1067(844-1286)  $\mu\text{m}$ ; compared to the specimens from Afghanistan 1251(1080-1408)  $\mu\text{m}$ ; the F/P ratio is very similar - 9.3 in Afghan forms and 9.8 in Pakistani forms. The subterminal tooth on the aedeagus is at a mean distance of 22 (14-26)  $\mu\text{m}$  from the tip in contrast to that of the specimens from Afghanistan 17 (12-20) $\mu\text{m}$ . Females showed no particular differences.

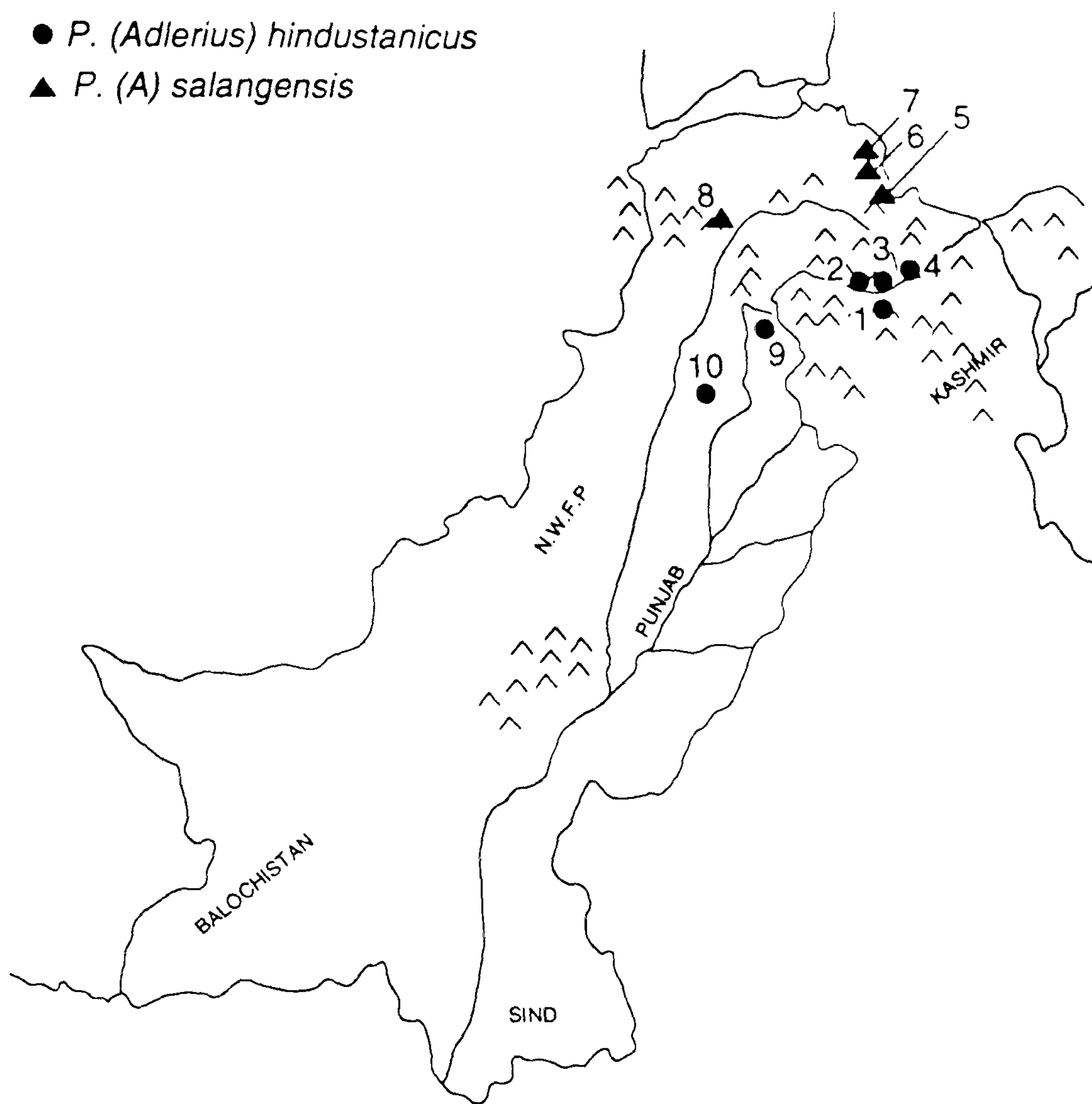
### **Distribution.**

Northern India (Theodor, 1958), south-east Afghanistan (Artemiev, 1978). Previously, in Pakistan this species has been recorded from Rawalpindi only (Lewis, 1967), but during the present survey it has been found widely distributed (Figure 5 7) in Azad Kashmir (Bagh, Malot, Bees Bagla, Banjosa) at an altitude ranging from

# Figure 5.7

## Geographical distribution of *Phlebotomus hindustanicus* & *P. salangensis* in Pakistan and AJK

- *P. (Adlerius) hindustanicus*
- ▲ *P. (A) salangensis*



- 1 = BAGH; 2 = GALI MALOT; 3 = BEESBAGLA;  
4 = BANJOSA; 5 = KURU; 6 = KERIS; 7 = MEHDIABAD (PERKUTA);  
8 = CHILAS; 9 = ISLAMABAD (MARGALLA HILLS); 10 = RAWALPINDI

**Table 5.11. Comparison of morphological measurements (um). of male *P(A)hindustanicus* and *P(A) salangensis* from Pakistan with that of Artemiev(1980).**

CHARACTERS	PHIN (Artemiev1980)	PHIN (AJK)	PSAL (Artemiev1980)	PSAL (N.Areas)
Ant.Formula	1/3-7;2/8-15	1/3-7;2/8-15	1/3-5;2/6-15	1/3-5;2/6-15
NoCXHRS	79(69-94)	88 ± 9.0 (45-96)	64(40-85)	72 ± 5.7 (56-76)
DISTCXHRS	0.54(0.51-0.61)	0.50 ± 0.04 (0.45-0.59)	0.44(0.4-0.48)	0.44 ± 0.02 (0.38-0.50)
DSTADTOOTH	17.5(12-20)	22 ± 0.002 (14-16)	15(10-20)	22 ± 2.7 (19-27)
GFL (µm)	1194(1024-1408)	1067 ± 0.113(844-1286)	972(752-1120)	814 ± (500-1029)
F/P RATIO	9.0(7.9-10.1)	9.8 ± 1.0 (7.9-11.8)	7.7(6.6-8.8)	7.5 ± 1.8(4.9-10)
A3 (µm).	402(332-468)	441 ± 0.03(387-498)	324(260-424)	332 ± 0.03(290-380)
A3/L RATIO	1.37(1.27-1.49)	1.42 ± 0.07(1.21-1.56)	1.28(1.11-1.51)	1.27 ± 0.08(1.12-1.43)
Asc4. (µm)	48(40-56)	49 ± 0.004 (30-50)	33(26-44)	34 ± 0.002 (31-39)
CXL (µm)	375(340-428)	399 ± 0.02 (338-448)	370(316-412)	381 ± 0.02 (350-410)
STL (µm)	187(168-212)	200 ± 0.009(183-223)	184(156-208)	179 ± 0.008(165-194)
ADL (µm)	181(152-208)	191 ± 0.008(171-205)	164(152-188)	170 ± 0.01(164-194)
CX/AD RATIO	2.08(1.84-2.24)	2.1 ± 0.1(1.76-2.36)	2.27(2.02-2.55)	2.13 ± 0.009 (2.0-2.27)

(µm) = micrometers.

PHIN = *Phlebotomus hindustanicus*.

PSAL = *Phlebotomus salangensis*.

Ant.Formula = Antennal formula

Table gives means, standard deviation (±) and ranges (in parentheses).

**Table. 5.12. Comparison of morphological measurements ( $\mu\text{m}$ ) of female *P(A)hindustanicus* and *P(A) salangensis* from Pakistan with that of Artemiev (1980).**

<b>PARAMETERS</b>	<b>PHIN (Artemiev 1980)</b>	<b>PHND (AJK)</b>	<b>PSAL (Artemiev 1980)</b>	<b>PSAL (N.Areas)</b>
<b>A3 (<math>\mu\text{m}</math>)</b>	440-480	373 $\pm$ 0.04(315-468)	264 (232-296)	294 $\pm$ 0.02(257-340)
<b>A3/L Ratio</b>	1.0	0.9 $\pm$ 0.09(0.7-1.06)	0.85(0.78-0.91)	0.80 $\pm$ 0.03(0.72-0.85)
<b>Asc 4</b>	-	59 $\pm$ 5.72(51-72)	41(34-48)	45 $\pm$ 3.06(39-49)

( $\mu\text{m}$ ) = micrometers

PHIN = *P. hindustanicus*

PSAL = *P. salangensis*

Table gives means, standard deviation ( $\pm$ ) and ranges (in parentheses).



1200-1890 metres. It was also found in the Margella Hills (Islamabad/Rawalpindi). This species was not captured from the Northern Areas.

### Medical Importance.

This thesis incriminates *Phlebotomus hindustanicus* as a vector of visceral leishmaniasis (caused by *Leishmania infantum*) to Man for the first time.

### *Phlebotomus salangensis* Artemiev, 1978.

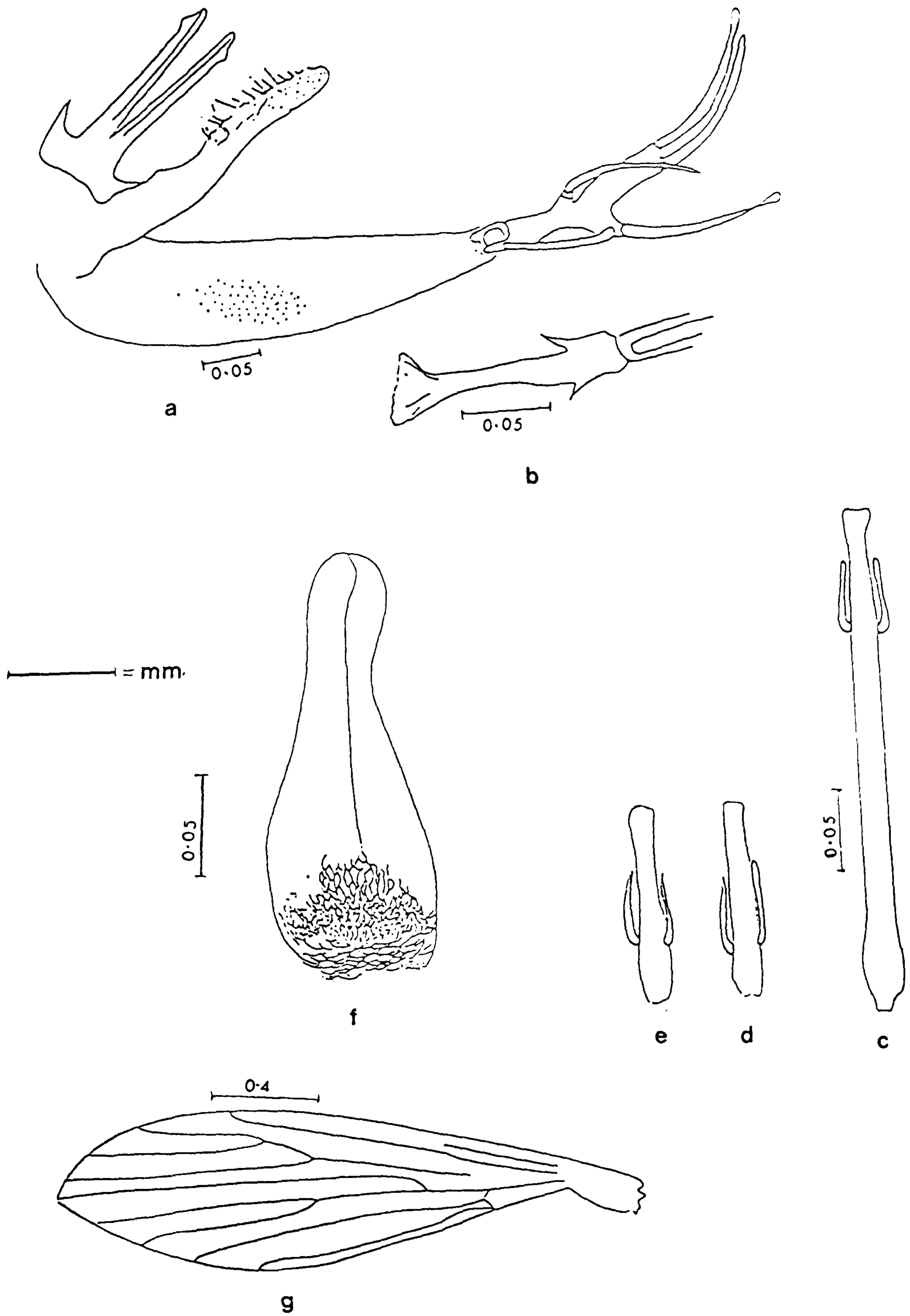
*Phlebotomus (Adlerius) salangensis* Artemiev, 1978: 22[♂ ♀]; 1980: 1189, Holotype ♂. Afghanistan (MI, Moscow) [not examined]. Paratypes [♂ ♀] BMNH, London [examined]. Material examined. 10♂ and 10 ♀ from Northern Areas.

*Phlebotomus salangensis* was first described from Afghanistan by Artemiev (1978). The species is close to *Phlebotomus angustus* from which it differs in the number of hairs on the coxite, width of coxite, length of genital filaments and F/P ratio (Artemiev, 1978). It is being described for the first time from northern Pakistan. Table 5.9 & 5.10 gives the morphological measurements of various male and female characters.

### Male.

Antennal formula 2/3-5, 1/6-15. Coxite (Figure 5.8a) moderately wide with group of 72 (56-76) hairs. Distal border of the hairy patch at 0.44 (0.38-0.50) of coxite length. Paramere with long neck. Aedeagus with sub-terminal tooth at 22 (19-27)  $\mu\text{m}$ . from the tip. Genital filaments 0.814 (0.500-1.029)  $\mu\text{m}$  long. F/P ratio=7.2 (4.2-7.7) Style 0.18 (0.17-0.19)  $\mu\text{m}$  long with short ventral process.

FIGURE 5.8



*Phlebotomus salangensis*, a-b: ♂; c-g: ♀. a: terminalia; b: sperm pump; c, d, e: antennal segment 3, 4, 5; f: Pharynx; g: wing.

**Females.**

Pharyngeal armature projecting forward and consists of numerous long scales, basal concentric lines well developed (Figure 5.8c). A3/L, 0.8 (0.7-0.9); Ascoid 4, 0.45 (0.04-0.05)  $\mu\text{m}$  long; Asc4/A4, 0.4 (0.35-0.45).

**Discussion.**

Nearly all the measurements of specimens from Pakistan are close to those of the early description of *P. salangensis* by Artemiev (1980) (table 5.11 & 12). The only noticeable difference between the specimens from Pakistan and Afghanistan are the number of hairs on the coxite, and the distance of the aedeagus tooth from the tip. Pakistani specimens have 72 (56-76) hairs compared to 64 (35-69) hairs on Afghanistan specimens. These two characters are important for the identification of males belonging to the sub-genus *Adlerius* (Artemiev, 1978). However, the variations in the male specimens from Pakistan with respect to these characters seems to be no more than geographic variation.

Females from Pakistan also show close affinity to Afghan *P. salangensis* in the key characters of the pharyngeal armature, A3/labrum ratio, length of Ascoids on A4 and Asc4/A4 ratio.

**Distribution.**

This species has been recorded from central and southern Afghanistan and this is the first record of *P. salangensis* from northern Pakistan (Figure 5.7).

**Medical Importance.** Its role in the transmission of leishmaniasis is not known.

## CHAPTER 6

### BIOLOGY OF PHLEBOTOMINE SANDFLIES IN RELATION TO VISCERAL LEISHMANIASIS TRANSMISSION IN NORTHERN PAKISTAN.

#### 6.1. INTRODUCTION.

The status of visceral leishmaniasis and the associated sandfly fauna in northern Pakistan and neighbouring countries has been discussed in detail in the previous chapters (chapter 1 & 4). Briefly, visceral leishmaniasis (caused by *Leishmania infantum*) has been known to be endemic in Northern Areas since the 1960's, and in Azad Jammu & Kashmir (AJK) more recently, in the early eighties. Intensive control measures involving mass treatment of the cases as well as insecticide spraying (mainly DDT) were undertaken in Baltistan (Northern Areas). Subsequent surveys did not reveal any active cases and it was therefore thought that the disease had been controlled.

The early studies were of paramount importance in establishing the disease foci, estimating numbers of cases, and listing the sandfly species in the endemic area, but detailed information on the epidemiology, including vector (s) and reservoir (s) is scarce. Past entomological studies on phlebotomine sandflies in Pakistan are particularly limited and the only comprehensive information available in the literature is the inventory of sandflies (Lewis 1967) which mainly concentrates on taxonomy and distribution. Sampling of sandflies has never been undertaken seriously in an organized way to incriminate the vector and to understand their population dynamics and behaviour in relation to disease. *Phlebotomus kandelakii burneyi* was suspected as the most likely vector involved in the transmission of disease in Baltistan (Northern Areas) since this fly was found in close association with human habitations and was

caught resting indoors. All sandflies dissected for natural infections were negative. No information is available on the seasonality or biting cycle of the suspected vectors or any other sandfly species from the visceral leishmaniasis foci of Pakistan.

The recent resurgence of visceral leishmaniasis cases in the foci where the disease has been thought to be eliminated and the appearance of fresh cases from the areas previously not known to be endemic formed the basis for undertaking in depth studies on the disease epidemiology (by Dr M. A Rab) and systematics and biology of phlebotomine sandflies (by myself) in Northern Areas and Azad Jammu & Kashmir.

## **6.2. MATERIALS AND METHODS.**

The study sites have been detailed in chapter 3 and the overall sampling rationale and methodology in chapter 4. To recapitulate: Longitudinal entomological studies were carried out in two contrasting areas, Northern Areas (NA) and Azad Jammu & Kashmir (AJK). Two localities were selected in Northern Areas, one representing lower altitudes (Hudur village 1200m) and the other higher altitudes (Thor village, 1700 m), both in the Chilas district. In AJK, one locality, Rehra village (1200m) was selected in the Bagh District. The two areas (N.A. & AJK) are different in respect to landscape, climate, and vegetation. The province of northern Areas has high mountains, dry rugged terrain, sparse vegetation, heavy winds in the afternoons and very few rains, whereas Azad Jammu & Kashmir, is characterized by hills covered with green vegetation, rains are abundant and floods are not uncommon.

## 6.3. RESULTS

### 6.3.1. SPECIES COMPOSITION.

During the present investigations a total of 8797 sandflies belonging to the genus *Phlebotomus* were collected and identified, of which 6920 were from Northern Areas and 1877 from Azad Jammu & Kashmir. The results, collected from different localities using a variety of methods are summarized in Table 6.1. A total of 8 species of *Phlebotomus* belonging to four subgenera: *Phlebotomus (Phlebotomus)*, *P. (Paraphlebotomus)*; *P. (Adlerius)* and *P. (Larroussius)* were collected. With the exception of the sub-genus *Phlebotomus*, all other sub-genera represented in this collection contain species which transmit zoonotic visceral leishmaniasis in the Old World, particularly the Mediterranean region. It is interesting to note that the species belonging to the sub-genus *Phlebotomus* were not found anywhere in Azad Jammu & Kashmir or at altitudes above 6000ft in Northern Areas. The species composition in the two areas is as follows:

#### 6.3.1.1 Northern Areas.

A total of 6920 sandflies belonging to six species representing four subgenera were collected from this area. These in order of abundance were *Phlebotomus (Phlebotomus) papatasi*, *Phlebotomus (Paraphlebotomus) sergenti*, *Phlebotomus (Paraphlebotomus) alexandri*, *Phlebotomus (Larroussius) keshishiani*, *Phlebotomus (Larroussius) kandelakii burneyi* and *Phlebotomus (Adlerius) salangensis*.

##### i) Hudur Village (Altitude 1200 m).

A total of 2809 sandflies were collected from this locality (2331 in light traps

**Table 6.1. Percentage species composition of *Phlebotomus* spp. (n=8797) from Northern Areas and Azad Jammu & Kashmir.**

Species	Areas		
	Northern Areas		Azad Jammu & Kashmir
	Hudur* n=3979	Thor* n=2941	Rehra n=1877
<i>Phlebotomus papatasi</i>	35.75	1.13	0
<i>Phlebotomus sergenti</i>	19	8.2	32.65
<i>Phlebotomus alexandri</i>	0.63	1.59	0
<i>Phlebotomus salangensis</i>	0.62	1.74	0
<i>Phlebotomus hindustanicus</i>	0.00	0	64.62
<i>Phlebotomus keshishiani</i>	1.48	29.66	0
<i>Phlebotomus kandelakii burneyi</i>	0.00	0.16	0.11
<i>Phlebotomus major</i>	0	0	2.26

NA=Northern area.

AJK=Azad Jammu and Kashmir.

\*Percentages for Hudur and Thor villages are calculated from the entire Northern Area collection (n=6920).

and 478 in sticky trap collections) (Tables 6.2). Using pooled data (inside/outside houses) five species of sandflies were captured which in order of descending abundance were *P.(P) papatasi* (1575:56.1%), *P (Par) sergenti* (1107:39.40%), *P.(L) keshishiani* (62:2.34%), *P.(A) salangensis* (38:1.35%) and *P. alexandri* (23:0.81%).

ii) Thor Village (Altitude 1700 m.)

Total number of sandflies collected from this locality were 2446 (1784 in light trap and 662 in sticky trap collections) (Table 6.2). Based on pooled data from inside and outside houses six species were found five of which were the same as in Hudur village. These species in order of decreasing abundance were *P.(L). keshishiani* (1641:67.08%), *P.(Par) sergenti* (531:21.7%), *P.(A) salangensis* (110:4.5%), *P.(Par) alexandri* (93:3.8%), *P.(P) papatasi* (66:2.69%) and *P.(L) kandelakii burneyi* (5:0.28%).

The relative abundance of individual species in different sites was as follows:

1) *Phlebotomus (Phlebotomus) papatasi* (Scopoli). A total of 2552 *P.papatasi* (both sexes) were collected from Northern Areas. It was the predominant species in Hudur village from where 2474 (97%) specimens were caught which constituted 35.75 percent of the *Phlebotomus* species from Northern Areas. Out of these 1339 (54.12%) were females. In contrast in Thor village this species was never found in abundance. Only 78 specimens (42 males and 38 females) were collected which made up to 1.13% of the collection from Northern Areas.

2) *Phlebotomus (Paraphlebotomus) sergenti* Parrot. Altogether 1883 *P.sergenti* were captured in from Northern Areas. This was the second most abundant species in Hudur village. A total of 1315 specimens were collected from this locality out of



**Table 6.2. Species, sex and number of *Phlebotomus* collected by various methods in Hudur & Thor villages in Northern Areas and Rehra village of Azad Jammu & Kashmir.**

Subgenus	Species	Sex	Localities and collection methods												Total	Grand Total
			Hudur(Chilas) 1990-91				Thor(Chilas) 1991-92				Rehra(Bagh) 1991-93					
			LT	ST	HB	DB	LT	ST	HB	DB	LT	ST	HB	DB		
<i>Phlebotomus</i>	<i>papatasi</i>	M	767	149	138	81	32	5	3	0	-	-	-	-	1175	
		F	579	80	564	116	28	1	9	0	-	-	-	-	1377	2552
<i>Paraphlebotomus</i>	<i>sergenti</i>	M	501	176	21	96	112	272	4	5	383	16	18	1604		
		F	368	62	36	56	89	58	17	11	183	3	10	892	2496	
	<i>alexandri</i>	M	7	0	2	4	16	50	0	4	-	-	-	83		
		F	13	3	0	15	22	5	2	11	-	-	-	71	154	
<i>Adlerius</i>	<i>salangensis</i>	M	24	3	0	2	16	4	0	3	-	-	-	52		
		F	10	1	1	2	85	5	2	6	-	-	-	112	164	
	<i>hindustanicus</i>	M	-	-	-	-	-	-	-	-	469	139	87	695		
		F	-	-	-	-	-	-	-	-	379	36	104	518	1213	
<i>Larrousius</i>	<i>keshishiani</i>	M	36	3	0	17	241	165	10	37	-	-	-	508		
		F	27	1	1	19	1138	97	184	181	-	-	-	1648	2156	
	<i>k. burneyi</i>	M	-	-	-	-	2	0	0	0	2	-	-	4		
		F	-	-	-	-	3	0	4	2	-	-	-	9	13	
	<i>major</i>	M	-	-	-	-	-	-	-	-	29	2	6	37		
		F	-	-	-	-	-	-	-	-	10	-	2	12	49	
			2331	478	762	408	1784	662	235	260	1455	195	227	8797		

LT=CDC Light Trap; ST=Sticky paper trap; HB=Human biting; DB=Dog baited sticky paper traps; M=Males; F=Females.

which 521 (39.7%) were females. The species comprised 19.0 % of the collection from Northern Areas. Although the density of *P. sergenti* in Thor village was less than half that in Hudur village it was still the second most abundant species in this locality. A total of 568 specimens were captured which made up to 8.2% of the Northern Area collection and 7.25% of the total sandflies collected.

3) *Phlebotomus (Paraphlebotomus) alexandri* Sinton. *P. alexandri* was rare both in Hudur and Thor village although the number of specimens collected in the latter locality was more than twice the collection made in the former. A total of 44 (13 males and 31 females) specimens comprising 0.63% of all *Phlebotomus*, were from Hudur village and 110 (40 males and 70 females) (1.6%) were from Thor village.

4) *Phlebotomus (Larroussius) keshishiani* Schurenkova. This species was the dominant species in Thor village where 2053 specimens were collected, out of which 1600 (78%) were females. *P. keshishiani* from Thor village constituted 29.66% of the collections from Northern Areas. In Hudur village this species was rare. Only 103 (54 males and 49 females) specimens were captured by all methods, which made up to 1.48% of the Northern Areas collection. Significantly more females than males were caught in Thor whereas almost equal numbers were caught in Hudur.

5) *Phlebotomus (Larroussius) kandelakii burneyi* Lewis. This species was only found in Thor village where a total of 11 (2 males and 9 females) specimens were captured. This was the least abundant species which made up to 0.16% of Northern Areas collection. *P. kandelakii burneyi* has been recorded for the first time from this area; the previous record of this species is from Baltistan (Lewis, 1967) which is also the part of Northern Areas.

6. *Phlebotomus (Adlerius) salangensis* Artemiev. This is again one of the less dominant species in both the localities. Of only 164 collected from Northern Areas, 43 (29 males and 14 females) specimens were from Hudur village, which constituted 0.62% of the collection from Northern Areas. *P.salangensis* was almost two times more abundant in Thor village than Hudur. From this locality 121 sandflies (23 males and 98 females) were captured in light-traps which comprised 1.74% of the *Phlebotomus* collection from NA.

#### 6.3.1.2. Azad Jammu & Kashmir.

The overall density (per unit catch) of phlebotomine sandflies in this area was found to be significantly lower than in Northern Areas (Table 6.1). A total of 1877 specimens was collected from Rehra village in Bagh district during the entire two seasons collection. Four species belonging to three subgenera were identified. These in order of abundance were *Phlebotomus (Adlerius) hindustanicus*, *P.(Paraphlebotomus) sergenti*, *P.(Larroussius) major*, and *P.(Larroussius) kandelakii burneyi*.

A total of 1650 specimens of *Phlebotomus* (1455 in light traps and 195 in sticky trap collections) (Tables 6.2) were collected from this locality. Four species were caught, in descending order of abundance *P.hindustanicus* (1022:61.94%), *P.sergenti* (585:35.45%), *P.major* (41:2.5%) and *P.kandelakii burneyi* (2:0.1%). The abundance of sandflies did not vary between years except that 2 specimens of *Phlebotomus kandelakii burneyi* were caught in the second year and none during the first year of sampling in AJK.

The relative abundance of individual species in different sites was as follows:

1). *Phlebotomus (Adlerius) hindustanicus* Theodor. This was the most abundant species in Bagh District. A total of 1213 specimens were collected using various methods. Out of these 518 (42.7%) were females. The species comprised 64.62% of the collections from AJK. The number of males (469) was higher than females (379).

2). *Phlebotomus (Paraphlebotomus) sergenti* Parrot. This is a common species both in Northern Areas and Azad Jammu & Kashmir, it was the second most abundant species in AJK. 613 specimens were collected in Azad Jammu & Kashmir, (64%) of which were males. Overall this species represented 32.65% of the AJK collection.

3). *Phlebotomus (Larrousius) major* Annandale. This species was much less abundant than *P.hindustanicus* and *P.sergenti*. A total number of 49 specimens was captured, 24.5% of which were females. Overall proportions were 2.26% of the collections from AJK.

4). *Phlebotomus (Larrousius) kandelakii burneyi* Lewis. This species is recorded from Azad Jammu & Kashmir for the first time. Only two male specimens were captured.

### 6.3.2. METEOROLOGICAL OBSERVATIONS

The monthly temperature (°C) and relative humidity (RH%) were recorded both in Hudur and Thor villages (N.A.) and Rehra village (AJK), between March 1991- November 1991 and in the same months in 1992 (Figures, 6.4, 6.7, & 6.13). In addition, temperature, humidity and rainfall, records were also obtained from the Meteorology department (Figures 6.3, 6.12 and 6.14) for Chilas (N.A) and

Muzafarabad (AJK) districts. No records were available on meteorology for Bagh district, the information was therefore obtained for the nearest district (Muzafarabad) which has similar climatic conditions to Bagh.

The main climatic feature which distinguishes the two areas (N.A & AJK) is that Northern Areas are extremely dry with low annual precipitation (maximum precipitation recorded is 30 mm), in contrast AJK is humid and rains are abundant throughout the year. The maximum precipitation recorded in AJK is 276 mm. (Figure 6.14). The temperature in both the areas varies with altitude. Between 800-1200 metres the temperature is generally warm, whereas it is cool and temperate between 1200-2400 metres.

### 6.3.3 SEASONAL ABUNDANCE.

Seasonal abundance of sandflies belonging to the genus *Phlebotomus* in the Northern Areas and Azad Jammu & Kashmir were determined by sandfly density based on pooled collections from CDC light-traps (indoor and outdoor) and castor-oil coated sticky paper traps (outdoors only). Sampling was made throughout the sandfly season i.e. April to November 1991 (Hudur) and between May and October 1992 (Thor) in the Northern Areas, whereas in Kashmir the one locality (Rehra Village) was sampled for two consecutive seasons i.e. between April and November 1991 and in the same months in 1992. The difference in the sampling routine in two areas was because of the behaviour of the human population. In Northern Areas most of the people migrate from lower altitudes such as Hudur village to high pastures in search of green pastures for their animals. Based on this and also to determine if there were

any differences in the composition of sandfly species between the lowland and high pastures, with particular reference to disease transmission, it was decided to change the sampling routine and a village (1700m.) which represented high pasture was selected for sampling in 1992 (Thor). Routine sampling at altitudes higher than Thor was not practical for logistic reasons. In Azad Jammu & Kashmir there is no such human movement and hence the same locality sampled for two consecutive seasons.

Sampling was not possible in September in either Northern Areas or Azad Jammu & Kashmir as a result of heavy land slides and floods. However as will be seen below, sandfly numbers at the beginning and end of the season were zero or almost zero.

#### **6.3.3.1. Seasonal abundance of *Phlebotomus* species in Northern Areas.**

Seasonal changes in abundance were measured principally by light-traps and sticky traps, both represented here. However some seasonal changes were also observed in human bait and dog bait catches which are discussed below (section 6.3.7.2 and 6.3.8.3).

A). Seasonal Changes: Light-trap collections.

i) Hudur Village.

The mean monthly distribution of the *Phlebotomus* species both inside and outside houses caught in CDC light-traps from Hudur village is shown in Table 6.3. The total number of trapping nights each month (both sexes) was pooled and divided by the total number of traps employed and expressed as mean number of flies/CDC light-trap/night both for indoor and outdoor collections.

The outdoor catches showed (Fig. 6.1A) that *P.papatasi* is active from May

Table 6.3. Densities of *Phlebotomus* in CDC light traps collected from indoors and compounds in Hudur, Northern Areas (1991).

Month	Location	<i>P. papatasi</i>		<i>P. sergenti</i>		<i>P. alexandri</i>		<i>P. salangensis</i>		<i>P. keshishiani</i>		Total traps
		n	X	n	X	n	X	n	X	n	X	
April	Indoors	0	0	0	0	0	0	0	0	0	0	9
	Compounds	0	0	0	0	0	0	0	0	0	0	9
May	Indoors	334	37.1	139	15.4	2	0.2	14	1.5	6	0.6	9
	Compounds	113	12.5	35	3.8	0	0	4	0.4	6	0.6	9
June	Indoors	127	14.1	116	12.9	2	0.2	2	0.2	0	0	9
	Compounds	285	31.6	144	16.0	1	0.1	3	0.3	0	0	9
July	Indoors	119	19.8	43	7.2	1	0.16	1	0.16	0	0	6
	Compounds	230	38.3	98	16.3	6	1.0	0	0	0	0	6
August	Indoors	70	7.7	169	18.8	5	0.5	2	0.2	2	0.2	9
	Compounds	59	6.5	122	13.5	3	0.3	0	0	0	0	9
September*	-	-	-	-	-	-	-	-	-	-	-	-
October	Indoors	5	0.5	3	0.3	0	0	3	0.3	21	2.3	9
	Compounds	4	0.4	0	0	0	0	5	0.5	27	3	9
November	Indoors	0	0	0	0	0	0	0	0	0	0	9
	Compounds	0	0	0	0	0	0	0	0	0	0	9

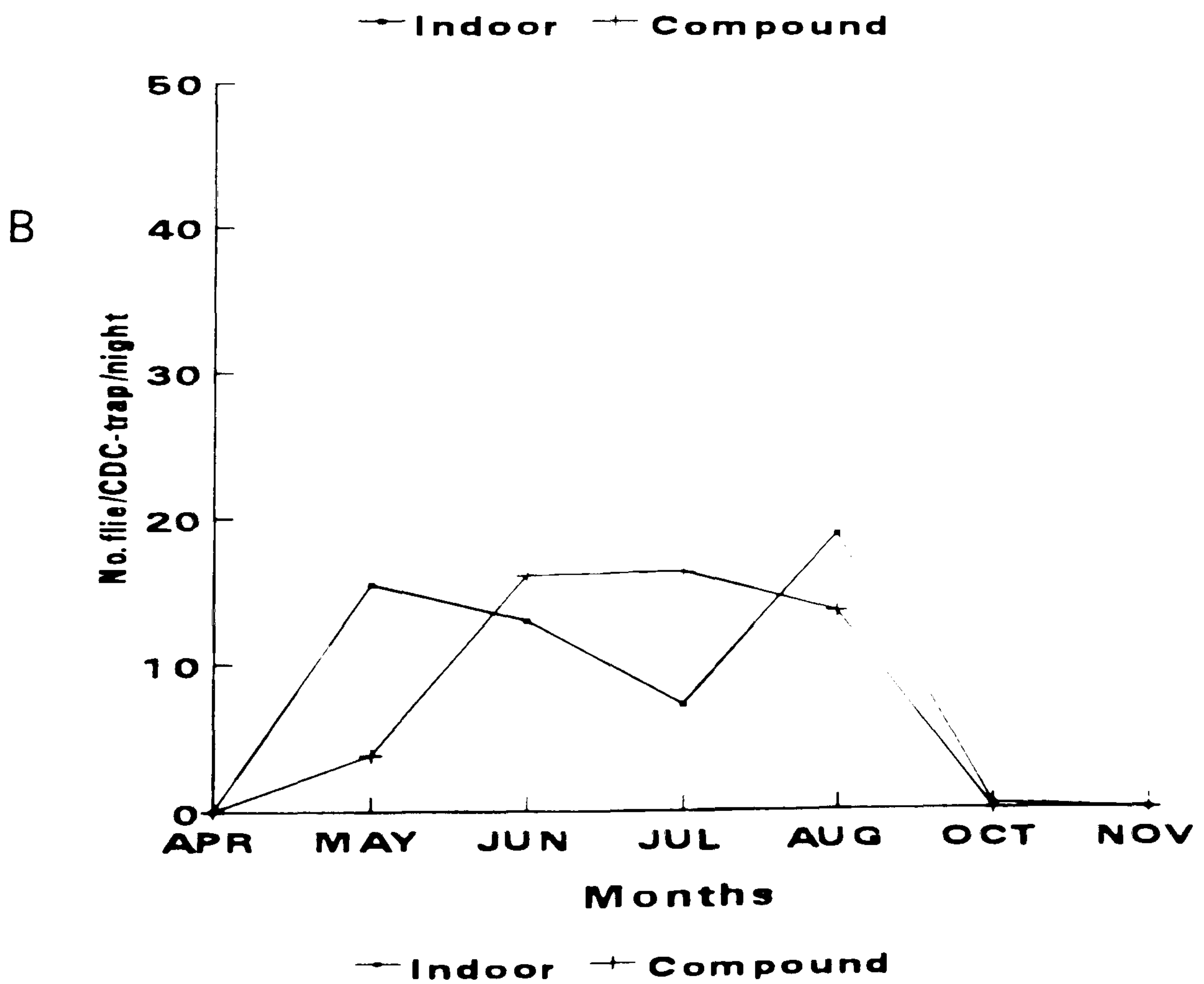
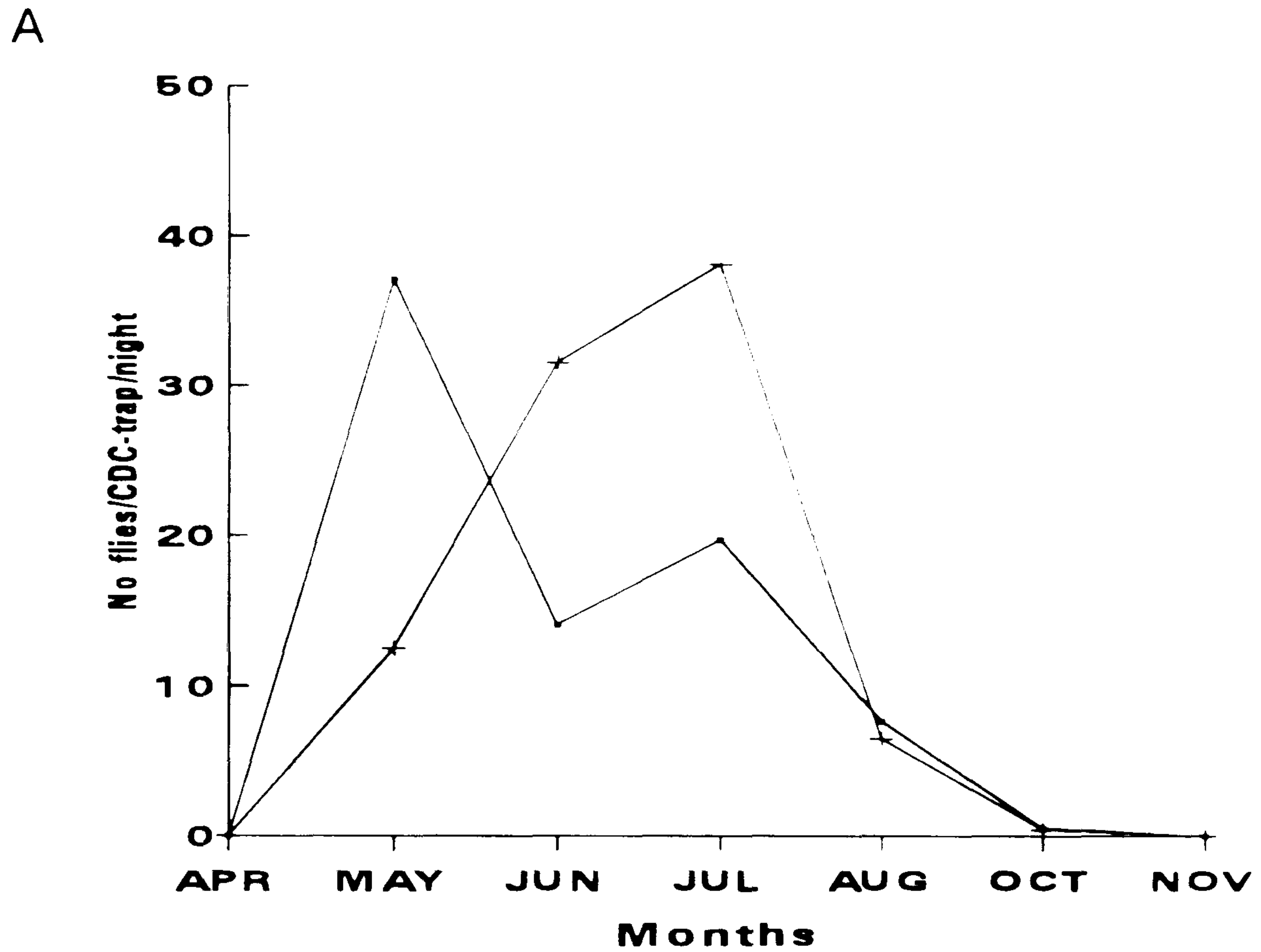
n=Total number of flies.

X=Mean number of flies /CDC light trap.

\*=Flies could not be sampled during this month due to inaccessibility to the field area.

Figure 6.1

Densities of *P.papatasi* (A) and *P.sergenti* (B) in CDC light-traps from Hudur village, Northern Areas





through to August. There is a gradual increase in the number of flies with the rising temperature (Figures 6.3 & 6.4) ( $r= 0.72196$ ;  $P < 0.02$ ), depicting peak activity in June and July with the maximum number of flies caught in July. Whereas indoor activity peaks in May when the outside temperature is low after which there is then an abrupt decline in June through to August. Compared to the outdoor activity, the numbers of flies caught inside houses were less in June and July which may be associated with the increased human activity outdoors. The total number of flies caught indoors and outdoors however did not show any difference ( $t=0.24$ ;  $P > 0.1$ ). In *P. sergenti* the second most abundant species, a similar temperature dependent-trend is found ( $r= 0.915$ ,  $P < 0.01$ ) except that the peak activity of *P. sergenti* (Fig 6.1 B) both inside and outside houses extends till August, one month longer than *P. papatasi*. The population of *P. sergenti* outside houses was a minimum in April, rising to peak in June and July, from which it declined slightly in August and completely diminished in October. In contrast to outdoors, the indoor activity of this species clearly showed two prominent peaks one in May and other in August. No significant differences ( $t=0.27$ ,  $P > 0.1$ ) were however found between the mean number of flies caught inside and outside houses.

*P. keshishiani*, *P. salangensis* and *P. alexandri* were rare species in Hudur village (Fig 6.2), and the number of flies caught were too small (Table, 6.3) for meaningful interpretation of seasonality other than slight evidence for *P. salangensis* being trapped more during the early part of the season and *P. keshishiani* at the end of the season. Similarly, *P. alexandri* was mainly trapped between July (outdoors) and August (indoors). *P. salangensis* and *P. keshishiani* seem to be active in the lowlands when the

Figure 6.2

Densities of *P.salangensis* (A), *P.keshishiani* (B) and *P.alexandri* (C) in CDC light-traps from Hudur village, Northern Areas

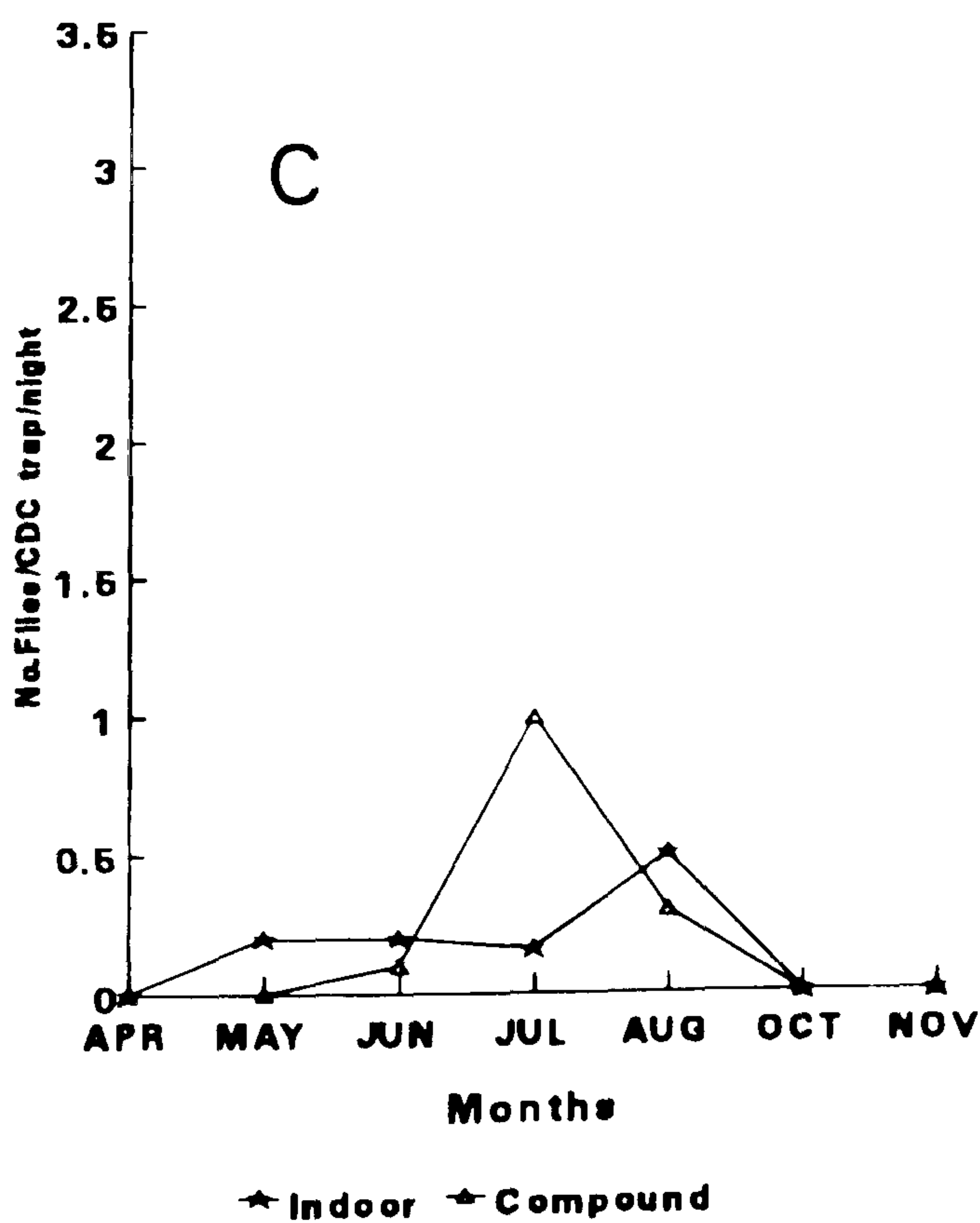
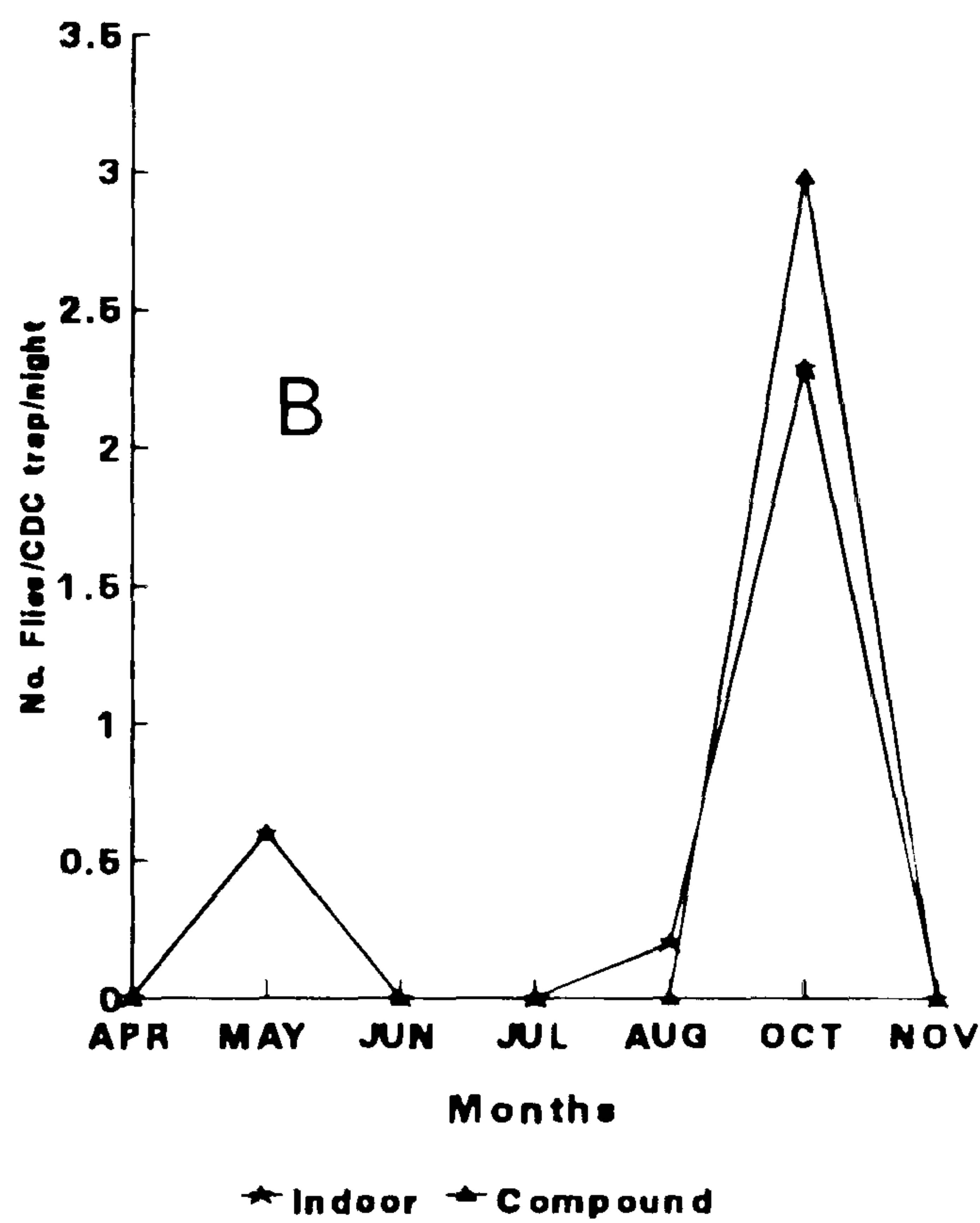
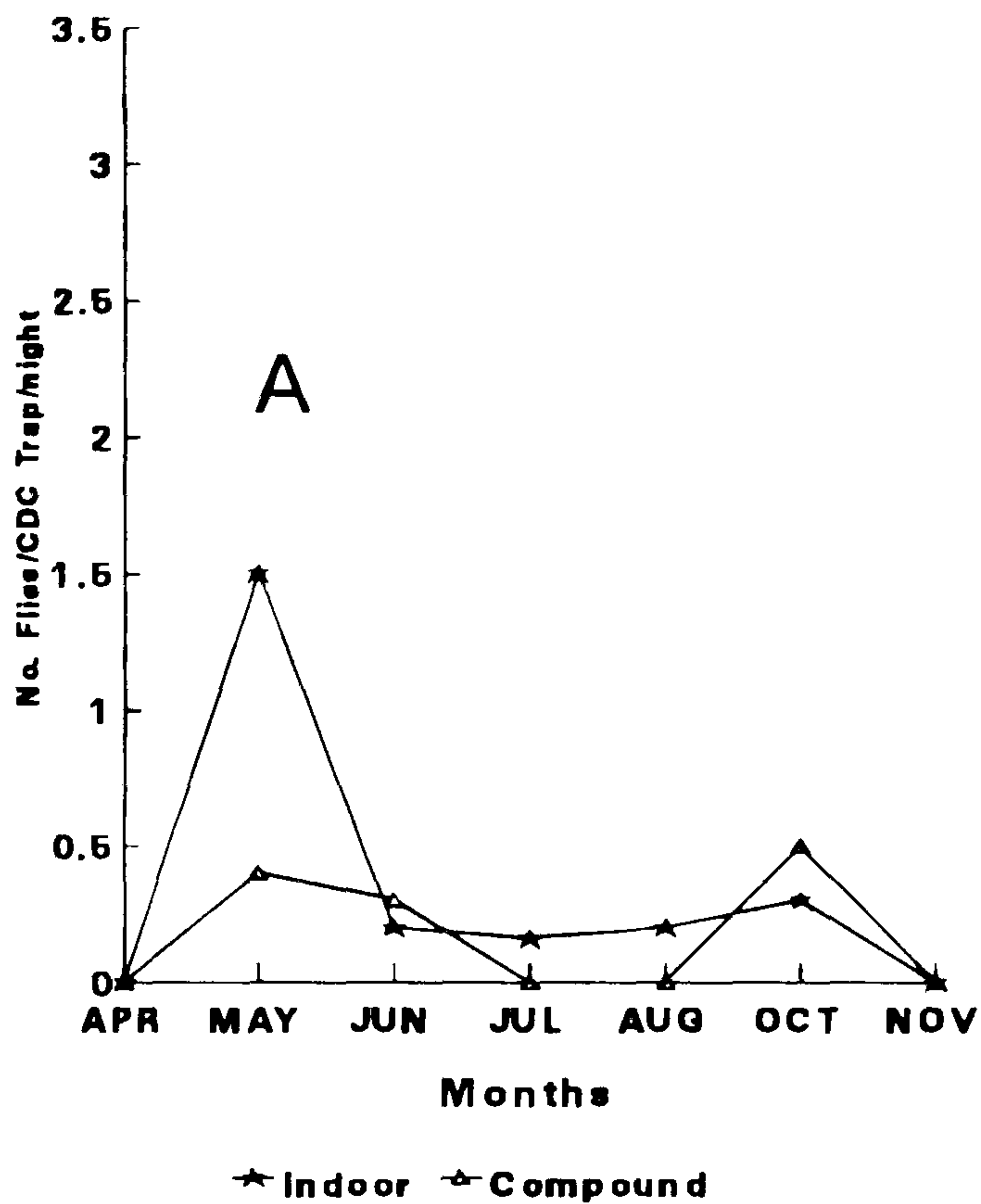
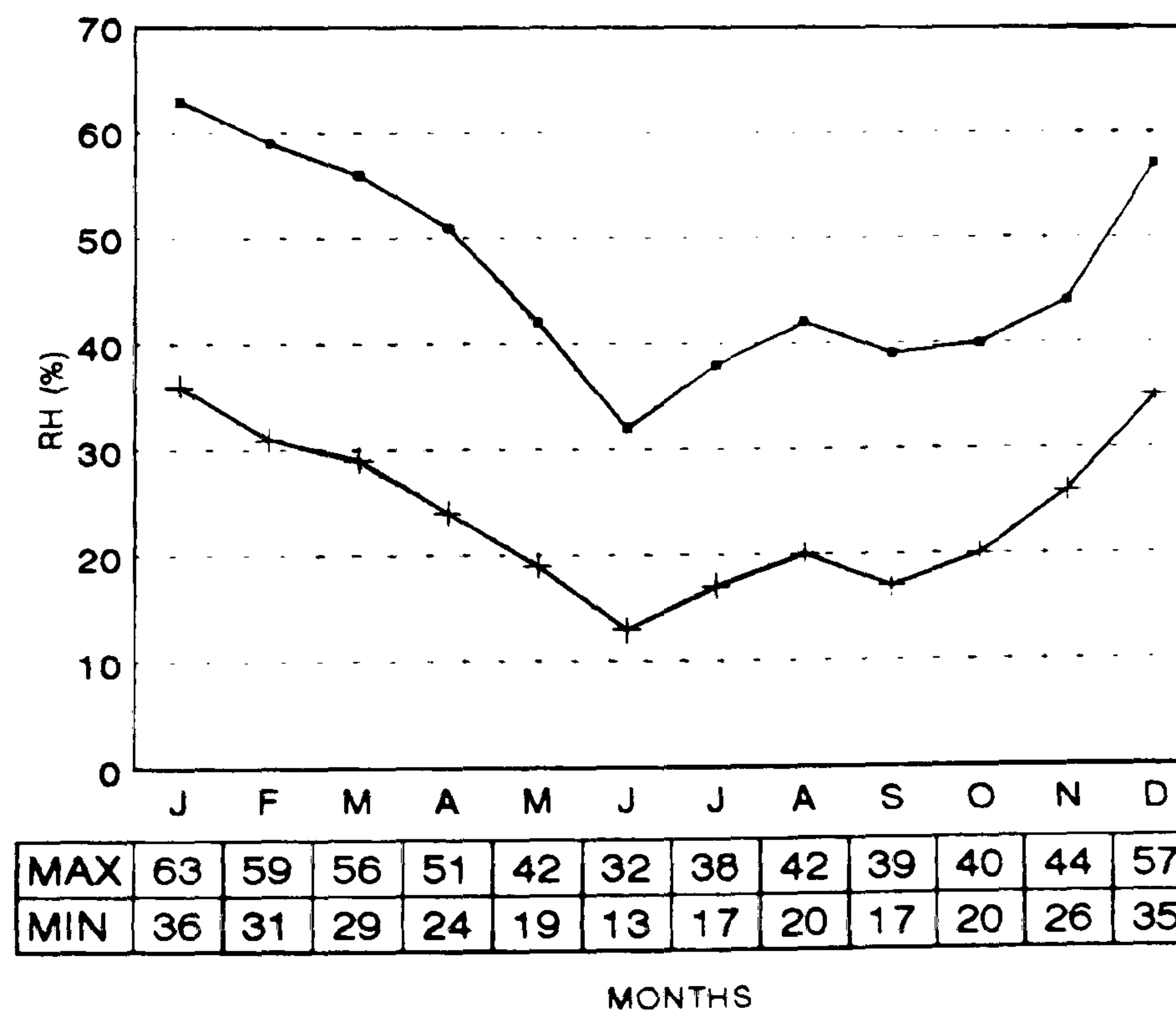
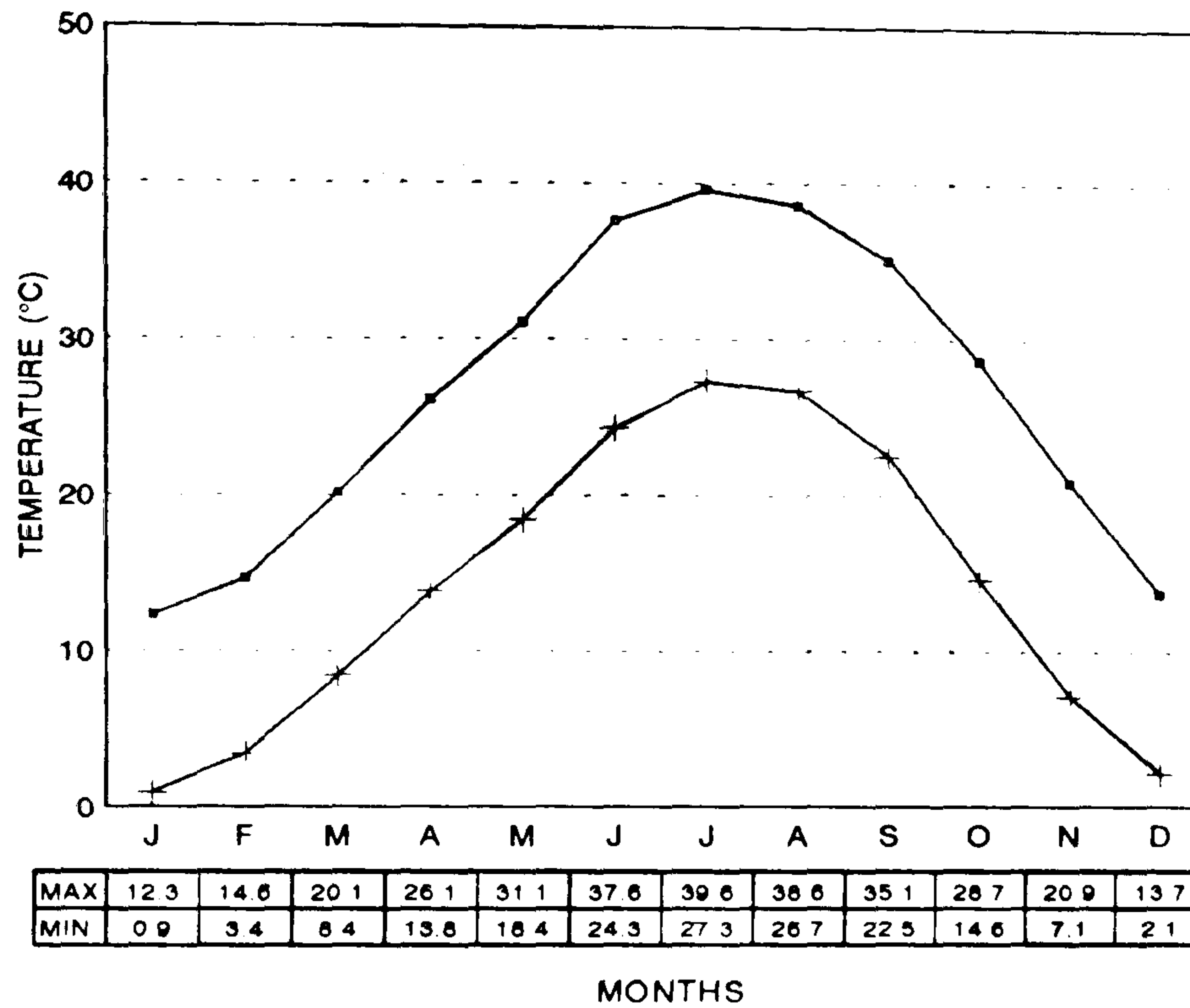


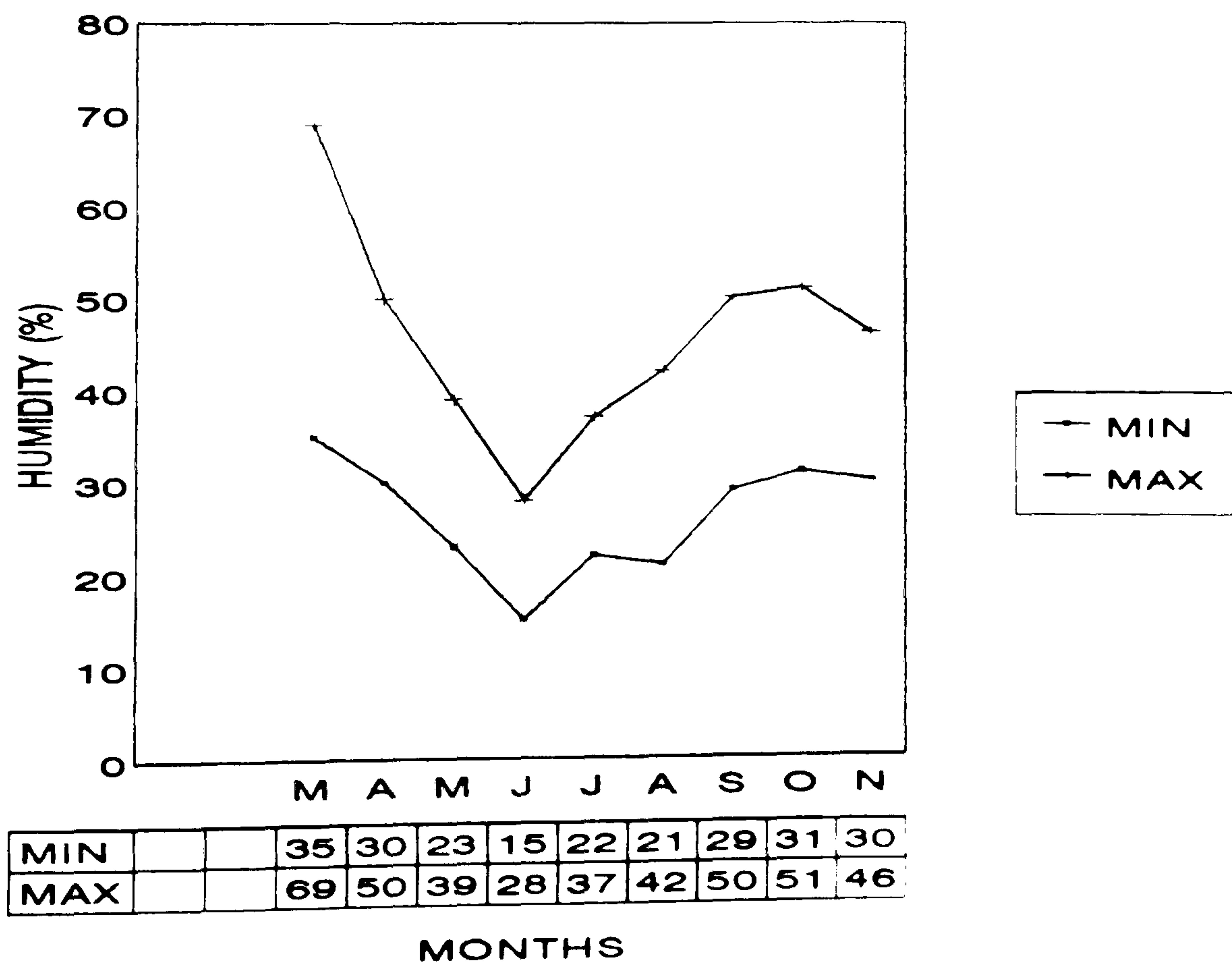
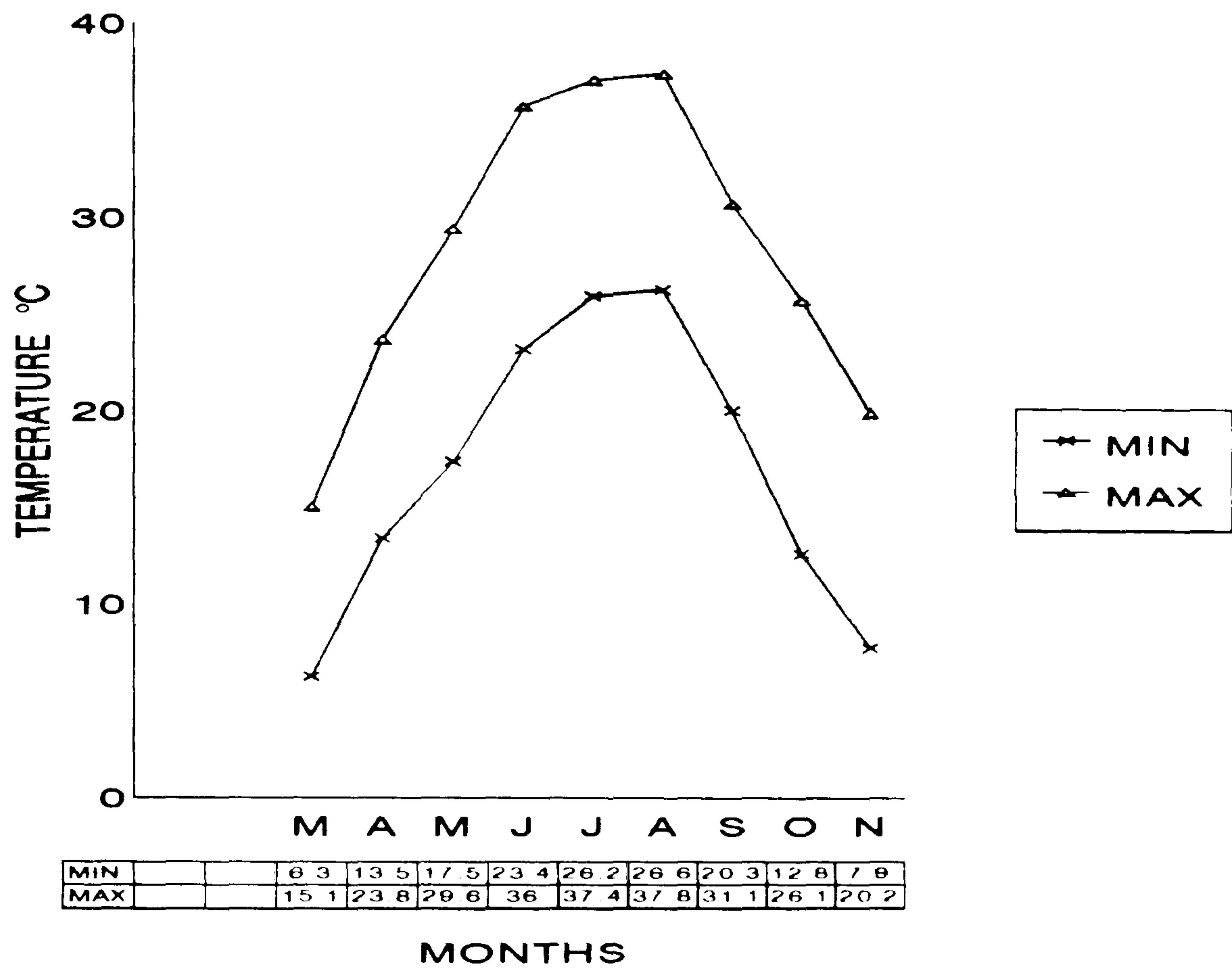
Figure 6.3

MEAN MONTHLY TEMPERATURE (°C) AND PERCENT RELATIVE HUMIDITY FOR CHILAS N.A.



# Figure 6.4

MEAN MONTHLY TEMPERATURE (°C) AND  
RELATIVE HUMIDITY(%) FOR HUDUR VILLAGE  
(N.A).



average temperature is low.

ii) Thor Village.

In Thor village, at a higher altitude, *P.keshishiani* was the dominant species (Tables 6.4). Maximum number were caught during the warm months when the temperature (mean minimum, 21.1°C and mean maximum, 32.6°C) (Figure 6.7) is highest ( $r= 0.793$ ;  $P < 0.054$ ). Rising from June (Fig. 6.5) the density (both indoors and outdoors) remains more or less stable in July and August with a sudden decline in September and completely vanishing in October (mean minimum and maximum temperature; 9.5°C - 24.6°C). Apart from temperature, a positive linear correlation has also been found between humidity and the activity of *P.keshishiani*, but this is not statistically significant ( $r= 0.873$ ;  $P > 0.05$ ). The mean number of flies caught outdoors was significantly higher compared to indoors ( $t=2.008$ ;  $P < 0.05$ ).

In *P.sergenti*, the second most common species in Thor village, the seasonal distribution was clearly bimodal both indoors and outdoors (Fig. 6.6B), with a peak in June and another in August, although the peak seen in June is less prominent compared to that seen in lowland (Hudur village).

Figure 6.6 A, C & D shows the seasonal abundance of *P.alexandri*, *P.salangensis*, *P.kandelakii burneyi* and *P.papatasi* in Thor village. These species were rare in Thor (as they were in Hudur village) (Table 6.4) and as such a meaningful analysis of seasonality cannot be made. Briefly, maximum numbers of *P.alexandri* and *P.salangensis* were caught in June and of *P.kandelakii burneyi* in July whereas *P.papatasi* was mainly trapped in August.

**Table 6.4. Densities of *Phlebotomus* in CDC light traps collected from indoors and compounds in Thor, Northern Areas (1992).**

Month	Location	<i>P.papatasi</i>		<i>P.sergenti</i>		<i>P.alexandri</i>		<i>P.salangensis</i>		<i>P.keshishiani</i>		<i>P.kandelakii burneyi</i>		Total traps
		n	X	n	X	n	X	n	X	n	X	n	X	
<b>May</b>	Unsampled													
<b>June</b>	Indoors	0	0	26	2.8	5	0.5	43	4.8	108	12	0	0.1	9
	Compounds	0	0	27	3.0	26	2.8	47	5.2	201	22.3	0	0	9
<b>July</b>	Indoors	11	1.2	10	1.1	0	0	1	0.1	176	19.5	2	0.2	9
	Compounds	8	0.88	14	1.5	3	0.3	2	0.2	339	41.0	0	1.2	9
<b>August</b>	Indoors	17	1.8	63	7.0	3	0.3	3	0.3	200	22.2	2	0.2	9
	Compounds	24	2.7	59	6.5	1	0.3	5	0.5	312	34.6	1	0.1	9
<b>September</b>	Indoors	0	0	2	0.2	0	0	0	0	33	3.6	0	0	9
	Compounds	0	0	0	0	0	0	0	0	10	1.3	0	0	9
<b>October</b>	Indoors	0	0	0	0	0	0	0	0	0	0	0	0	9
	Compounds	0	0	0	0	0	0	0	0	0	0	0	0	9

n=Total number of flies  
X=means number of flies/CDC light-trap/night.  
N=Total number of flies caught.  
n= Number of flies /CDC light trap/night.

Figure 6.5

Density of *P. keshishiani* in CDC light-traps from Thor village, Northern Areas

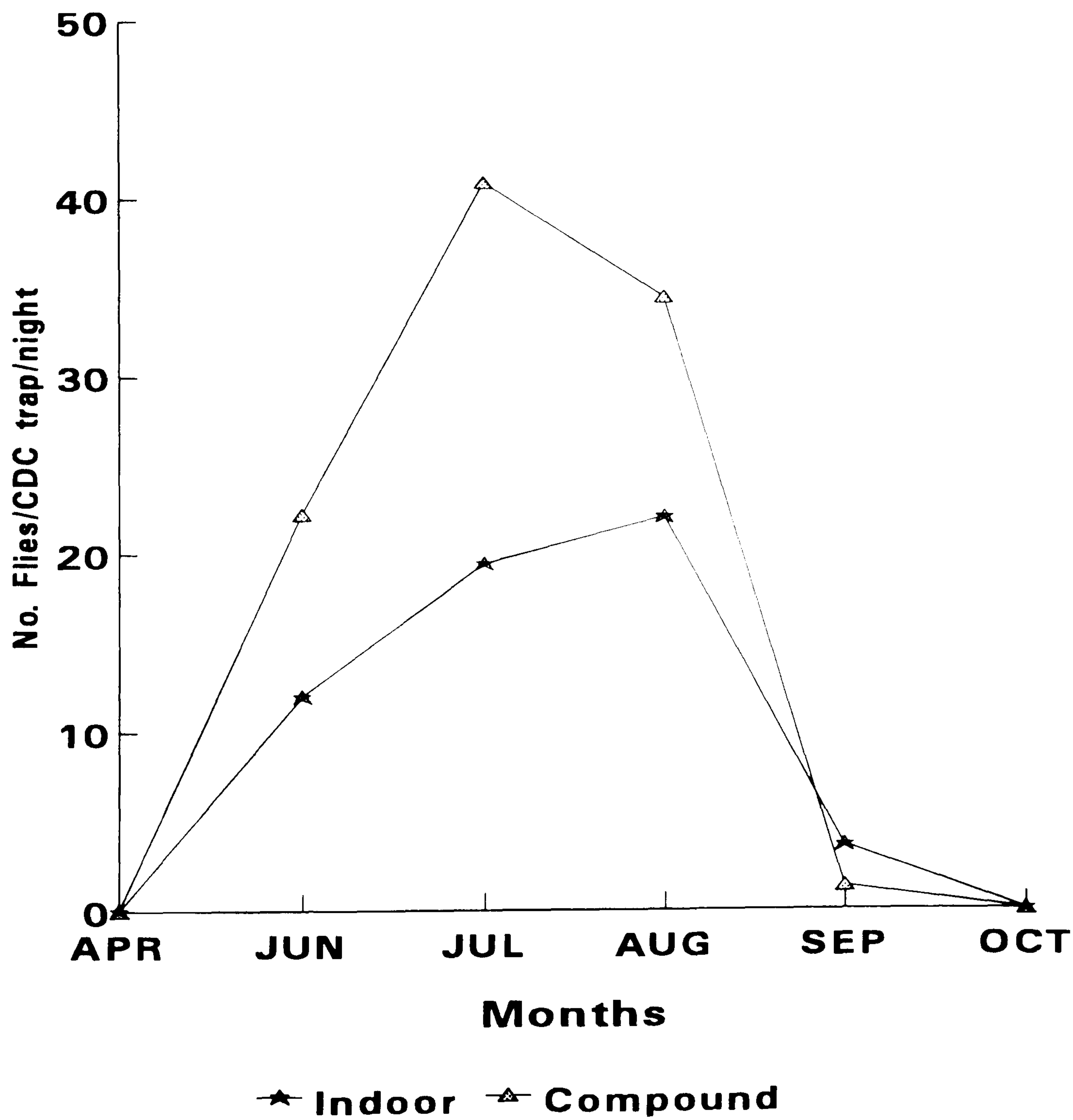


Figure 6.6

Densities of *P.papatasi* (A), *P.sergenti* (B), *P.alexandri* (C), *P.kandelakii burneyi* (D) and *P.salangensis* in CDC light-traps from Thor village, Northern Areas

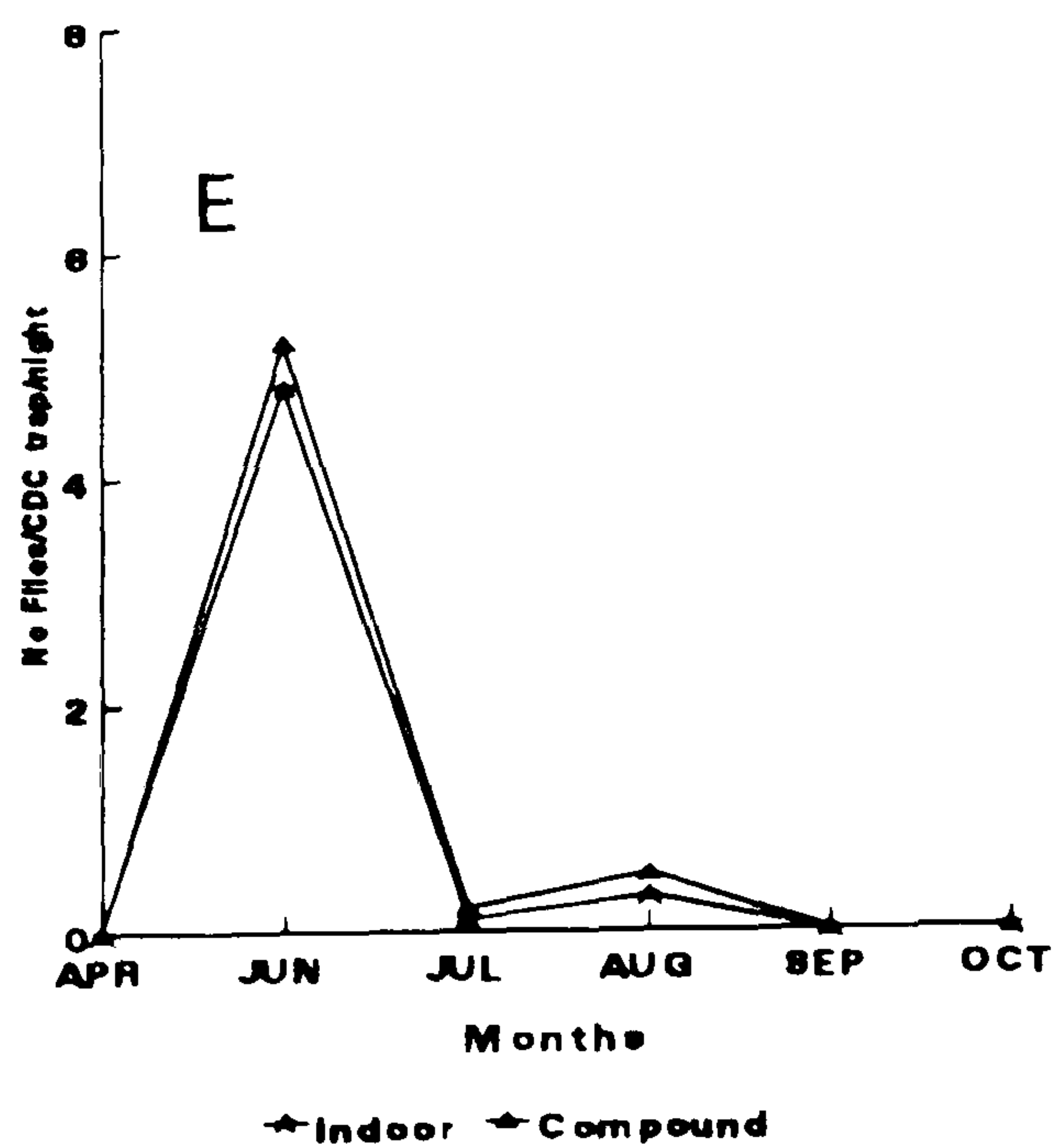
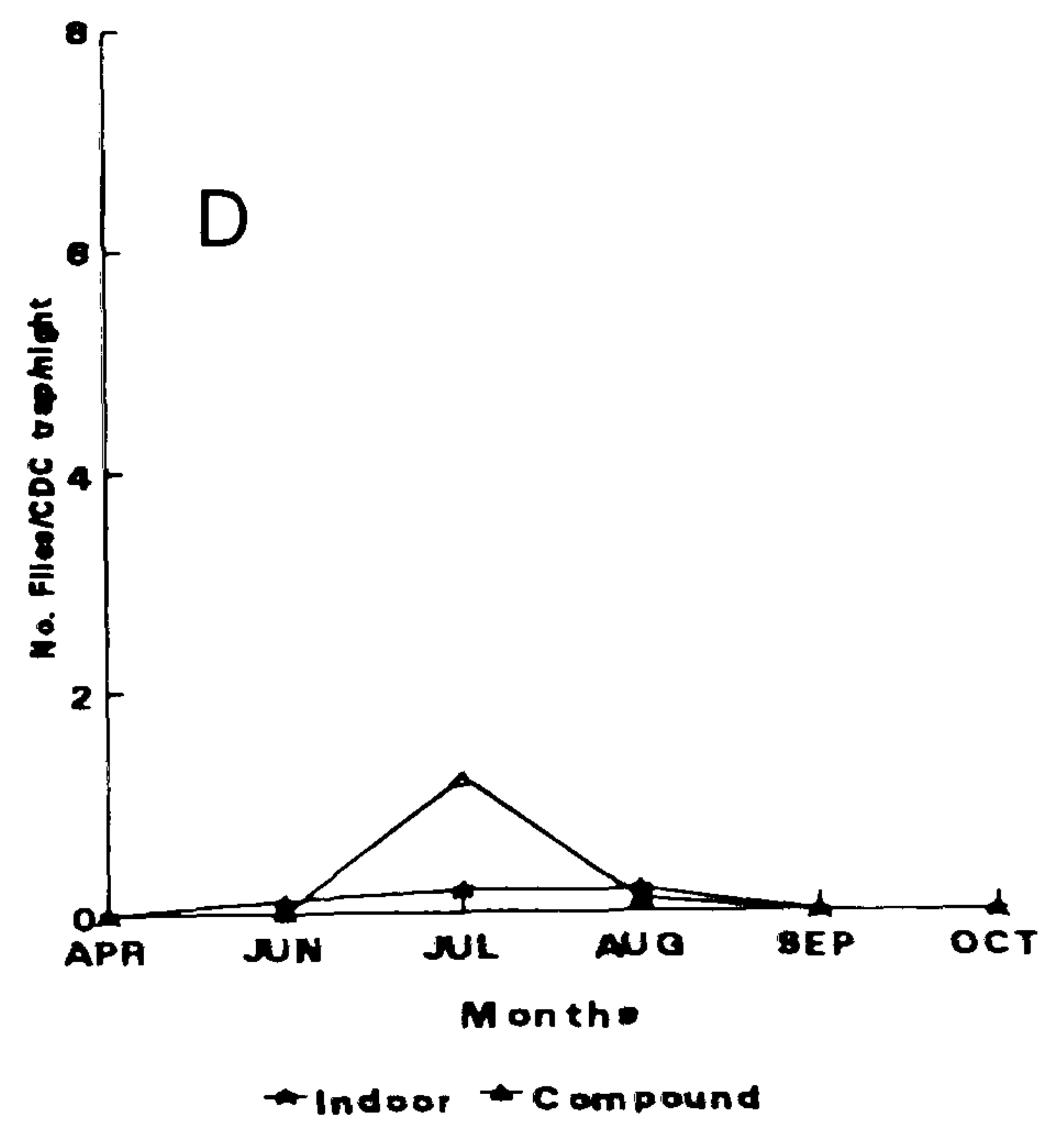
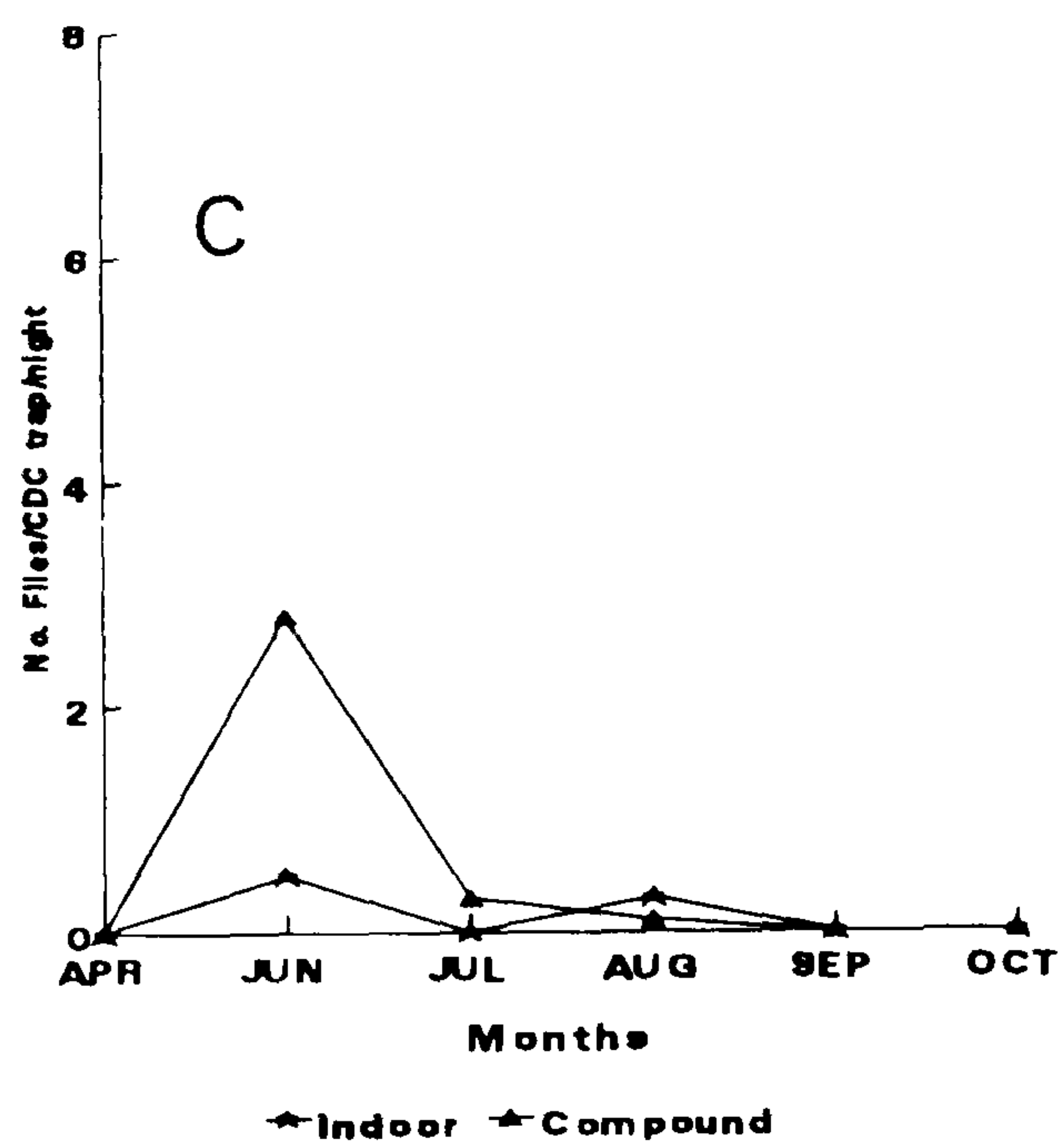
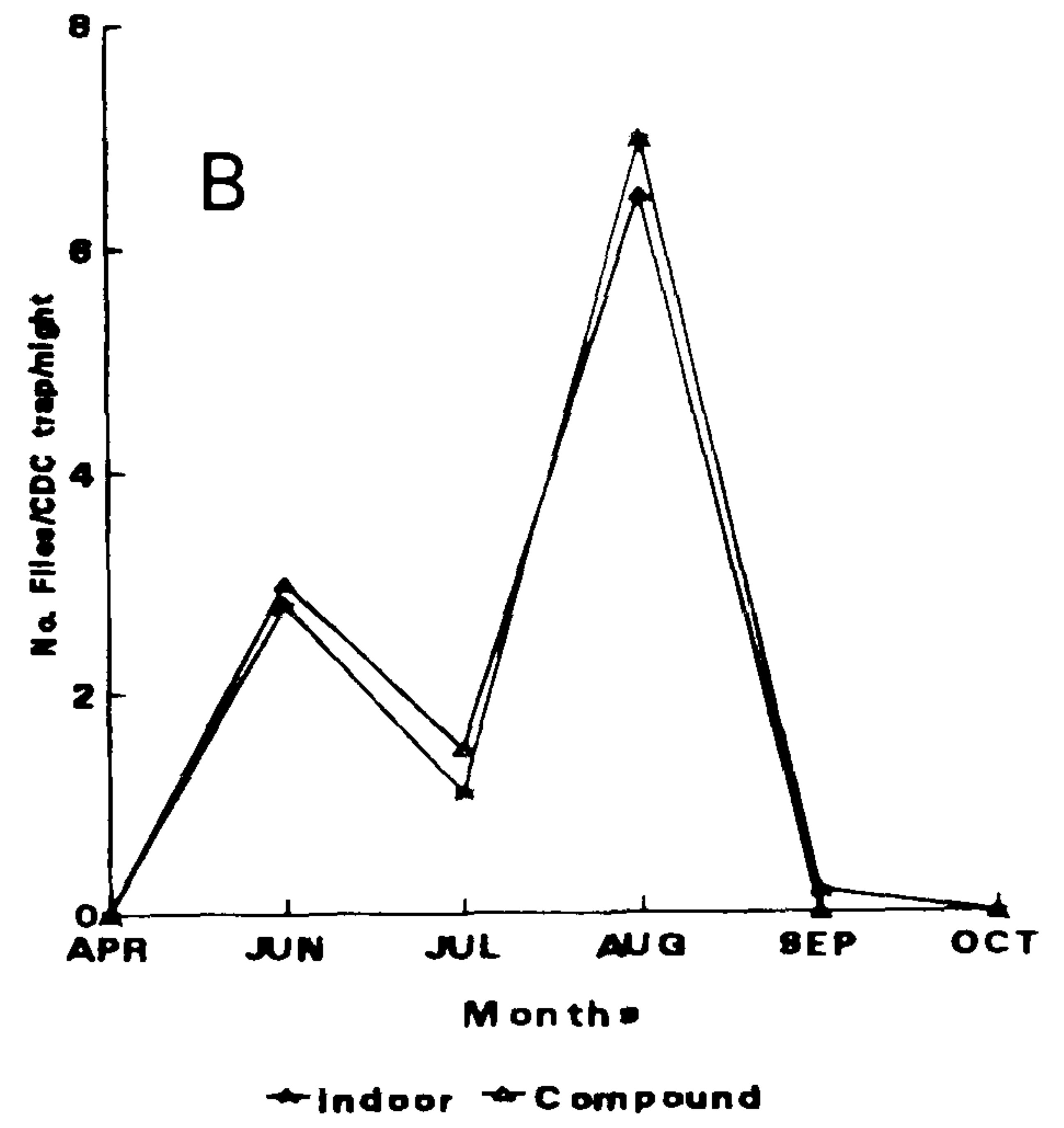
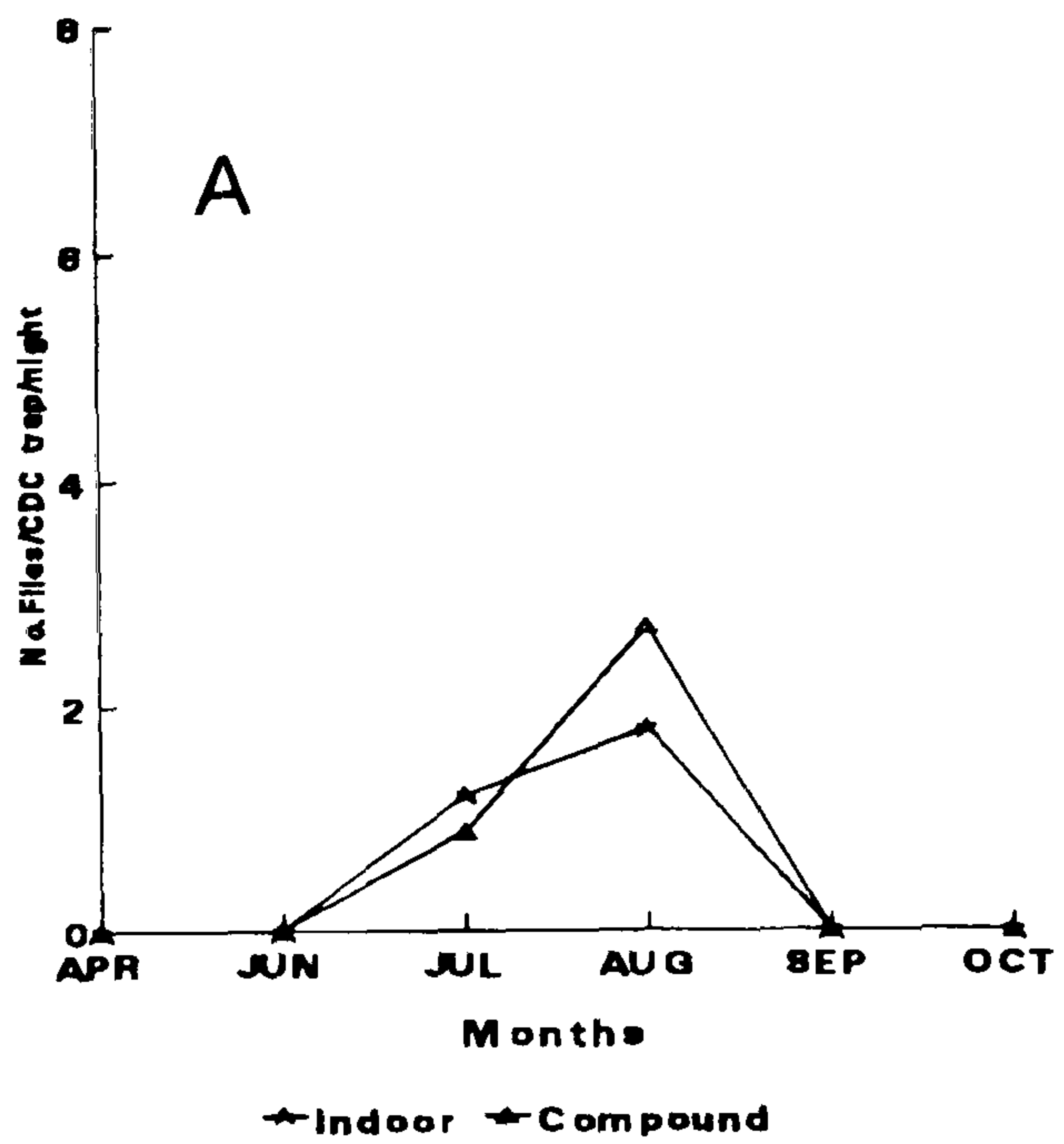
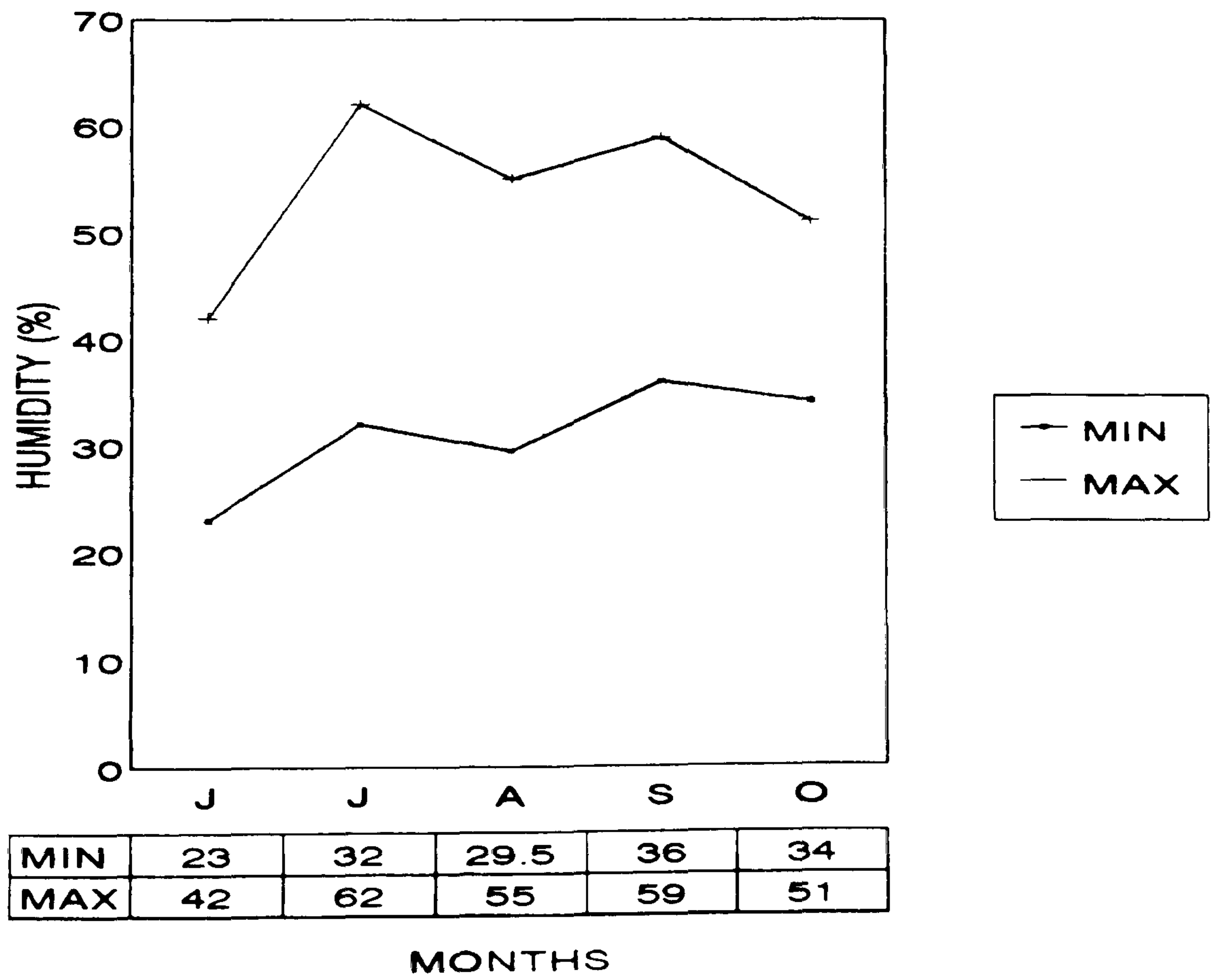
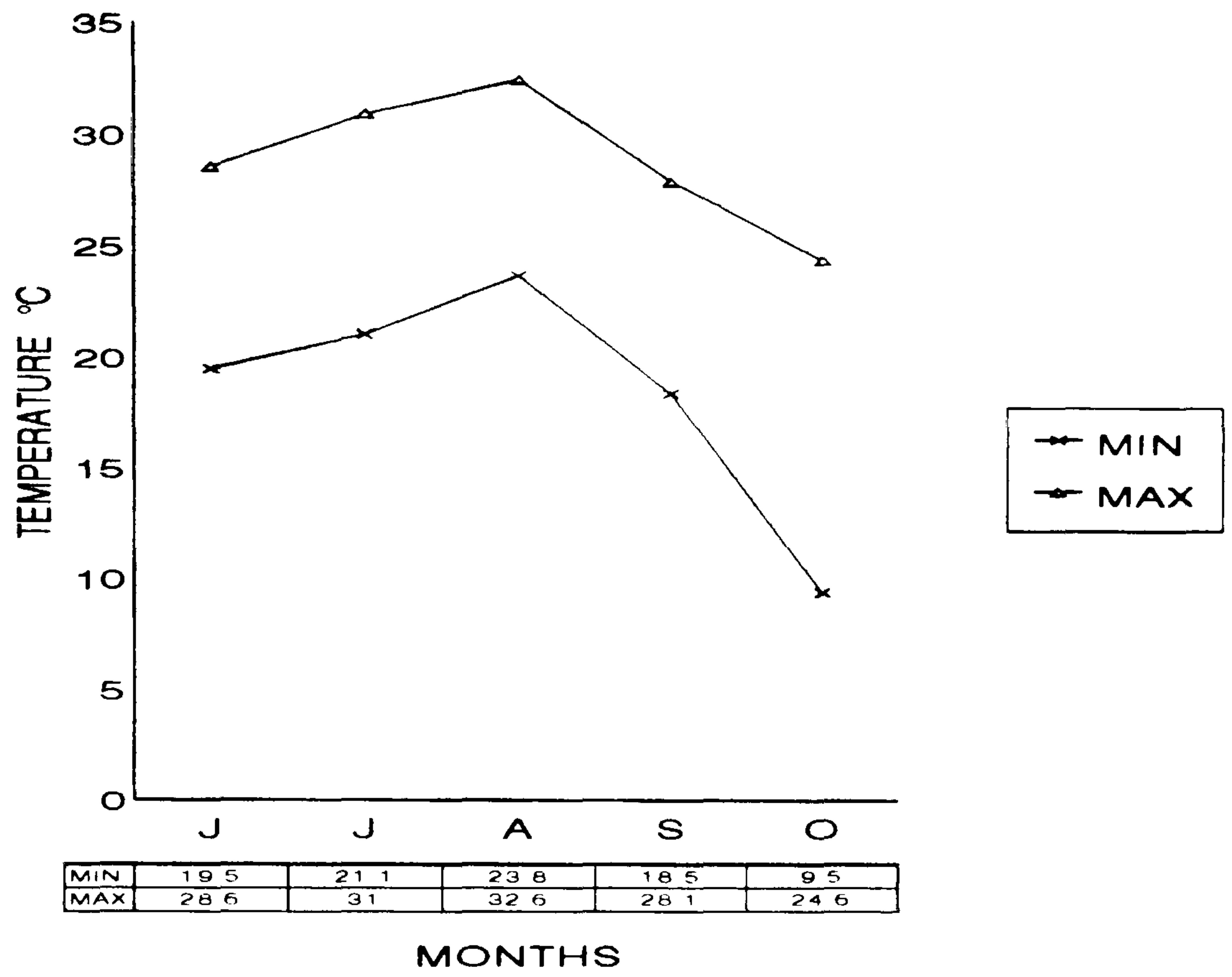




Figure 6.7

MEAN MONTHLY TEMPERATURE (°C) AND RELATIVE HUMIDITY (%) FOR THOR (N.A).



### B). Seasonal changes: Sticky trap collections

A total of 1140 sandflies were collected from Northern Areas (Table 6.2) by sticky traps. Out of these 478 (41.9%) were from Hudur Village and 662 (58.07%) were from Thor Village respectively. A total of five species of *Phlebotomus* were caught by this method both from higher and lower altitudes: *P.sergenti*, *P.papatasi*, *P.keshishiani*, *P.salangensis* and *P.alexandri* (Table 6.5, 6.6).

*Phlebotomus papatasi* and *P.sergenti* were the two dominant sandflies caught in sticky paper traps from lower altitudes (Hudur village). Despite the fact that low number of flies was captured on sticky traps, a general trend in their seasonal abundance is evident (Fig 6.8) which corresponds to the peak outdoor activity of these flies in CDC light-trap collections. The maximum number of *P.papatasi* caught on sticky traps was in July and of *P.sergenti* in June and July. These two months are the hottest months in Hudur when the temperature is at its peak (Figure 6.4). Other sandflies species were caught in too small numbers, to show any noticeable pattern.

At higher altitudes (Thor village, Table 6.6), sandfly densities were low in sticky traps compared to CDC light-trap collections but again their seasonal activities clearly correspond to catches in the outdoor light-trap collections. *Phlebotomus sergenti* was found on sticky traps from June till September with maximum numbers caught in August (Fig. 6.9B) which corresponds well with CDC light-trap collections. The species was more common in the wild than the domestic compounds. In contrast to *P.sergenti*, *P.keshishiani* was collected more on the sticky papers from the compounds than in the wild (Table 6.6). Maximum numbers were caught in July.

Table 6.5. Densities of *Phlebotomus* in sticky paper traps collected from compounds in Hudur, Northern Areas (1991).

Month	<i>P.papatasi</i>		<i>P.sergenti</i>		<i>P.alexandri</i>		<i>P.salangensis</i>		<i>P.keshishiani</i>		Total traps
	n	X	n	X	n	X	n	X	n	X	
April	-	-	-	-	-	-	-	-	-	-	-
May	18	0.1	58	0.36	-	-	-	-	2	0.01	162
June	106	0.6	46	0.28	-	-	1	0.006	-	-	166
July	93	0.7	97	0.76	3	0.02	1	0.007	-	-	127
August	12	0.06	37	0.20	-	-	-	-	-	-	193
September	Unsampled										
October	-	-	-	-	-	-	40	0.02	2	0.01	175
November	-	-	-	-	-	-	-	-	-	-	130

n=Total number of flies caught.

X=Mean number of flies caught/sticky paper trap/night.

Table 6.6. Densities of *Phlebotomus* in sticky paper traps collected from compounds and wild in Thor, Northern Areas (1992).

Month	Location of Sticky traps	<i>P.papatasi</i>		<i>P.sergenti</i>		<i>P.alexandri</i>		<i>P.salangensis</i>		<i>P.keshishiani</i>		Total traps
		n	X	n	X	n	X	n	X	n	X	
April	Compound	-	-	-	-	-	-	-	-	-	-	75
	Wild	-	-	-	-	-	-	-	-	-	-	50
May	Unsampled											
June	Compound	-	-	46	0.29	12	0.08	8	0.05	64	0.41	154
	Wild	-	-	32	0.18	5	0.03	1	0.006	20	0.11	177
July	Compound	-	-	-	-	-	-	-	-	81	0.60	137
	Wild	-	-	72	0.41	30	0.17	-	-	-	-	172
August	Compound	5	0.03	59	0.32	-	-	-	-	76	0.40	184
	Wild	1	0.005	102	0.53	8	0.04	-	-	6	0.03	191
September	Compound	-	-	9	0.06	-	-	-	-	10	0.06	156
	Wild	-	-	10	0.04	-	-	-	-	5	0.02	208
October	Compound	-	-	-	-	-	-	-	-	-	-	130
	Wild	-	-	-	-	-	-	-	-	-	-	155

n=Total number of flies caught on sticky paper traps.

X=Mean number of flies caught/sticky trap/night.

Figure 6.8

Densities of *P. papatasi* and *P. sergenti* on sticky paper traps from Hudur village, Northern Areas

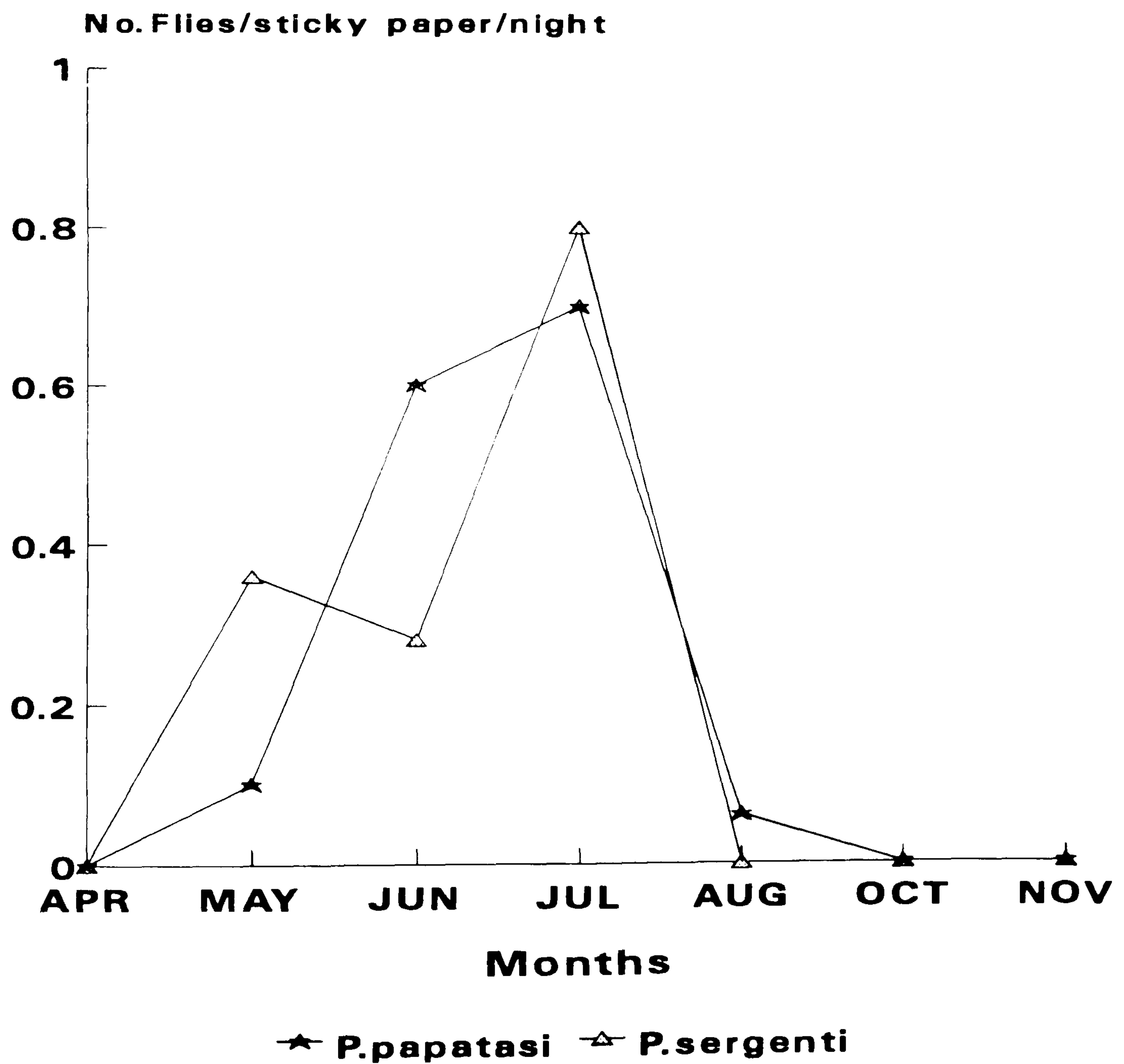
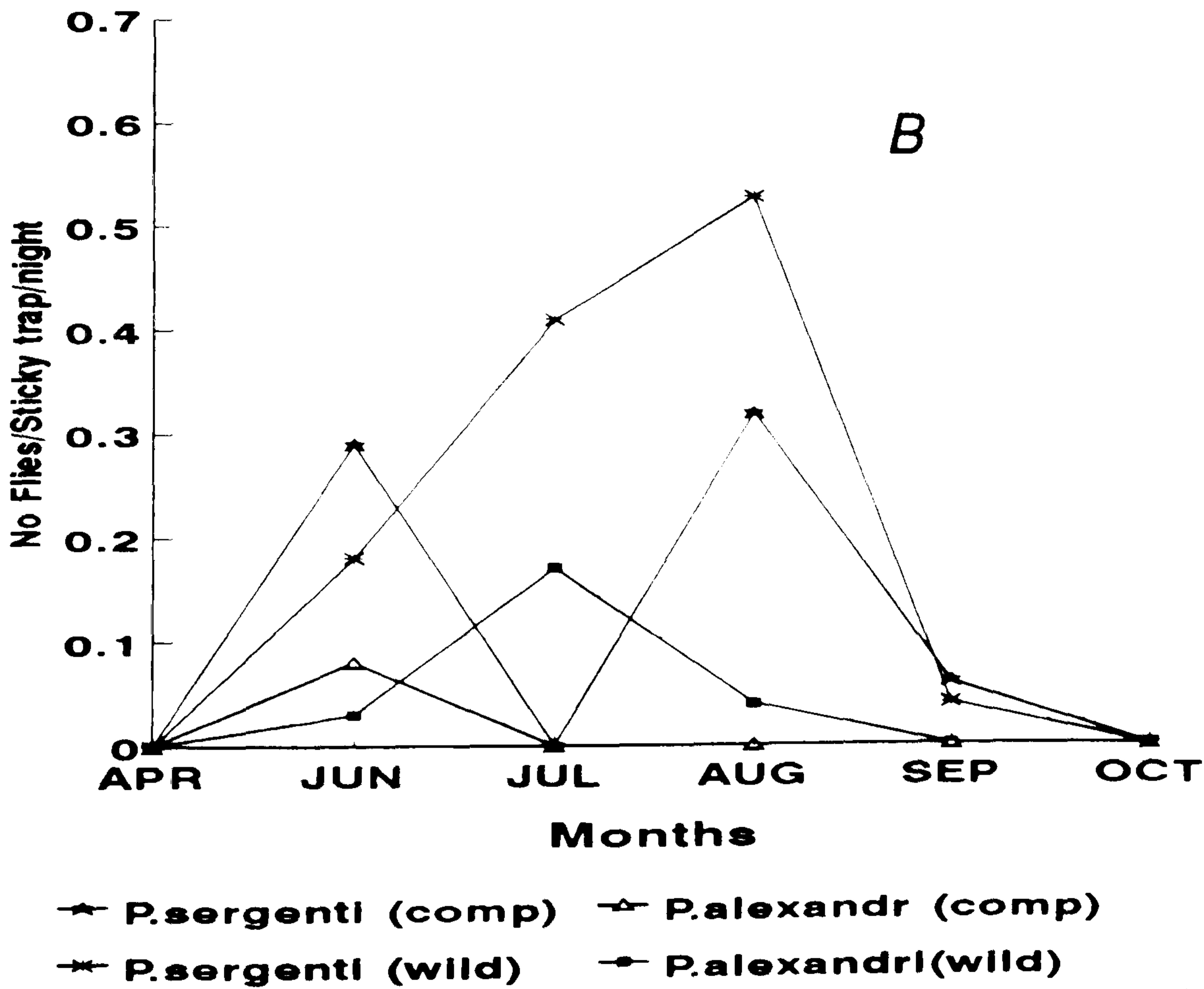
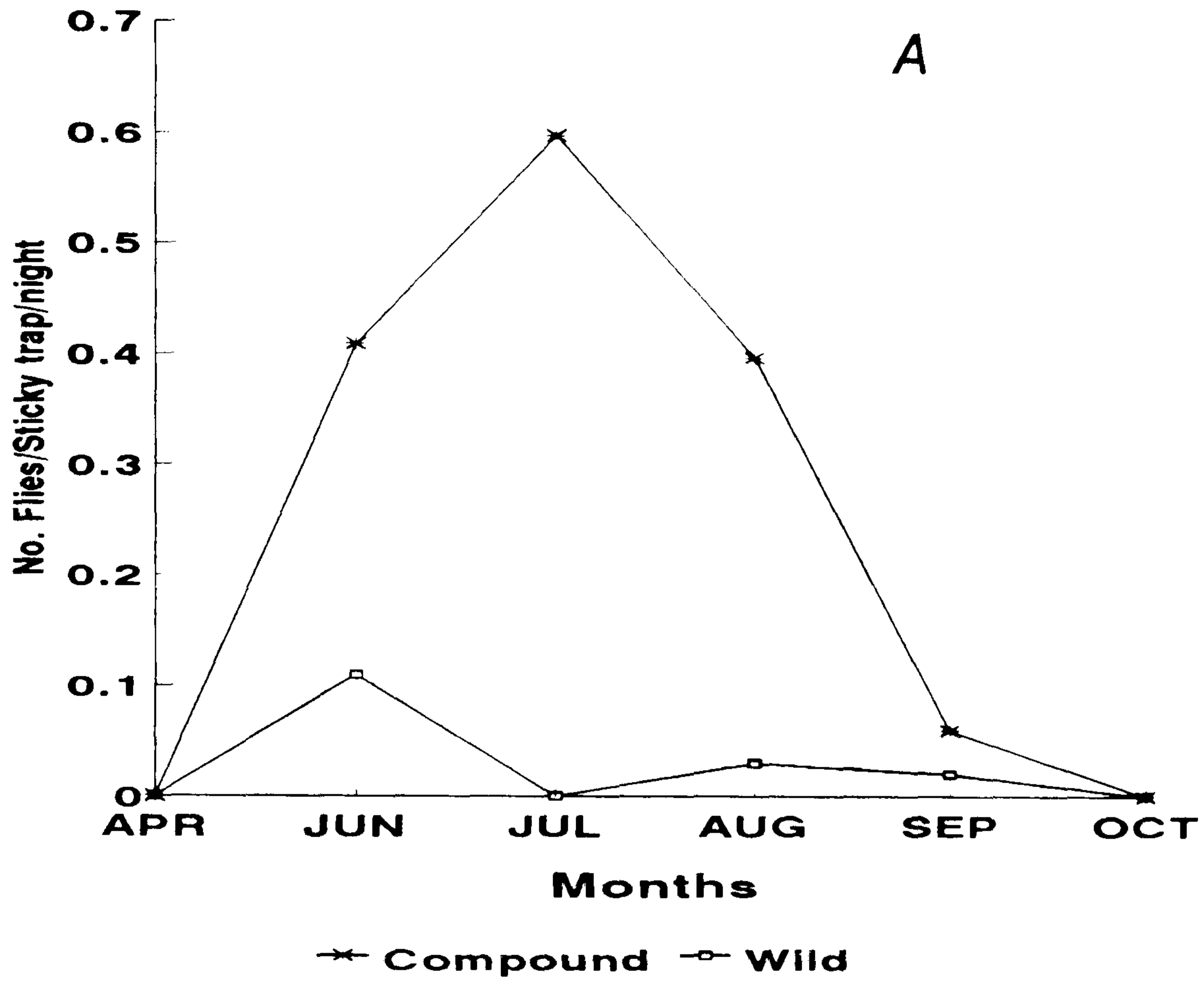


Figure 6.9

Densities of *P.keshishiani*(A), *P.sergenti* and *P.alexandri*(B) sticky traps from Thor village, Northern Areas.



*Phlebotomus alexandri* (Fig 6.9B) was mainly found on sticky traps from June through August and more numbers were caught from wild than domestic compounds. Only 8 flies of *P. salangensis* were caught on sticky traps (in June).

### 6.3.3.2 SEASONAL ABUNDANCE OF *PHLEBOTOMUS* SPECIES IN AZAD JAMMU & KASHMIR.

In contrast to Northern Areas, longitudinal sampling took place in AJK in one village only, Rehra, for 2 years.

#### A. Seasonal changes: Light-trap collections.

*Phlebotomus* species were found from May through<sup>to</sup> October (Fig. 6.10, 6.11) which is a longer period than in the Northern Areas. The peak activity of the dominant sandfly, *Phlebotomus hindustanicus* (Fig, 6.10) is in June inside houses, whereas light-trap collections made outside houses in the compounds do not show a distinct pattern because of the low number of flies caught and more or less the same numbers were caught from June till October. *Phlebotomus sergenti* and *P. major* also show a similar pattern of activity both inside houses and outdoors as *P. hindustanicus* (Figures 6.11 A & B). The peak activity of these flies was again in June (when the temperature is high Fig 6.13) with much lower outdoor collections. The indoor densities of *P. hindustanicus* ( $t=3.16$ ;  $P < 0.001$ ) and *P. sergenti* ( $t= 2.61$ ;  $P < 0.01$ ) were significantly higher than outdoors (Tables 6.7, 6.8).

Unlike Northern Areas, in Azad Jammu & Kashmir rains are more abundant through out the year with maximum rainfall recorded in the month of July and September (Fig. 6.14). The peak activity of all species of sandflies found in this locality strongly indicate that these flies are strictly seasonal here with peak activity

Figure 6.10

Density of *P. hindustanicus* in CDC light-traps from Rehra village, AJK (1991-92)

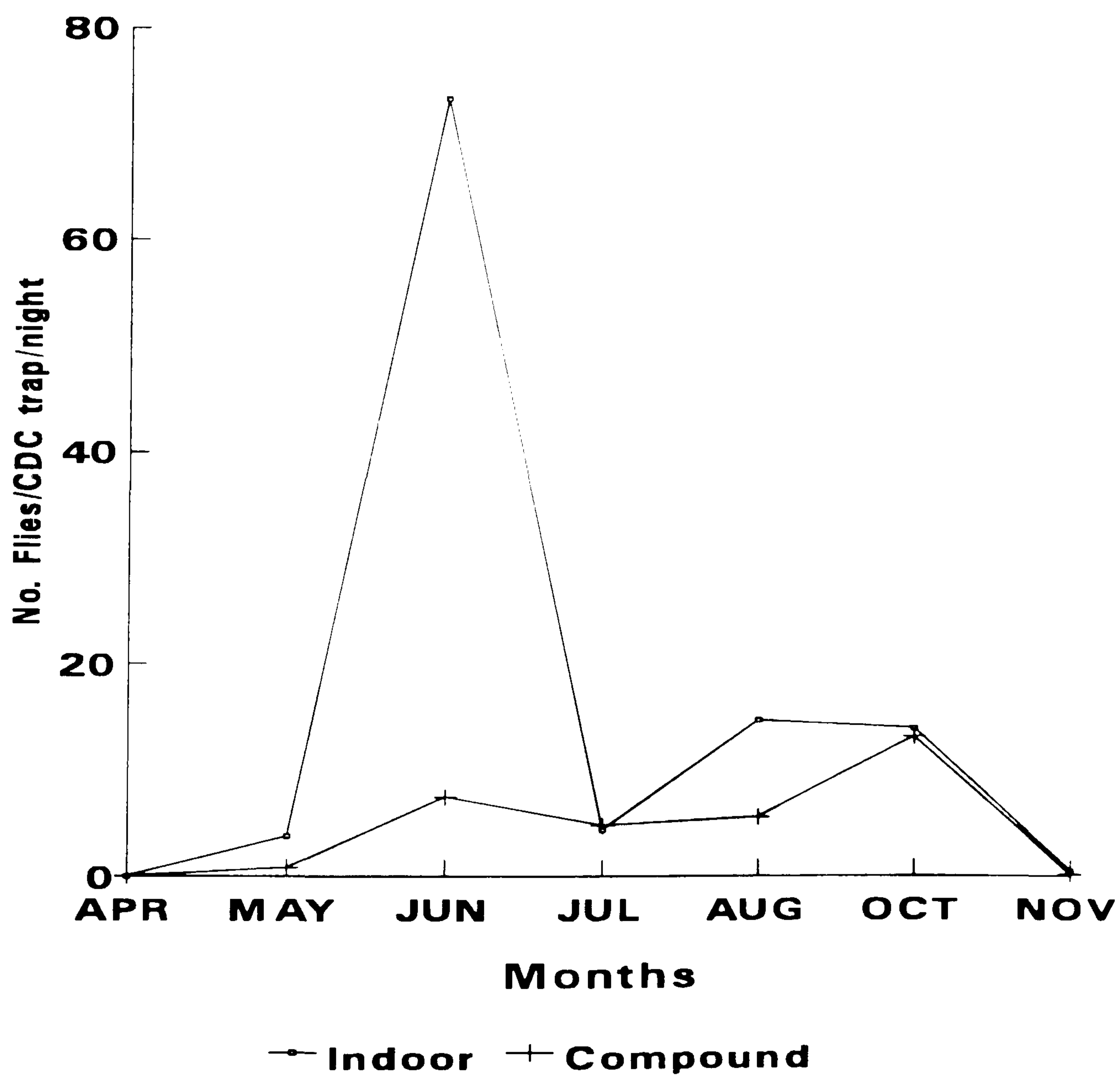
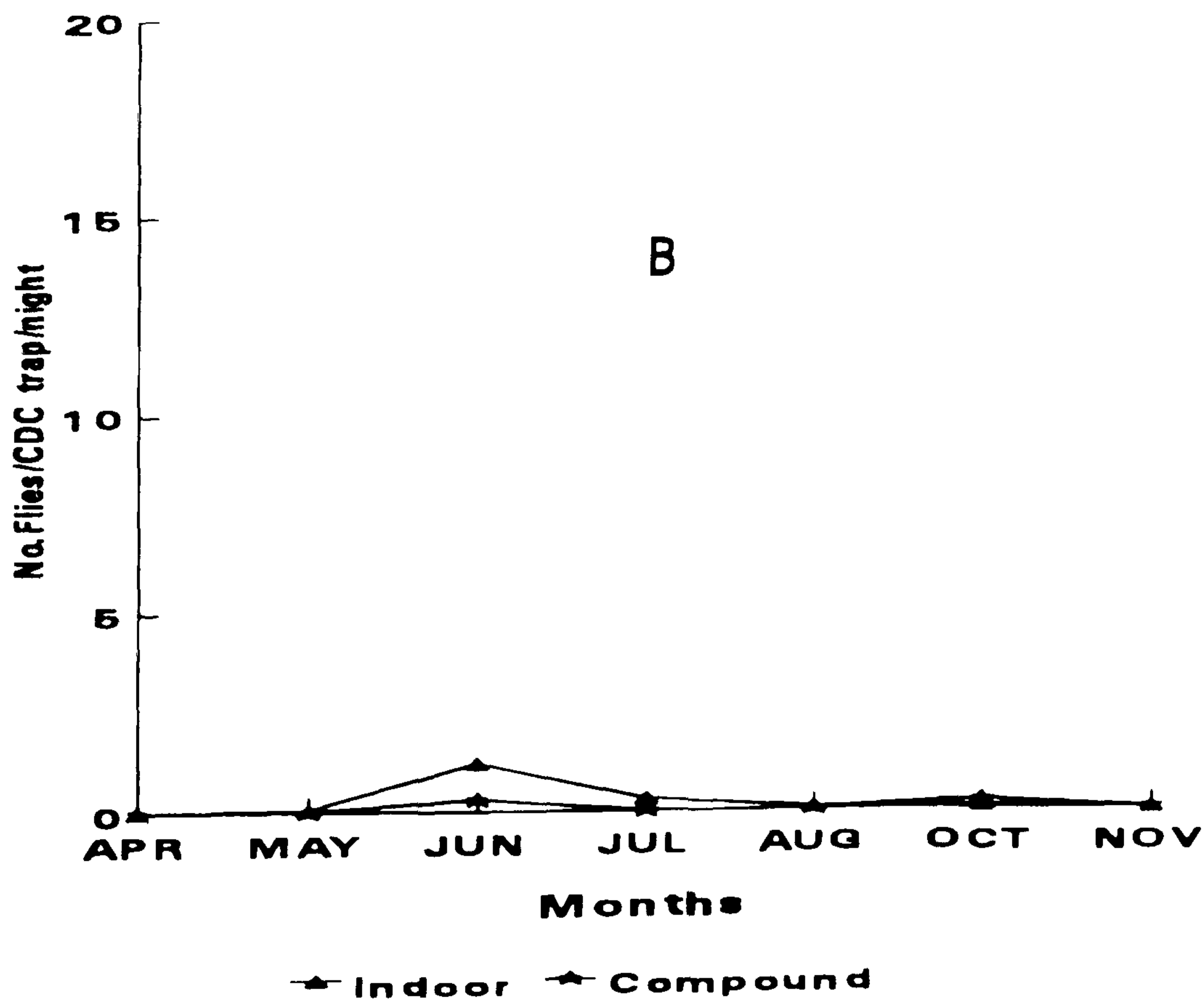
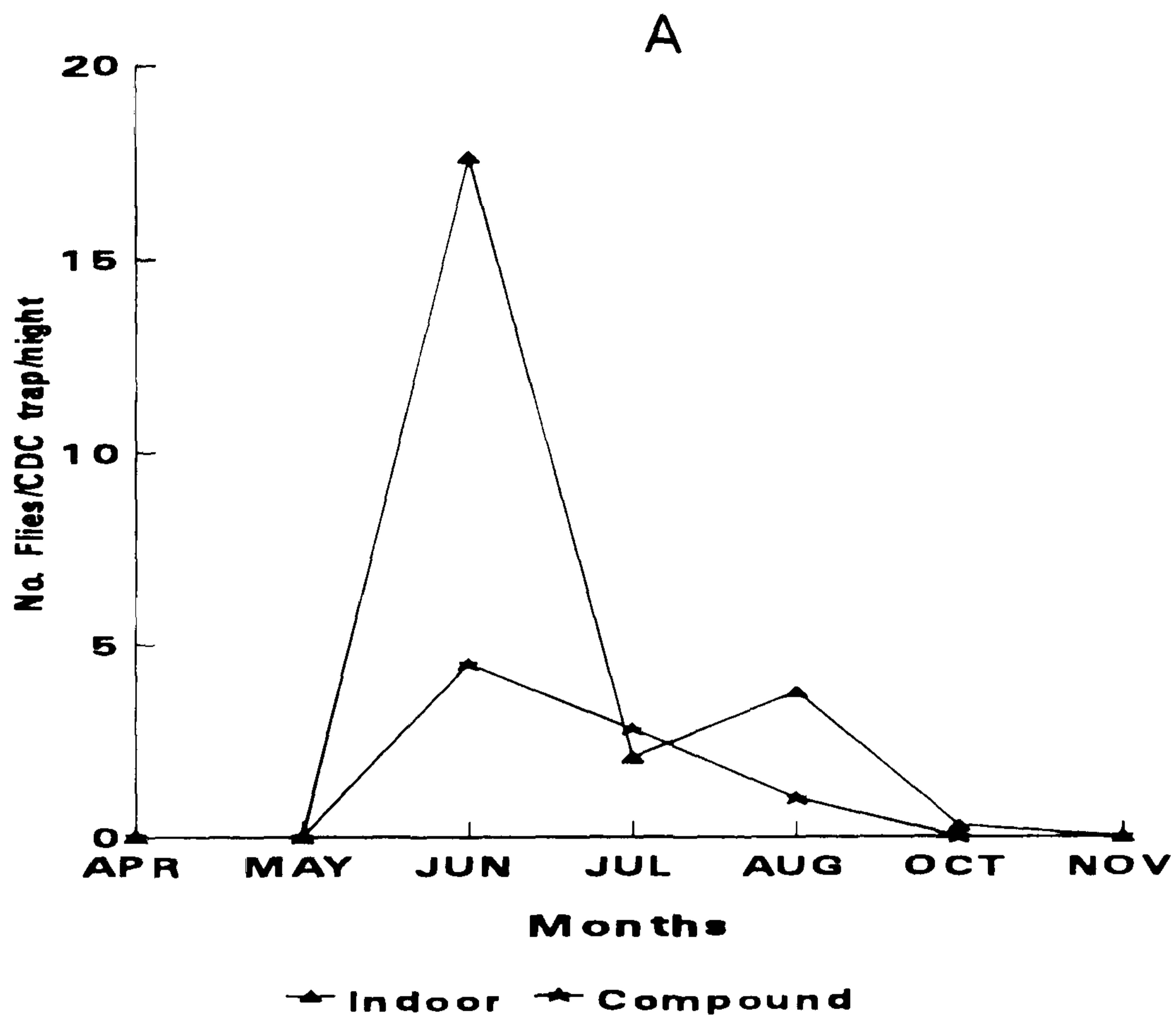




Figure 6.11

Densities of *P. sergenti*(A) and *P. major* (B) in CDC light-traps from Rehra village, Azad Jammu & Kashmir pooled data (1991-92)



# Figure 6.12

## MEAN MONTHLY TEMPERATURE (°C) AND RELATIVE HUMIDITY (%) FOR AJK

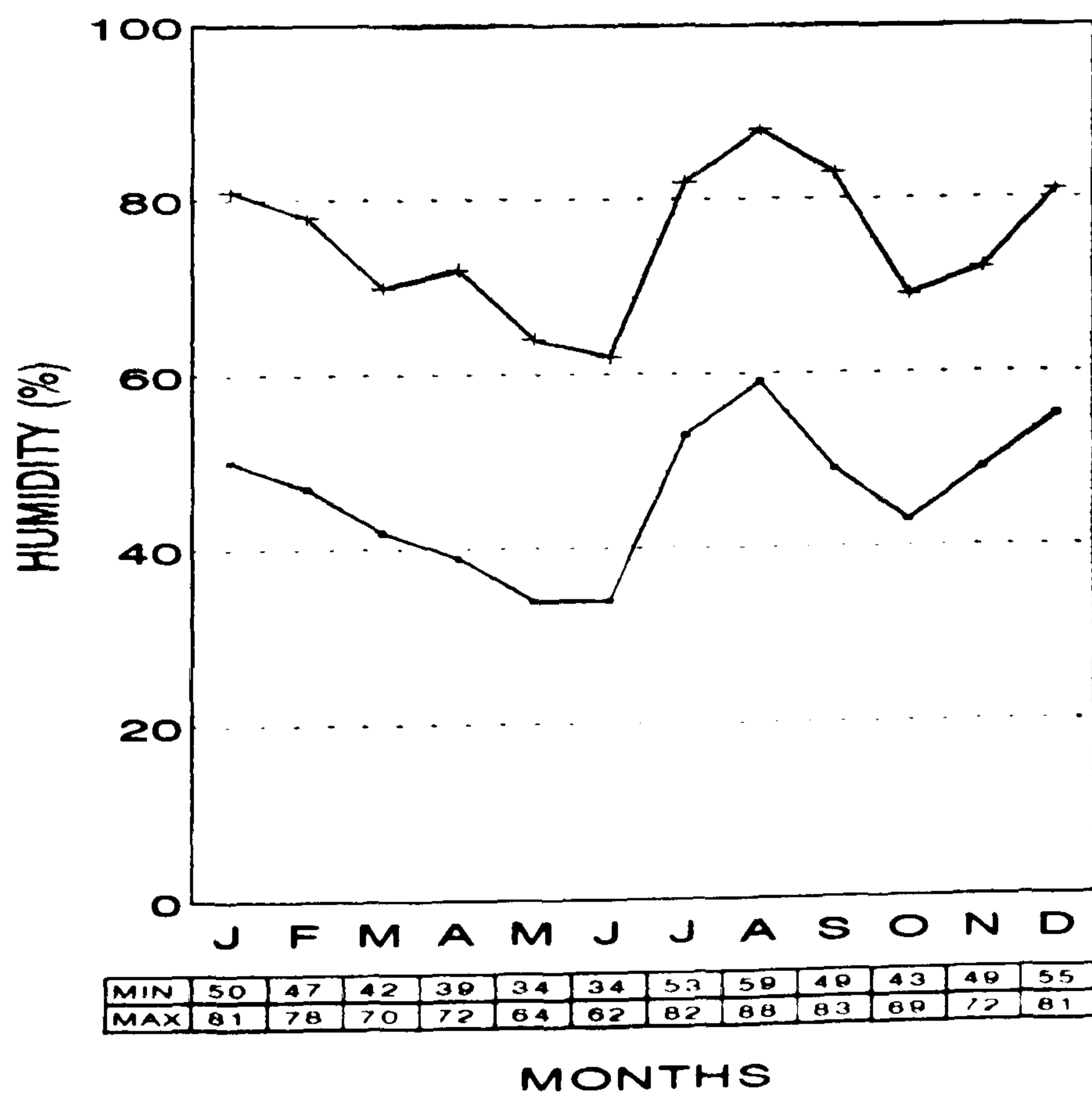
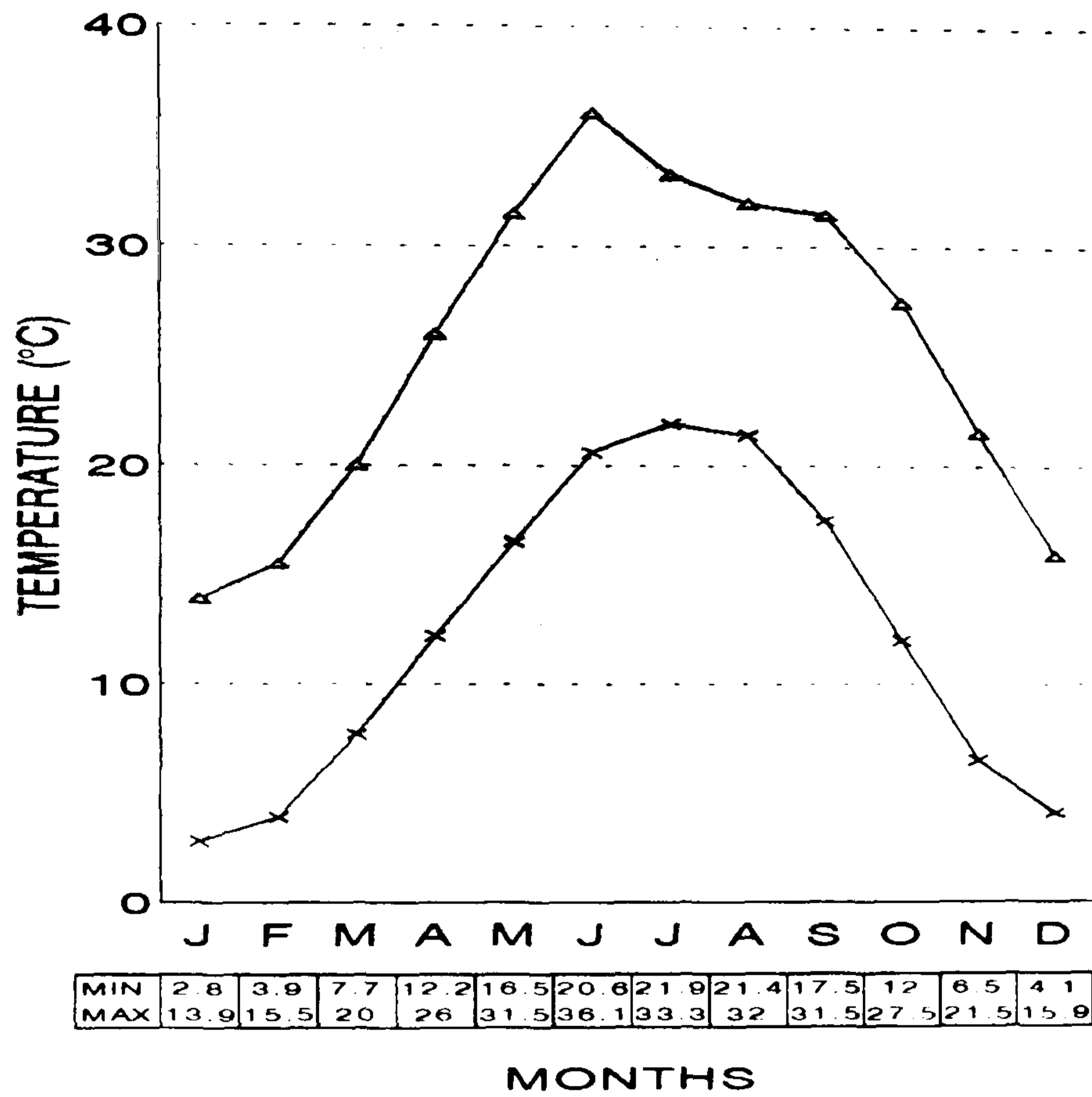
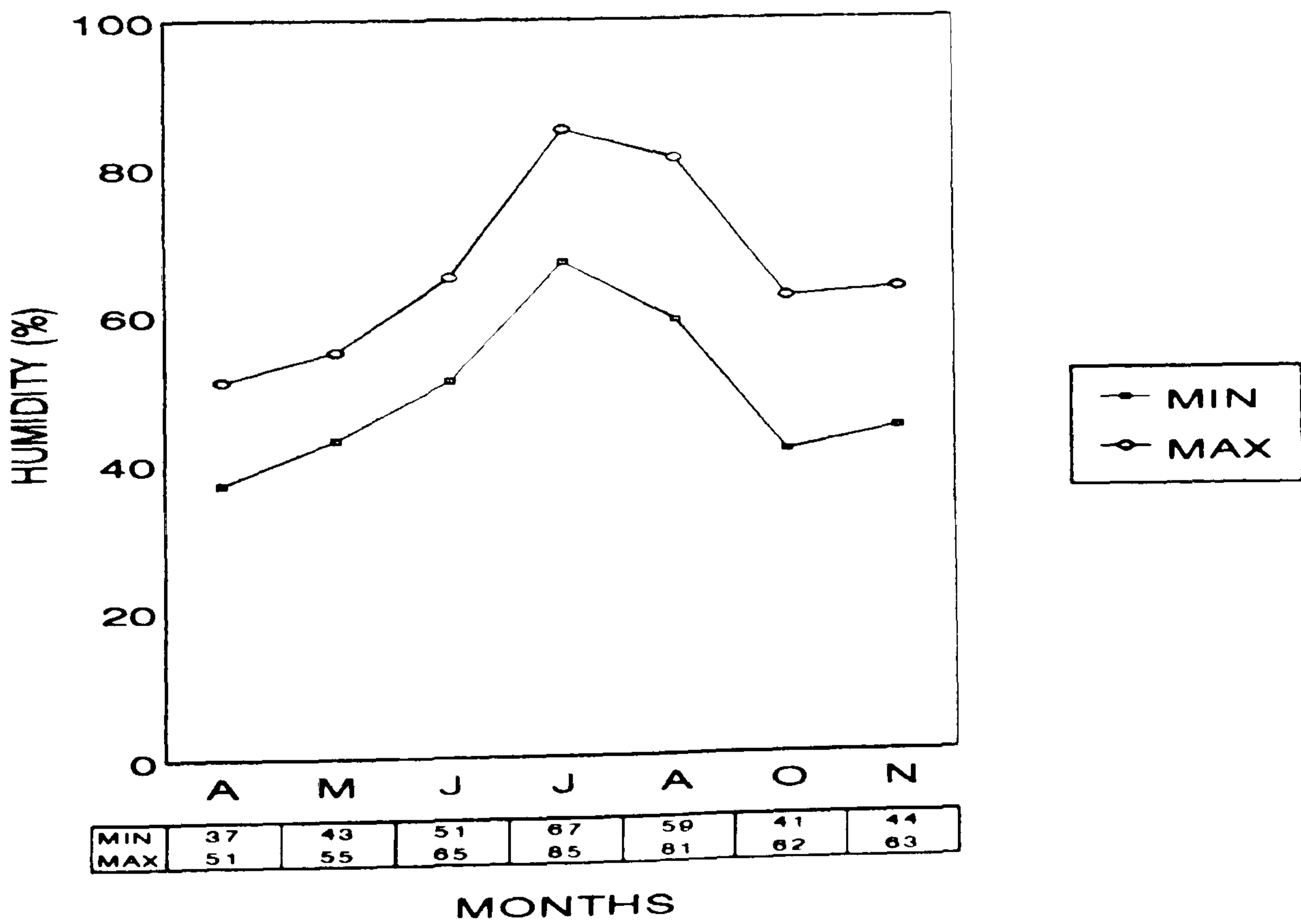
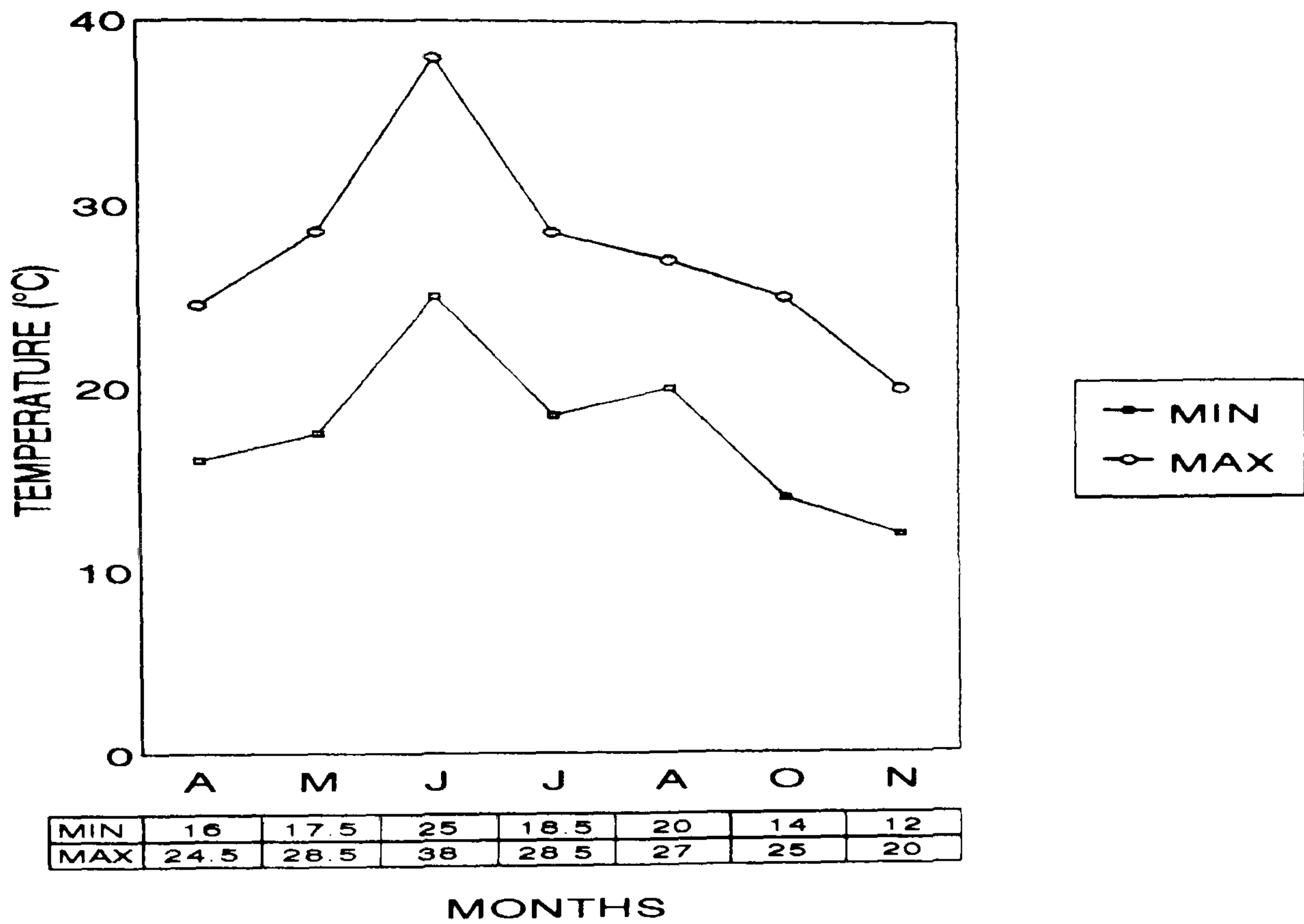


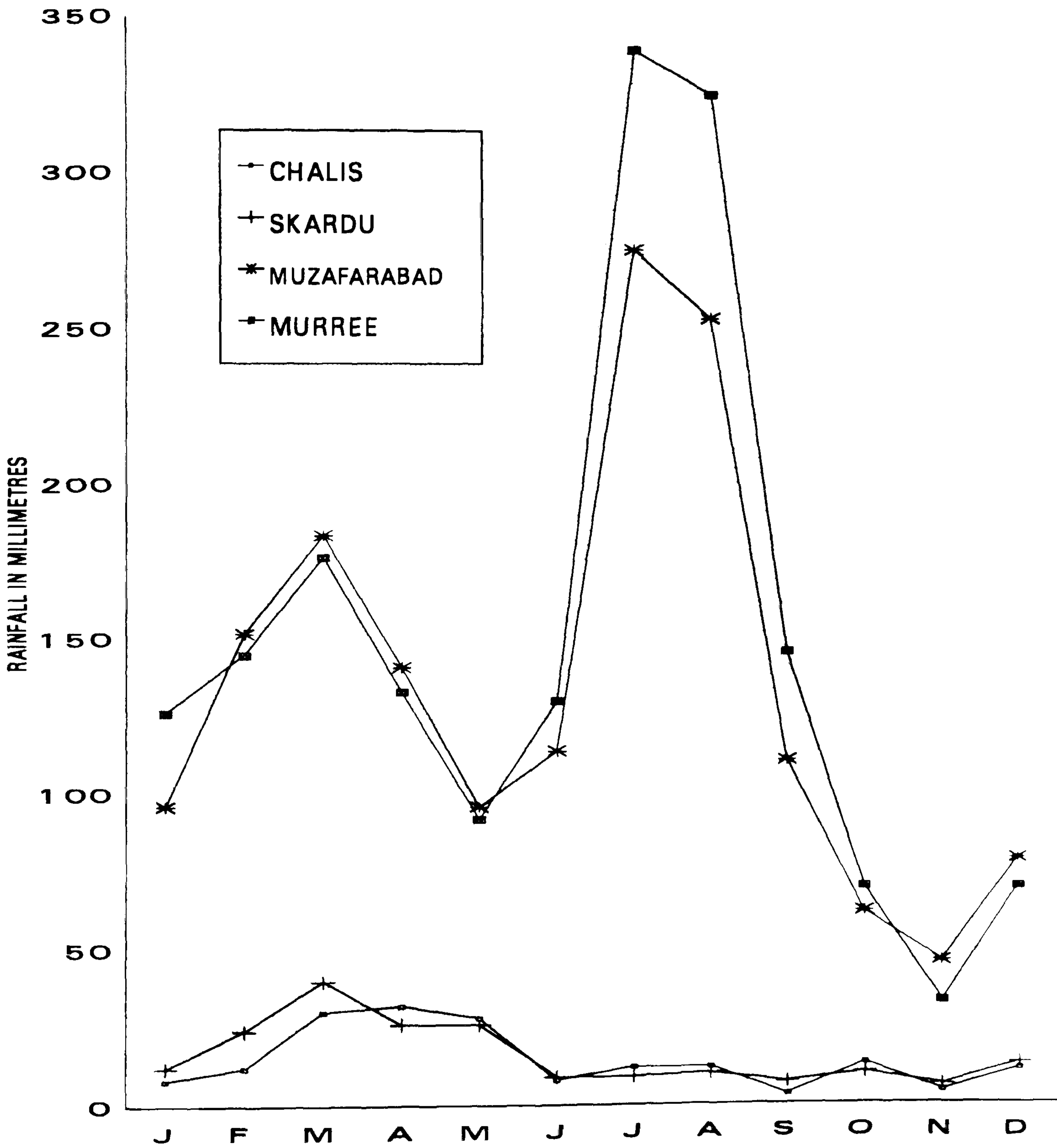
Figure 6.13

MEAN MONTHLY TEMPERATURE (°C) AND RELATIVE HUMIDITY IN REHRA VILLAGE (AJK).



# Figure 6.14

Precipitation in N.A. and AJK averages (Period 1961-1991).



CHALIS	8	12	30	22	28	8	12	12	3	13	4	11
SKARDU	12	24	40	28	28	9	9	10	7	10	8	12
MUZAFARABAD	98	152	184	141	98	114	278	254	111	62	48	78
MURREE	128	148	177	132	92	130	340	328	148	70	32	70

MONTHS

SOURCE:

Dept. of Meteorology

Govt. of Pakistan. Shadman,

**Table 6.7. Densities of *Phlebotomus* in CDC light traps collected from indoors and compounds in Rehra (Bagh) Azad Jammu & Kashmir (pooled data 1991-92).**

Month	Location	<i>P.hindustanicus</i>		<i>P.sergenti</i>		<i>P.major</i>		Total traps
		n	X	n	X	n	X	
April	Indoors	0	0	0	0	0	0	18
	Compounds	0	0	0	0	0	0	18
May	Indoors	23	1.3	0	0	1	0.05	18
	Compounds	5	0.3	0	0	0	0	18
June	Indoors	481	27.0	319	17.7	22	1.2	18
	Compounds	45	2.5	82	4.5	6	0.3	18
July	Indoors	22	1.5	31	2.06	5	0.3	15
	Compounds	29	1.9	42	2.8	0	0	15
August	Indoors	89	4.9	68	3.8	1	0.05	18
	Compounds	17	0.9	18	1	0	0	18
September*	Sampling could not be done during this month due to inaccessibility to the field area because of heavy floods.							
October	Indoors	85	4.7	6	0.3	1	0.05	18
	Compounds	80	4.4	0	0	3	0.16	18
November	Indoors	9	0.5	0	0	0	0	18
	Compounds	3	0.2	0	0	0	0	18

**n=Total number of flies.**

**X=Mean number of flies /CDC light trap.**

**Note: In addition to the above *Phlebotomus* species 2 males *P.kandelakii burneyi* were also captured in CDC light trap during 1992.**

**Table 6.8. Densities of *Phlebotomus* in CDC light traps collected from indoors and compounds in Rehra (Bagh) Azad Jammu & Kashmir (1991 & 1992).**

Month	Location	<i>P. hindustanicus</i>				<i>P. sergenti</i>				<i>P. major</i>				Total traps			
		1991		1992		1991		1992		1991		1992		1991	1992		
		n	X	n	X	n	X	n	X	n	X	n	X				
April	Indoors	0		0		0		0		0		0		0	9	9	
	Compounds	0		0		0		0		0		0		0	9	9	
May	Indoors	12	1.3	11	1.2	0		0		0		0		1	0.1	9	9
	Compounds	5	0.5	0		0		0		0		0		0		9	9
June	Indoors	119	13.2	322	35.7	156	17.3	163	18.1	19	6.3	3	0.3	9	9	9	
	Compounds	10	1.1	35	3.9	16	1.8	66	7.3	3	1.0	3	0.3	9	9	9	
July	Indoors	13	2.2	9	1	22	3.7	9	1	4	2.0	1	0.1	6	9	9	
	Compounds	0		29	3.2	1	0.17	41	4.5	0		0		6	9	9	
August	Indoors	34	3.8	55	6.1	36	4.0	32	3.5	1	0.1	0	0	9	9	9	
	Compounds	11	1.2	6	0.7	13	1.4	5	0.5	0		0		9	9	9	
September	Sampling could not be done during this month due to inaccessibility to the field area because of heavy floods.																
October	Indoors	47	5.2	38	4.2	1	0.1	5	0.5	1	0.1	0	0	9	9	9	
	Compounds	63	7.0	17	1.9	0		0		3	0.3	0	0	9	9	9	
November	Indoors	9	1.0	0		0		0		0		0		9	9	9	
	Compounds	3	0.3	0		0		0		0		0		9	9	9	

n=Total number of flies.

X=Mean number of flies /CDC light trap.

Note: In addition to the above *Phlebotomus* species 2 males of *P. kandelakii burneyi* were also captured in CDC light trap during 1992.

preceding the monsoon. Statistical analysis also showed that the activity of *P.hindustanicus* is significantly correlated with temperature ( $r= 0.725$ ;  $P < 0.03$ ) and negatively correlated with rainfall ( $r= -0.402$ ;  $P > 0.05$ ) although this later relationship is not statistically significant..

#### **B. Seasonal changes: Sticky trap collections.**

The number of sandflies caught from Rehra Village by sticky traps were very few (Table 6.9, 6.10), a total of only 195 sandflies were collected belonging to three species: *P.hindustanicus*, *P. sergenti* and *P. major*.

In Azad Jammu & Kashmir only *P.hindustanicus* was collected on sticky traps and in very low density (Table 6.9). In Kashmir the inefficiency of sticky traps in intercepting sandflies can be clearly attributed to the heavy rains in this area.

#### **6.3.4. SEX RATIOS.**

There were substantial differences in the sex ratios between species in both the CDC light traps and sticky trap collections from Northern Areas (Table 6.11). Male to female proportions were significantly higher for *Phlebotomus papatasi* and *P.sergenti*, whereas the ratio was converse in *P.keshishiani* and *P.salangensis* which showed significantly higher proportions of females than males. The sex ratio in *P.alexandri* was however almost equal. In Azad Jammu & Kashmir males were always significantly higher in number than females for all *Phlebotomus* species collected in light-traps.

In contrast to light-trap collections the sticky paper traps always showed higher proportions of males than females for all species captured in both the areas (N.A &

Table 6.9. Densities of *Phlebotomus* in sticky paper traps collected from Rehra (Bagh), Azad Jammu & Kashmir.

Month	<i>P.hindustanicus</i>				<i>P.sergenti</i>				<i>P.major</i>				Total traps	
	1991		1992		1991		1992		1991		1992		1991	1992
	n	X	n	X	n	X	n	X	n	X	n	X		
April	-	-	-	-	-	-	-	-	-	-	-	-	-	-
May	-	-	-	-	-	-	-	-	-	-	-	-	210	212
June	21	0.10	52	0.24	9	0.04	4	0.02	-	-	-	211	217	
July	-	-	-	-	-	-	-	-	-	-	-	148	174	
August	9	0.04	13	0.06	-	-	2	0.01	-	-	2	225	215	
September	Unsampled													
October	17	0.07	28	0.13	-	-	-	-	-	-	-	221	210	
November	12	0.10	-	-	-	-	-	-	-	-	-	128	208	

n=Total number of flies caught..

- No sandflies caught.

X=Mean number of flies caught/sticky paper /night.



**Table 6.10. Densities of *Phlebotomus* in Sticky paper traps collected from Wild in Rehra (Bagh) Azad Jammu & Kashmir (1992).**

Month	<i>P.hindustanicus</i>		<i>P.sergenti</i>		<i>P.major</i>		Total traps
	n	X	n	X	n	X	
April	-	-	-	-	-	-	
May	3	0.01	-	-	-	-	209
June	11	0.05	4	0.02	-	-	204
July	-	-	-	-	-	-	151
August	7	0.03	-	-	-	-	200
September	Unsampled						
October	-	-	-	-	-	-	210

n=Total number of flies caught.  
X=No. sandflies caught/ sticky paper/night.

**Table 6.11. Sex ratios of *Phlebotomus* species in light-traps and sticky traps collections.**

METHODS	AREA	SPECIES	MALES (%)	FEMALES (%)	Z	P
CDC light-traps	NA	<i>P.papatasi</i>	799(56.82)	607(43.17)	5.1	< 0.001
		<i>P.sergenti</i>	613(57.28)	457(42.71)	4.76	< 0.001
		<i>P.keshishiani</i>	276(19.15)	1165(80.84)	23.4	< 0.001
		<i>P.alexandri</i>	23(39.65)	35(60.34)	1.57	> 0.1
		<i>P.salangensis</i>	40(29.6)	95(70.37)	4.73	< 0.001
	AJK	<i>P.hindustanicus</i>	469(55.30)	379(44.69)	3.09	< 0.05
		<i>P.sergenti</i>	383(67.66)	183(32.33)	8.40	< 0.001
		<i>P.major</i>	29(74.3)	10(25.64)	3.04	< 0.05
Sticky traps	NA	<i>P.papatasi</i>	154(65.5)	81(34.46)	4.76	< 0.001
		<i>P.sergenti</i>	448(78.87)	120(21.12)	13.76	< 0.001
		<i>P.keshishiani</i>	168(63.15)	98(36.84)	4.29	< 0.001
		<i>P.alexandri</i>	50(86.20)	8(13.7)	5.5	< 0.001
		<i>P.salangensis</i>	7(53.84)	6(46.15)	0.3	> 0.5
	AJK	<i>P.hindustanicus</i>	139(79.8)	35(20.1)	7.9	< 0.001
		<i>P.sergenti</i>	16(84.2)	3(15.78)	2.1	< 0.001

NA= Northern Areas.  
AJK = Azad Jammu & Kashmir  
Within parenthesis are the percentages.

AJK) (Table 6.11).

### 6.3.5. COMPARISON BETWEEN CATCHING METHODS.

In order to determine how similar different catching methods were in sampling sandflies over the whole period, the proportions of *Phlebotomus* species collected by one method was compared with another. Table 6.12 shows<sup>a</sup> comparison of various species caught in CDC light traps and sticky traps outdoors and Table 6.12 shows<sup>a</sup> comparison of *Phlebotomus* species collected indoors in CDC light traps and human bait catches.

There are substantial differences in the proportions of various species collected outdoors by the two methods: CDC light-traps and sticky trap. The proportions of *Phlebotomus papatasi* and *P.keshishiani* were significantly higher in light-trap collections than sticky traps whereas this was reverse in *P.sergenti*, *P.alexandri* and *P.hindustanicus*, which were collected in significantly higher proportions on Sticky traps than light-traps. Only one species, *P.salangensis*, was collected in almost equal numbers both in light traps as well as sticky traps.

Indoor catches (Table, 6.13) with CDC light traps when compared with human bait collections revealed that *P.papatasi* and *P.keshishiani* were caught in significantly higher proportions in human bait catches than light traps.

### 6.3.6. HUMAN BITING SPECIES.

To find out which species of sandflies bite humans in the two foci of visceral leishmaniasis two types of study were carried out: Human bait catches and blood-meal analysis.

**Table 6.12. Comparison of *Phlebotomus* species caught in CDC light-traps and sticky traps from Northern Areas and Azad Jammu & Kashmir.**

Locality	Species	LT(outdoors)	n	ST(outdoors)	n	Z	P
Hudur (N.A)	<i>P.papatasi</i>	691(63.39)	1090	229(49.03)	467	5.18	< 0.001
	<i>P.sergenti</i>	399(36.6)		238(50.96)		5.5	< 0.001
Thor (N.A)	<i>P.keshishiani</i>	802(82.42)	973	262(39.57)	662	18.39	< 0.001
	<i>P.sergenti</i>	100(10.27)		330(49.84)		19.9	< 0.001
	<i>P.papatasi</i>	32(3.28)		6(0.90)		3.28	< 0.005
	<i>P.alexandri</i>	30(3.08)		55(8.30)		5.22	< 0.001
	<i>P.salangensis</i>	9(0.9)		9(1.35)		0.9	> 0.4
REHRA (AJK)	<i>P.hindustanicus</i>	179(56.28)	195	174(89.2)	202	4.9	< 0.001
	<i>P.sergenti</i>	142(44.65)		28(20.5)		4.3	< 0.001

**Table 6.13. Comparison of *Phlebotomus* species collected in light-traps and on human baits in Northern Areas.**

Locality	Species	LT(indoors)	n	HB(indoors)	n	Z	P
Hudur (NA)	<i>P.papatasi</i>	655(58.22)	1125	702(93.35)	758	16.4	< 0.001
	<i>P.sergenti</i>	470(41.77)		56(7.38)		16	< 0.001
Thor(NA)	<i>P.keshishiani</i>	527(83.92)	628	194(90.23)	215	2.25	< 0.02
	<i>P.sergenti</i>	101(16.08)		21(9.76)		2.26	< 0.01

N.A.= Northern Areas.

AJK = Azad Jammu & Kashmir.

n= number of sandflies in a trap type.

LT= Light trap.

HB= Human bait catches.

ST= Sticky traps.

### 6.3.6.1. Human-bait catches.

All night biting catches on humans were carried out in Northern Areas and Azad Jammu & Kashmir. Curiously we were unable to catch sandflies biting human bait indoors or outdoors in the latter area at any time during the entire sandfly season. Therefore the data presented pertain only two localities (Hudur village and Thor village) sampled (indoors) in Northern Areas.

Throughout the study period, the accumulated data from indoor catches on human bait yielded 997 sandflies out of which 762 were from Hudur village and 235 from Thor village (Table 6.2). Seven *Phlebotomus* species were found biting man indoors which in descending order of abundance were: *P.papatasi* (714; 71%), *P.keshishiani* (195; 19.56%), *P.sergenti* (77; 7.7%), *P.alexandri* (4; 0.4%), *P.salangensis* (3; 0.3%), and *P. kandelakii burneyi* (4; 0.4%).

Within each locality, the percentages of the common man biting species however varied (Table 6.14 & 6.15); *P.papatasi* (702:92%) and *P.sergenti* (7.34%) were the dominant man biting species in Hudur and *P.keshishiani* (195: 83%) was the only man biting species in Thor village.

### 6.3.6.2. Seasonal changes in the common human-biting species.

In order to show the relative biting densities of the most common man biting species and their seasonal variation, the monthly human bait collections from indoors were expressed as mean number of flies per two-man team per night.

Seasonal variation in the biting densities of *Phlebotomus papatasi* and *P.sergenti* from Hudur village is shown in table 6.14 and Fig 6.15. *P.papatasi* was found biting humans throughout the season (May till August) with peak biting activity

**Table 6.14. The two hourly indoor biting density of *Phlebotomus papatasi* and *P.sergenti* per two men per night in Hudur village, Northern areas.**

Hour	<i>Phlebotomus</i> spp.	APR	MAY	JUN	JUL	AUG	OCT	Grand Total
18-20	<i>P.papatasi</i>	-	-	-	-	-	-	-
	<i>P.sergenti</i>	-	-	-	-	-	-	-
20-22	<i>P.papatasi</i>	-	21(10.5)	72(36)	12	15(7.5)	-	120(17.1)
	<i>P.sergenti</i>	-	-	1(0.5)	1	5(2.5)	-	7(1)
22-24	<i>P.papatasi</i>	-	39(19.5)	73(36.5)	16	34(17)	-	162(23.5)
	<i>P.sergenti</i>	-	2(1)	5(2.5)	3	7(3.5)	-	17(2.4)
24-02	<i>P.papatasi</i>	-	48(24)	65(32.5)	9	17(8.5)	-	139(19.8)
	<i>P.sergenti</i>	-	1(0.5)	3(1.5)	1	2(1)	-	7(1)
02-04	<i>P.papatasi</i>	-	29(14.5)	19(9.5)	6	7(3.5)	-	61(8.7)
	<i>P.sergenti</i>	-	2(1)	-	-	2(1)	-	4(0.6)
04-6	<i>P.papatasi</i>	-	5(2.5)	19(9.5)	12	46(23)	-	82(11.7)
	<i>P.sergenti</i>	-	-	-	-	-	-	-
Total	<i>P.papatasi</i>	-	142(71)	248(124)	55	119(59.5)		564(80.5)
	<i>P.sergenti</i>	-	5(2.5)	4.5(9)	5	16(8)		35(5)

Two night catches except in July when one night catches could be made only.

**Table 6.15. The two hourly indoor biting density of *Phlebotomus keshishiani* per two men per night in Thor village, Northern areas.**

Hour	Apr		Jun		Jul		Aug		Sep		Oct		Grand Total	
	n	X	n	X	n	X	n	X	n	X	n	X	n	X
18-20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20-22	-	-	6	3	9	4.5	11	5.5	-	-	-	-	28	4.3
22-24	-	-	24	12	22	11	27	13.5	-	-	-	-	73	12.1
24-02	-	-	12	6	13	6.5	16	8	-	-	-	-	41	6.8
02-04	-	-	-	-	12	6	20	10	-	-	-	-	32	5.3
04-6	-	-	-	-	-	-	12	6	-	-	-	-	12	2
Total	-	-	42	21	56	28	86	38	-	-	-	-	184	30.6

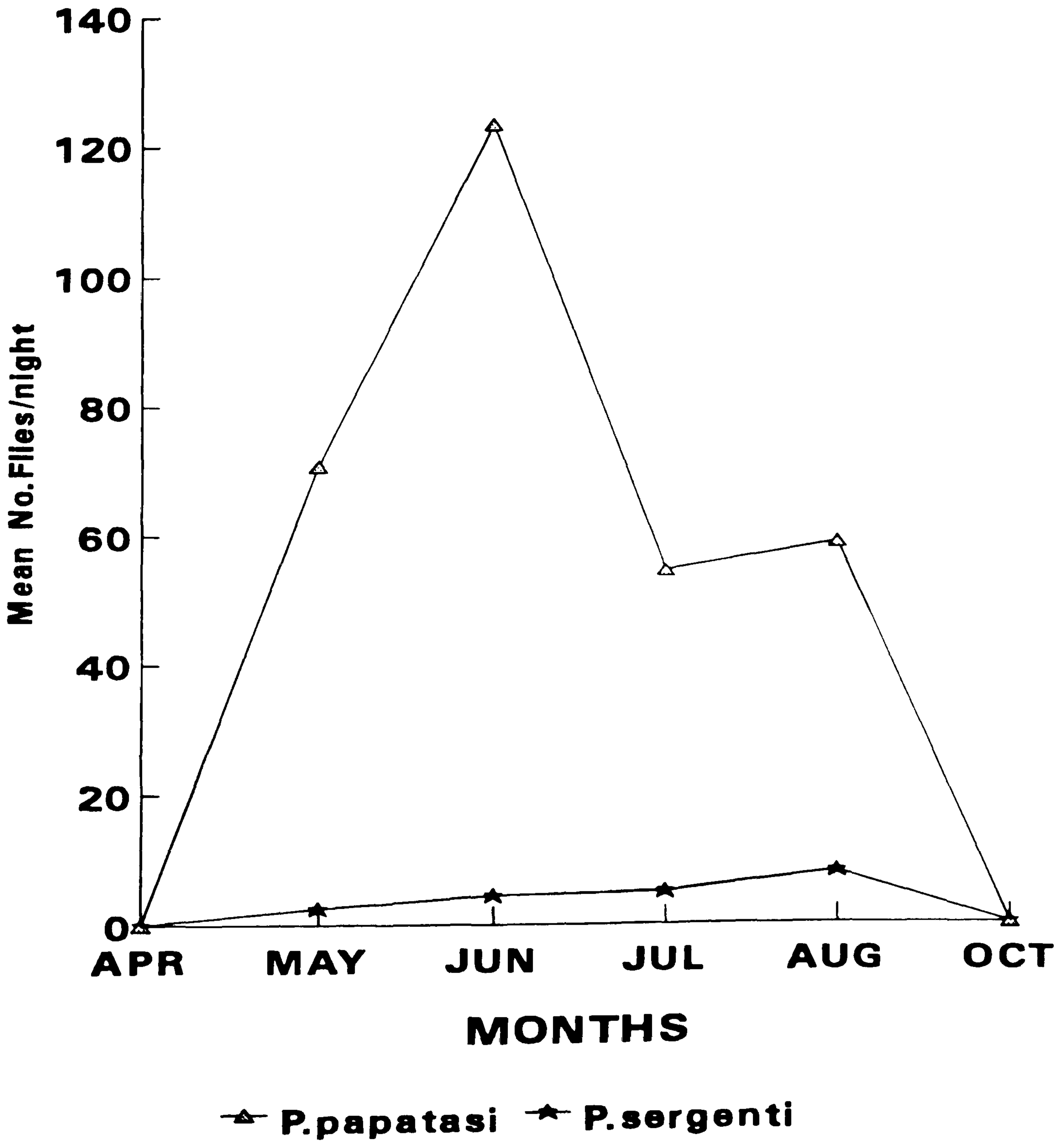
n=Total number of sandflies biting man.

X=Mean number of flies biting two man/night.

Note: Month of May could not be sampled.

Figure 6.15

Seasonal variation in the biting rates of *P. papatasi* and *P. sergenti* from Hudur village, Northern Areas



in June. This is in contrast to the peak activity in May as observed in CDC light trap collections. This difference is almost entirely due to males in the traps:- males dominated the indoor collections of *Phlebotomus* in May whereas female density did not show much variation between May and June.

In Thor village, *P.keshishiani* was found biting humans indoors (Table 6.15 & Fig 6.16) from June till August with a clear peak in August, which exactly corresponds to the pattern observed in the indoor collections by CDC light-traps. The biting densities of *P.alexandri*, *P.salangensis*, *P.papatasi*, *P.sergenti* and *P.kandelakii burneyi* were too low to reveal any significant seasonal trends.

In Thor village, at higher altitude (1700 metres), *Phlebotomus keshishiani* was the dominant sandfly biting man. Peak biting activity was observed between 22-24.00 hours, as the summer progressed the period of biting was extended with an additional peak between 04.00 and 06.00 hours in August (Fig. 6.17).

The monthly biting rhythm of *Phlebotomus papatasi* from Hudur village is shown in Fig 6.18. In May when the temperature is low, the biting activity started at 20.00 hours and continued till 06.00 hours with peak activity between 24.00 hours and 02.00 hours, whereas in June, consistently intense biting was observed between 20.00 and 02.00 hrs gradually declining from 04.00 to 06.00 hrs without a prominent peak. In July and August comparatively low numbers of flies were found biting, corresponding to the reduction in overall density of sandflies during these months. Two peaks of biting were observed, one between 22-24.00 hours and another between 04.00-06.00 hours, the early morning peak was more prominent in August (Fig. 6.18).

*Phlebotomus sergenti* on the other hand was found biting man throughout the

Figure 6.16

Seasonal variation in the biting rates of *P. keshishiani* (indoors) from Thor village, Northern Areas

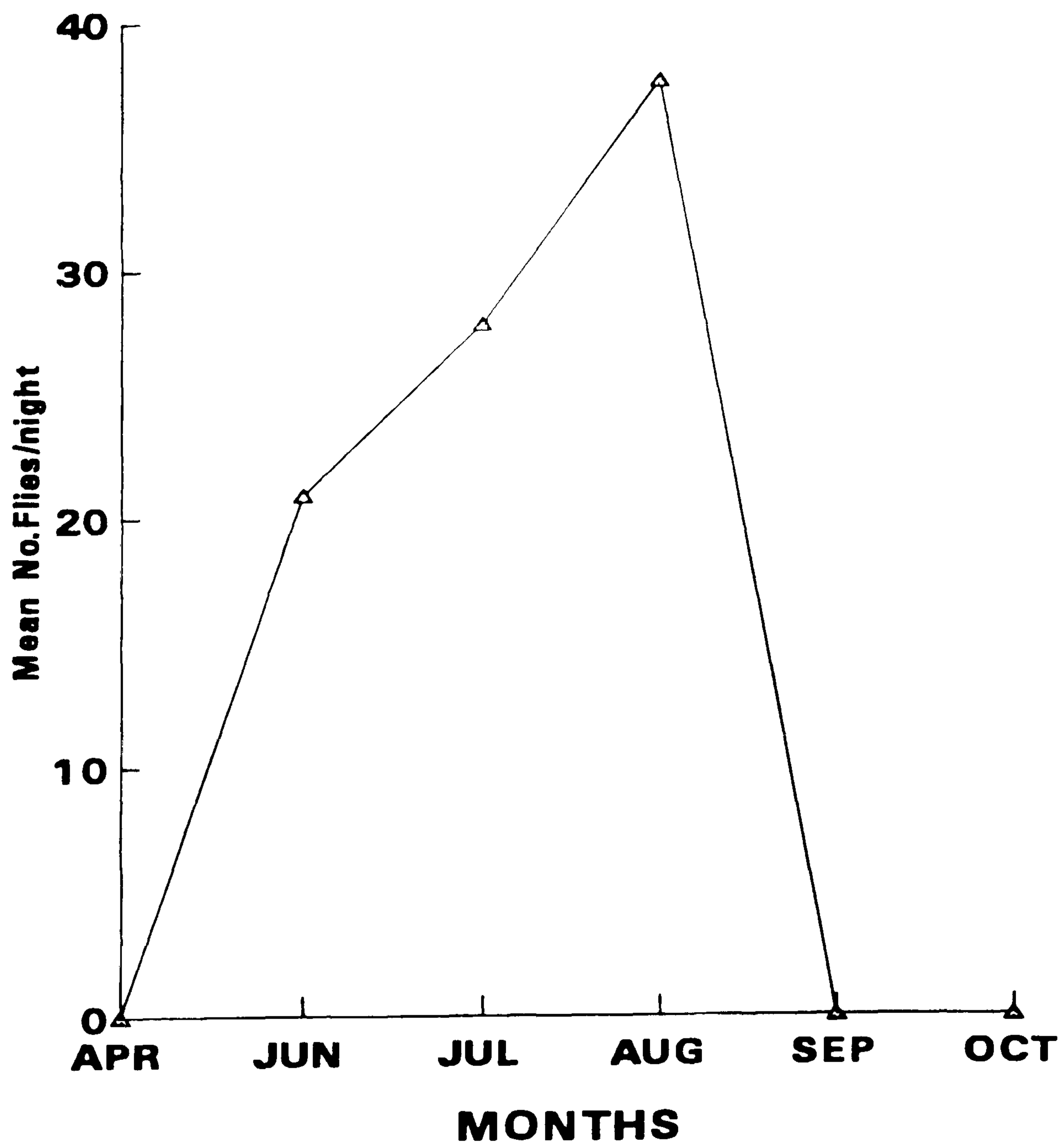
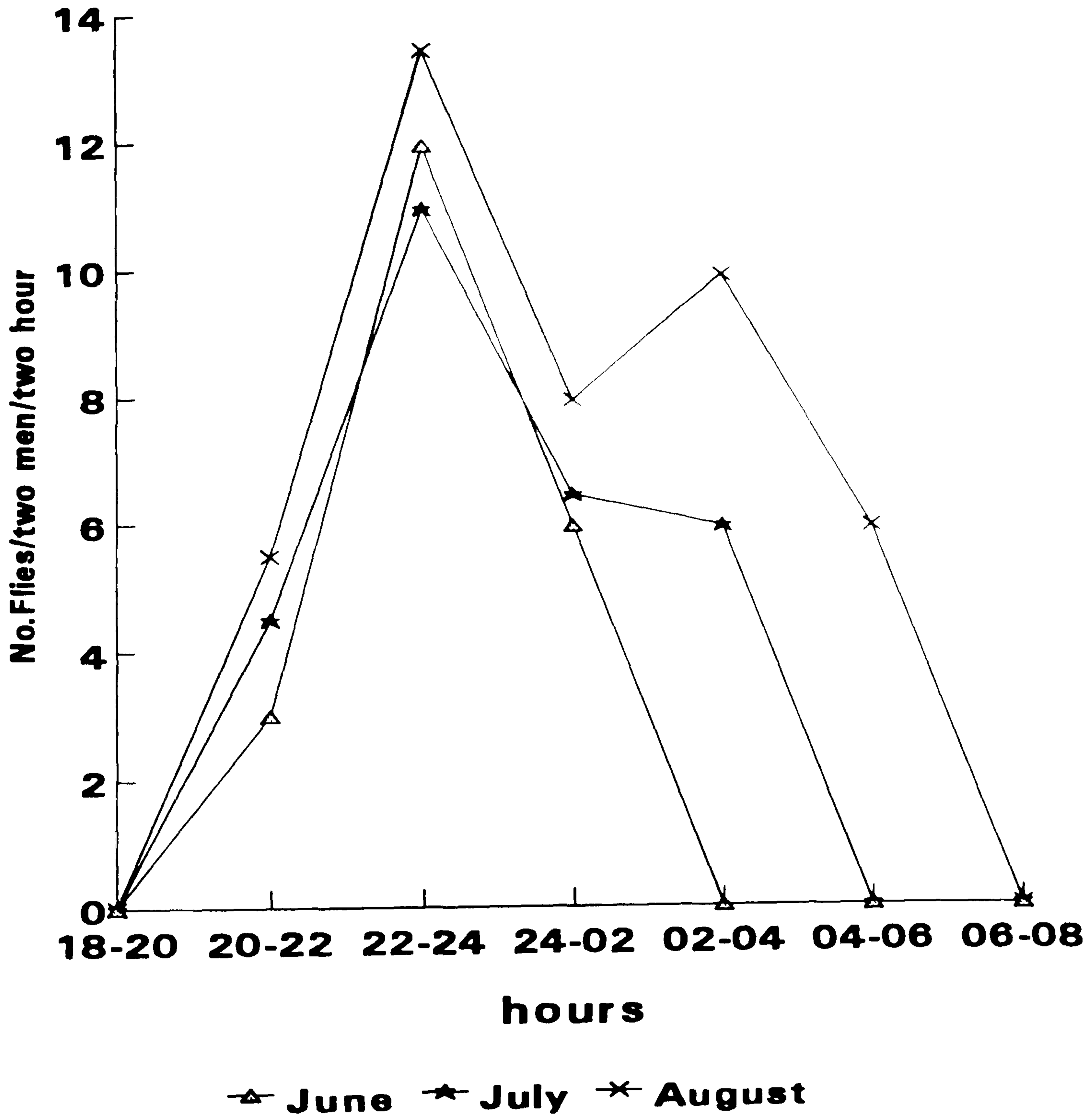




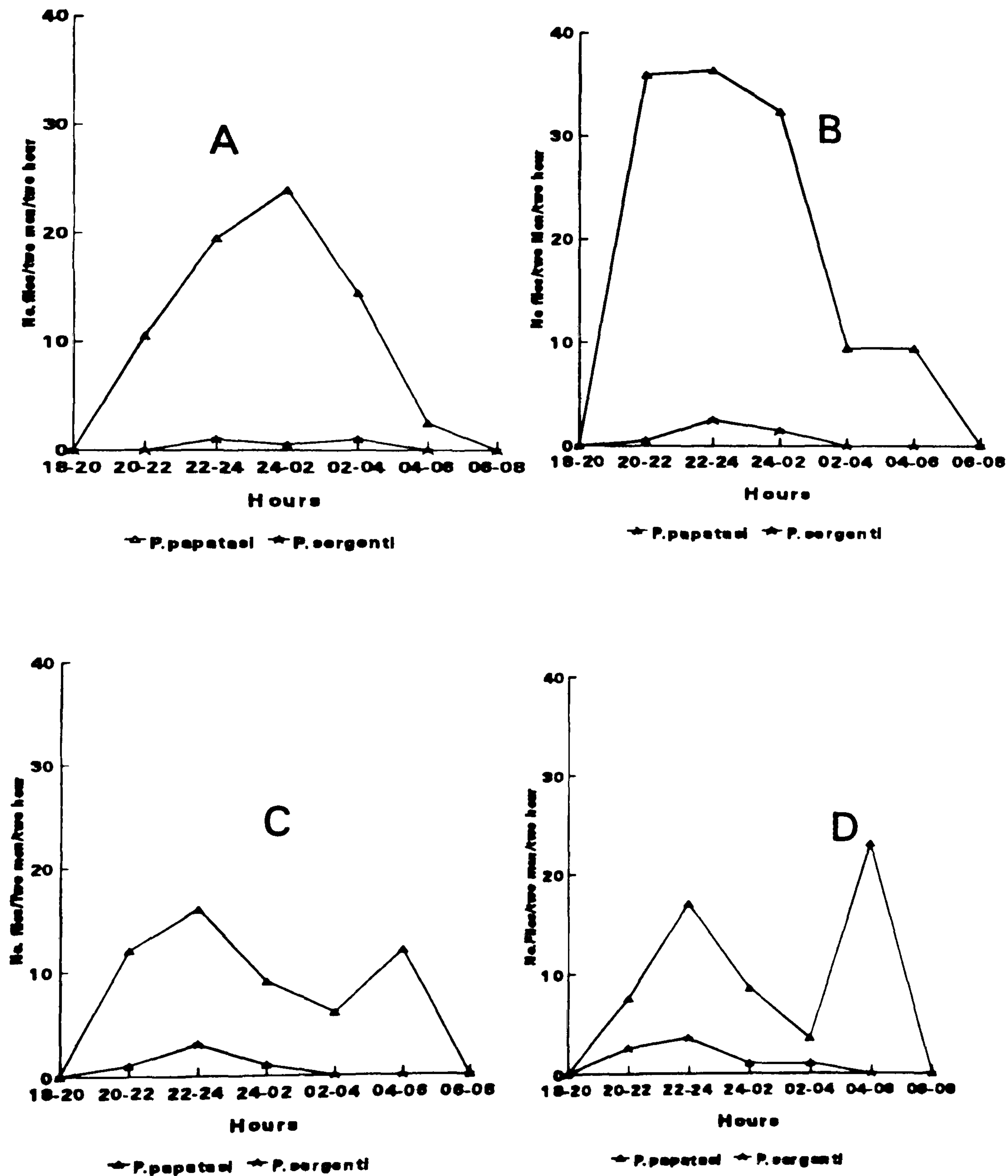
Figure 6.17

Biting rhythm of *P.keshishiani* (indoors) in Thor village, Northern Areas



# Figure 6.18

Indoor biting rhythm of *P. papatasi* and *P. sergenti* in May (A), June (B), July (C) and August (D) in Hudur village, Northern Areas



night with only a trend towards peak activity around midnight. However, numbers were low throughout the year.

### 6.3.6.3. Blood-meal analysis.

A total of 287 engorged specimens of *Phlebotomus* sandflies were captured in CDC light-traps from Northern Areas and Azad Jammu & Kashmir. The blood-meals were analyzed using Enzyme linked Immunosorbent Assay (Direct ELISA). ELISA kits were obtained from <sup>Dr.</sup> Alistair Voller's laboratory, London.

#### i) Northern Areas.

Table 6.16. shows the proportions of sandflies with positive blood meals of man and other vertebrate hosts. A total of 67 blood-fed sandflies were collected from Thor village (higher altitudes) out of which 59 (35 flies collected indoors and 24 outdoors) were *P.keshiahiani* and 8 (3 flies collected indoors and 5 outdoors) *P.sergenti*.

Of the 59 *P.keshishiani* collected 19 (32%) had fed on human, 5 (8.5%) on bovine and 18 (30.5%) on dog blood. Three flies showed double blood-meals: human/dog (2: 3.3%), human/bovine (1: 1.6%). Flies collected indoors showed the highest proportion of human blood whereas those from outdoors were mainly found to have fed on dogs. From the among 8 *P.sergenti* only 1(12.5%) collected from outdoors was positive with dog antisera. Blood-meals of 24 sandflies (17 *P.keshishiani* and 7 *P.sergenti*) were unidentified either because the blood was too well digested or alternatively they had fed on other vertebrate hosts.

In Hudur village (lower altitude) a total of 107 blood fed flies were captured.

Table 6.16 Blood meal analysis of *Phlebotomus* spp from Northern Areas and Azad Jammu & Kashmir.

Species n=287	Location	Blood Source										
		H	B	D	HD	HB	DB	HDB	UR			
<i>P.hindustanicus</i> (AJK) n=95	Indoors	17	38	20	0	2	1	1	16			
	Outdoors	0	3	1	1	0	0	0	0			
	Total	17(17.8)	41(43.1)	21(22.1)	1(1.05)	2(2.1)	1(1.05)	1(1.05)	16			
<i>P.sergenti</i> (AJK) n=15	Outdoors*	0	4 (26.6)	3 (20)	0	0	0	0	8 (53)			
	Outdoors*	0	0	1 (33.3)	0	0	0	0	2(66.6)			
<i>P.papatasi</i> (Hudur,NA) n=72	Indoors	11	6	3	1	1	1	1	47			
	Outdoors	2	0	3	1	0	0	0	0			
	Total	13(18.0)	6 (8.3)	6(8.3)	2 (2.8)	1 (1.4)	1 (1.4)	1 (1.4)	47			
<i>P.sergenti</i> (Hudur,NA) n=29	Indoors	3	7	1	2	0	2	0	17			
	Outdoors	1	0	0	0	0	0	0	0			
	Total	4(13.7)	7(24)	1(3.4)	2(6.8)	0	2(6.8)	0	17			
<i>P.alexandri</i> (Hudur,NA) n=2	Outdoors*	0	0	1(50)	0	0	0	0	1(50)			
	Outdoors*	0	0	1(25)	0	0	0	0	3 (75)			
<i>P.keshishiani</i> (Thor,NA) n=59	Indoors	15	3	6	2	1	0	0	17			
	Outdoors	4	2	12	0	0	0	0	0			
	Total	19(32.0)	5 (8.5)	18 (30.5)	2 (3.3)	1(1.6)	0	0	17			
<i>P.sergenti</i> (Thor,NA) n=8	Outdoors*	0	0	1 (12.5)	0	0	0	0	7 (87.5)			

H= Humans B= Bovine D= Dog HD= Human/Dog HB= Human/Bovine DB= Dog/Bovine HDB= Human/Dog/Bovine

UR= Unreactive

\*= Blood fed flies found only outdoors.

Out of these, 72 (54 indoors and 18 outdoors) were *P.papatasi*, 29 (13 indoors and 16 outdoors) *P.sergenti*, 4 (1 indoors and 3 outdoors) *P.salangensis* and 2 (1 indoor and 1 outdoors) *P.alexandri*. Of the 72 *P.papatasi*, 13 (18%) were positive with human blood, 6 (8.3%) with dog and 6 (8.3%) with bovine blood. 5 flies showed mixed blood-meals viz: human/dog (2: 2.8%), human/bovine (1: 1.4%), dog/bovine (1: 1.4%) and human/dog/bovine (1: 1.4%). Maximum numbers of flies caught indoors were positive with human and bovine blood, whereas only two out of 18 collected from outdoors reacted with human antisera.

Of the 29 *P.sergenti*, 4 (13.7%) were positive with human, 7 (24.1%) with bovine blood and 1 (3.4%) with dog blood. Two (6.8%) females showed mixed human/dog blood and 2 dog/bovine blood. Only 1 out of 16 flies collected outdoors was positive to human antisera. Out of the 4 *P.salangensis* and 2 *P.alexandri* only one each was found positive to dog blood. Both the positive flies were from outdoor light trap collections.

Over all, 47, *P.papatasi*, 17 *P.sergenti*, 3 *P.salangensis* and 1 *P.alexandri* were un-reactive to the antisera used perhaps for the same reasons as mentioned above.

## ii). Azad Jammu and Kashmir.

From this locality a total of 113 blood fed sandflies were captured out of which 95 were identified as *P.hindustanicus*, 15 as *P.sergenti* and 3 as *P.major*. The highest proportion of *P.hindustanicus* was found reactive to human, bovine and dog antisera (Table 6.16). Out of a total of 95 *P.hindustanicus*, 17 (17.8%) flies showed positive

blood-meals for humans, 41 (43%) for bovines and 21 (22.1%) for dogs. Four flies were found with double blood-meals in their guts: human/dog (1: 1.05%), human/bovine (2: 2.1%), dog/bovine (1: 1.05%). One fly was found with a triple blood-meal of human/bovine/dog origin. *P.sergenti* mainly showed positive blood meals of bovine (4: 26.6%) and dog (3: 20%) respectively. 1 out of 3 *P.major* was positive with dog antisera. In AJK only 4 blood fed flies (*P.hindustanicus*) were captured outdoors, 3 were positive to bovine and 1 to dog antisera.

Overall blood-meals of 16 *P.hindustanicus*, 8 *P.sergenti* and 2 *P.major* were unidentified because these did not react with the antisera used (Table 6.16).

### 6.3.7. PHLEBOTOMUS SPECIES BITING DOGS.

To understand which species of *Phlebotomus* sandflies bite dogs in the two foci, information was obtained in two ways: i) through regular monthly sampling of sandflies on dog-baited sticky paper traps and ii) by analysing the blood meals of the engorged flies captured in the disease foci.

#### 6.3.7.1. Dog-baited collections.

The objective of this experiment was to determine which species of *Phlebotomus* feed on dog the (reservoir of *Leishmania infantum*) in the two foci. For this purpose a dog was caged and sticky papers coated with deodorized castor oil were placed horizontally along side the cage. These were collected next morning before sun rise. The experiment was carried out in domestic compounds each month. To assess the comparative attraction of the dog the proportion of flies captured in baited traps were compared with the unbaited sticky traps used in the same area for sampling

sandflies outdoors. The abundance of flies is expressed as the mean number of flies captured per dog-trap per night. Sampling was done monthly in both the disease foci: Northern Areas (Hudur and Thor villages) and Azad Jammu & Kashmir (Rehra village).

### 6.3.7.2. Relative proportions of sandflies on dog baited and unbaited sticky traps.

For comparing the relative proportions of sandflies on dog baited and unbaited sticky paper traps the proportions were calculated from among the total number of flies caught during monthly sampling on both types of traps. Figure 6.19 & Tables 6.17 & 6.18 shows the relative proportions of male and female *Phlebotomus* species collected from Northern Areas. Four species: *P.papatasi*, *P.sergenti*, *P.alexandri* and *P.keshishiani* were caught on both the baited and unbaited traps. Compared to unbaited traps the proportions of females were higher on dog-baited traps suggesting some degree of attractiveness. This difference was highly significant with *P.keshishiani* ( $X^2 = 9.124, P < 0.001$ ). Similarly in Azad Jammu & Kashmir (Fig 6.19), the proportion of female *P.hindustanicus* were about two times higher (45%) on dog-baited traps ( $X^2 = 38.24, P < 0.001$ ) compared to its proportion unbaited traps (17.9%). Blood-meal analysis (see Table 6.15) also indicated higher proportions of *P.keshishiani* and *P.hindustanicus* positive to dog antisera.

### 6.3.7.3. Relative abundance and seasonal fluctuations of *Phlebotomus* species on dog-baited traps from Northern Areas and Azad Jammu & Kashmir.

#### i) Northern Areas.

Table 6.18 shows monthly collections of phlebotmine sandflies on dog-baited

**Table 6.17. Proportions of *Phlebotomus* spp. on control and dog baited sticky paper traps in Northern Areas.**

<i>Phlebotomus</i> spp.	SEX	Control sticky Traps (Pooled data) n=35	Dog baited sticky traps n=29
<i>P. papatasi</i>	M	12.1	12.16
	F	6.6	17.41
<i>P. sergenti</i>	M	42.7	15.16
	F	9.9	10.06
<i>P. alexandri</i>	M	4.1	1.2
	F	0.66	3.9
<i>P. keshishiani</i>	M	13.8	8.1
	F	8.1	30.03
<i>P. salangensis</i>	M	0.6	0.75
	F	0.5	1.2
<i>P. kandelakii burneyi</i>	-	-	-

**TABLE 6.18. Proportions of *Phlebotomus* spp. on control and dog baited sticky papertraps from Azad Jammu & Kashmir.**

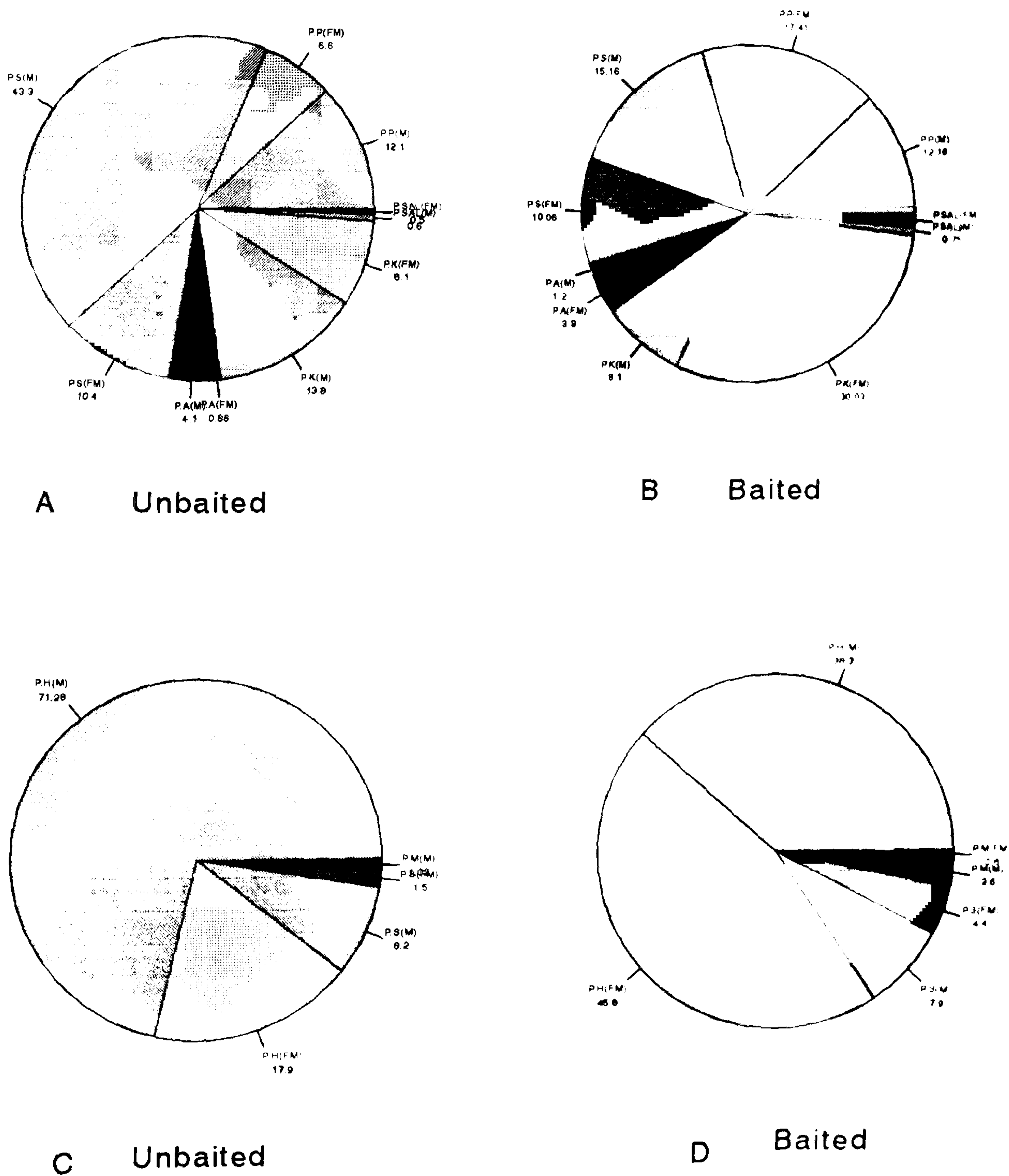
<i>Phlebotomus</i> spp.	SEX	Control Sticky traps (pooled data) n=39	Dog baited sticky traps n=20
<i>P. hindustanicus</i>	M	71.28	38.3
	F	17.9	45.8
<i>P. sergenti</i>	M	8.2	7.9
	F	1.5	4.4
<i>P. major</i>	M	1.02	2.6
	F	-	0.8

n= Number of Trap nigts. M=male; F= Female



Figure 6.19

Male and female proportions of *Phlebotomus* species on un-baited and dog-baited traps from Northern Areas(A,B) and AJK(C,D).



M= male, FM= female, P.P=*P.papatasi*, P.S=*P.sergenti*, P.A=*P.alexandri*, P.SAL=*P.salangensis*, P.K=*P.keshishiani*, P.H=*P.hindustanicus*, P.M=*P.major*

traps from Hudur village (lower altitude). A total number of 408 sandflies belonging to 5 *Phlebotomus* species were caught during the entire sampling period which include 10 nights sampling in Hudur and 13 nights in Thor village. The species in order of descending abundance were *P.papatasi* (197:48%), *P.sergenti* (152: 37.25%), *P.keshishiani* (36:8.8%), *P. alexandri* (19:4.6%) and *P.salangensis* (4:0.98%).

Figure 6.20 A & B. shows the seasonal variation in the sandfly species attracted to dog-baited traps in Hudur (1200 metres) (Table, 6.19). *Phlebotomus papatasi* was collected from May till August with peak activity in July, whereas *P.sergenti* did not show any obvious pattern but was more or less consistently found from May till July declining in August and diminishing in October. Other species, *P.keshishiani*, *P.alexandri* and *P.salangensis* were found in very low numbers and therefore did not show any mark seasonality.

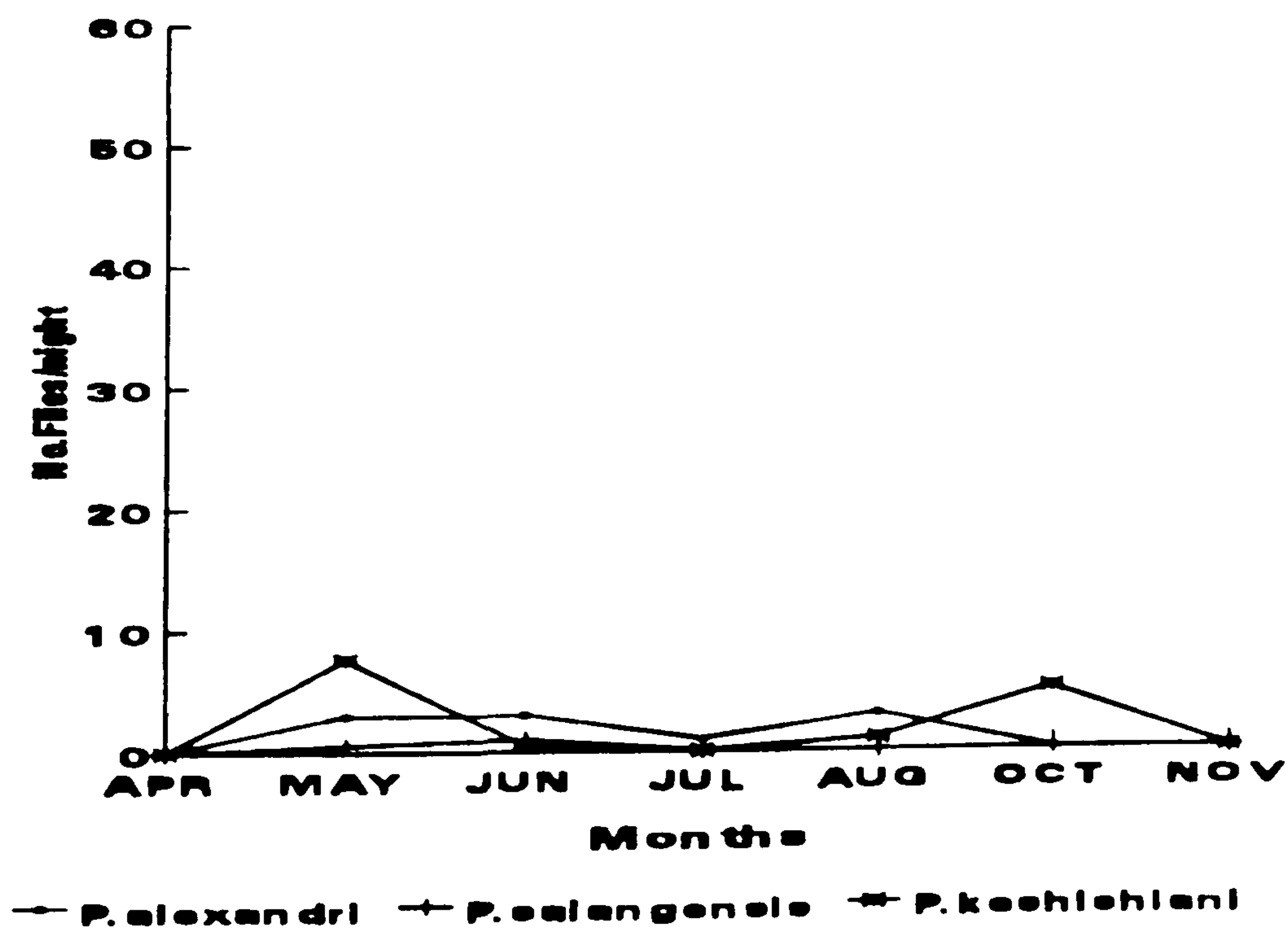
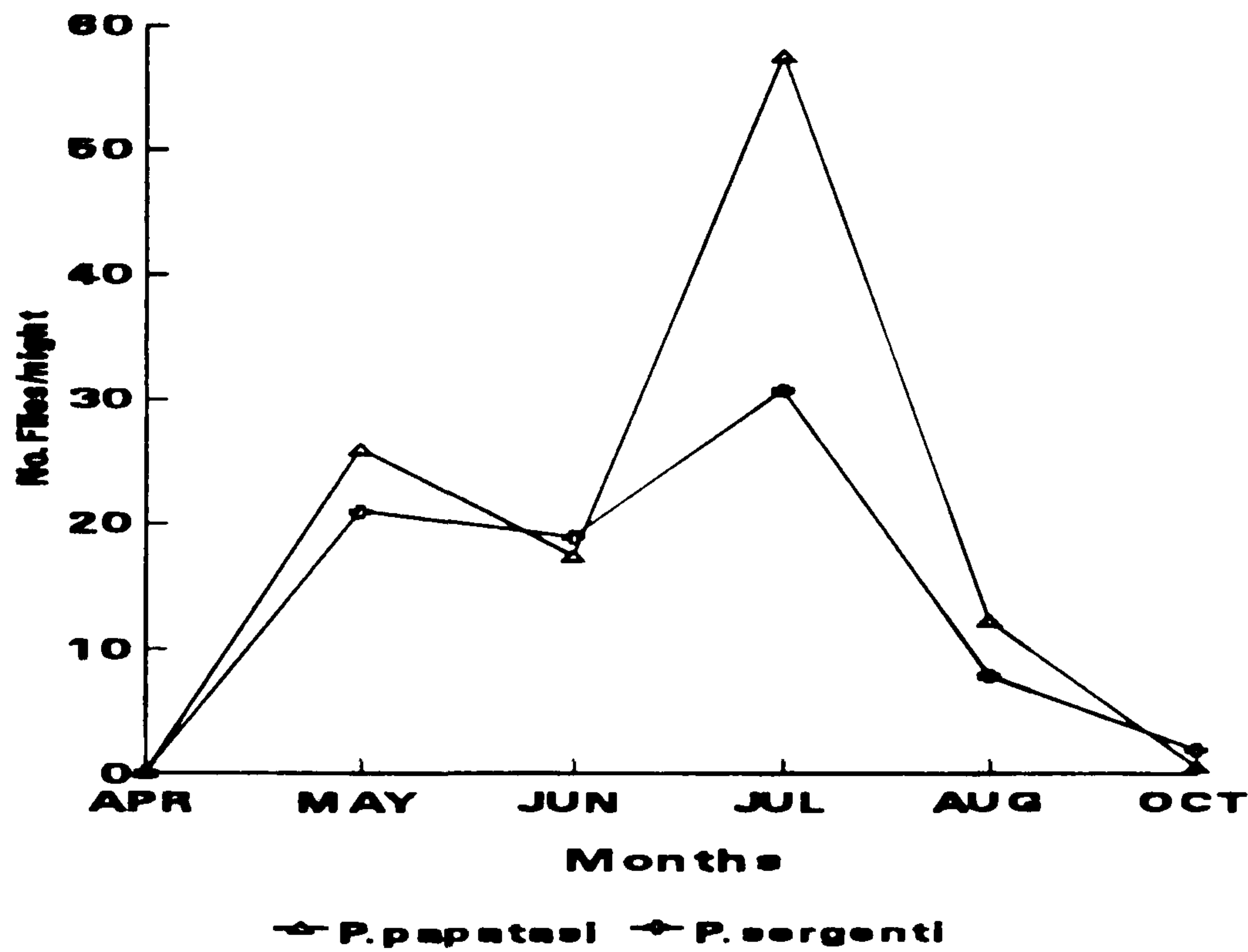
At higher altitudes in Thor village a total of 260 *Phlebotomus* specimens (Table 6.20) were captured out of which 218 (82%) were *P.keshishiani*, 16 (6%); *P.sergenti*, 15 (5.7%); *P.alexandri* and 9 (3.4%) *P.salangensis*. Females always outnumbered males. Figure 6.21 shows seasonal variation in the activity of these flies. *P.keshishiani* was found in more or less the same numbers in June and July gradually descending in August and September and completely diminishing in October. Other species, *P.alexandri*, *P.salangensis* and *P.sergenti* were too low in numbers to indicate any marked seasonality.

## ii). Azad Jammu & Kashmir.

In AJK a total of 227 (Table, 6.21) *Phlebotomus* specimens were collected on

Figure 6.20

Mean monthly variation *P.papatasi*, *P.sergenti*, *P.alexandri*, *P.salangensis* and *P.keshishiani* on dog baited sticky paper traps from Hudur village, Northern Areas.



**Table 6.19. Mean densities of *Phlebotomus* spp. collected on dog baited sticky papers per night in Hudur village, Northern Areas.**

Month	SEX	P.P	P.S	P.A	P.SAL	P.K	Total
April		-	-	-	-	-	-
May	M	40(13.3)	44(14.6)	1(0.3)	2(0.6)	11(4.3)	98(32.6)
	F	38(12.6)	19(6.3)	8(2.6)	-	12(3.3)	77(25.6)
June	M	12(6)	21(10.5)	1(0.5)	-	1(0.5)	35(17.5)
	F	23(12.5)	17(8.5)	5(2.5)	2(1)	-	47(23.5)
July	M	21	18	-	-	-	39
	F	37	13	1	-	-	51
Aug.	M	8(4)	13(6.5)	2(1)	-	-	23(11.5)
	F	17(8.5)	3(1.5)	1(0.5)	-	2(1)	23(11.5)
Sept.	Unsampled						
Oct.	M	-	1(0.5)	-	-	3(1.5)	4(2)
	F	1	3(1.5)	-	-	7(3.5)	11(5.5)
Total		197	152	19	4	36	408

Note: Month of May sampled for three nights; July for one night only and all other months for two nights each.

Within Paranthesis are the means.

P.P= *P.papatasi*; P.S= *P.sergenti*; P.A= *P.alexandri*; P.Sal= *P.salangensis*; P.K=*P.keshishiani*.

**Table 6.20. Mean number of *Phlebotomus* spp. collected on dog baited sticky papers per night in Thor village Northern Areas (1992).**

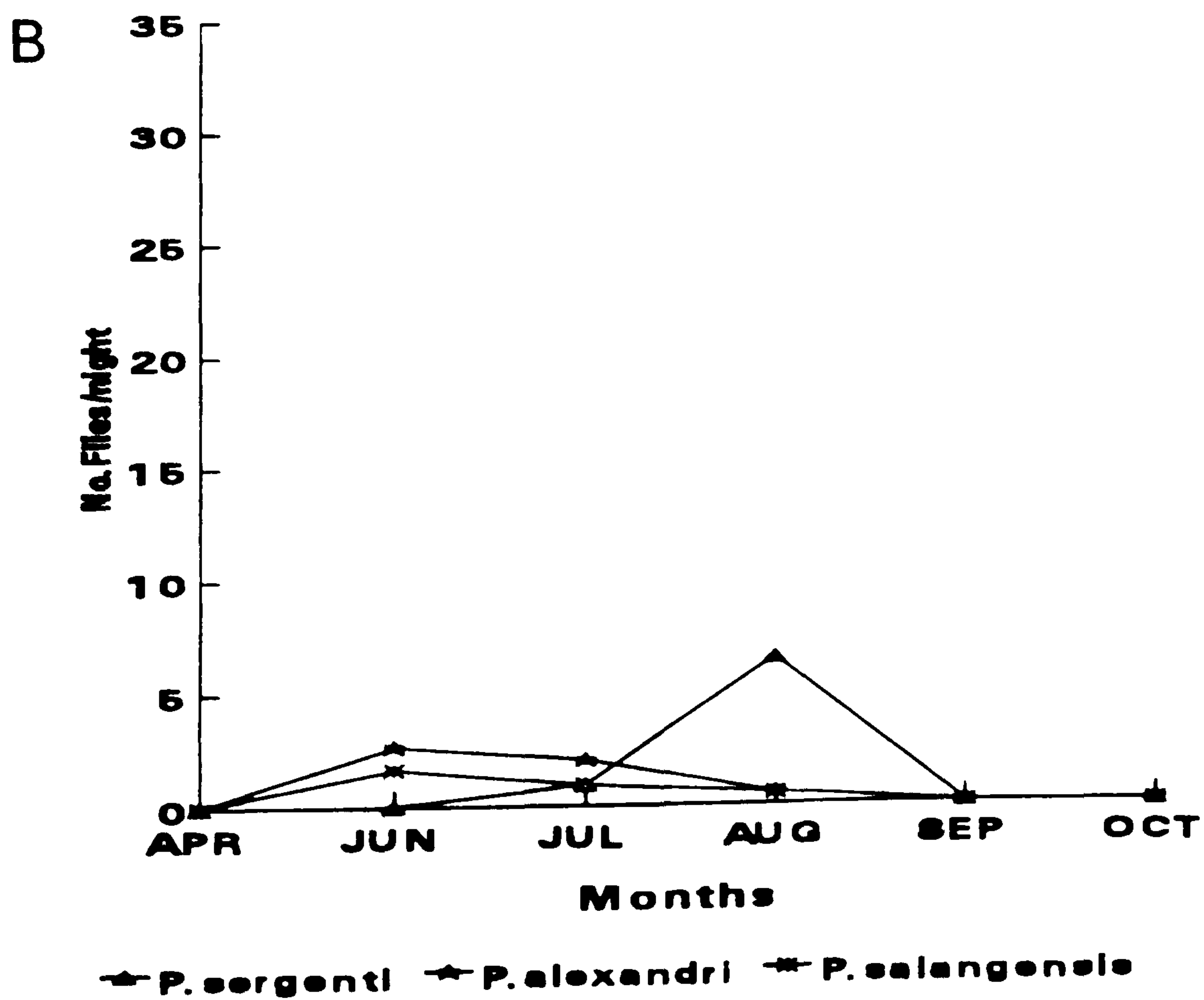
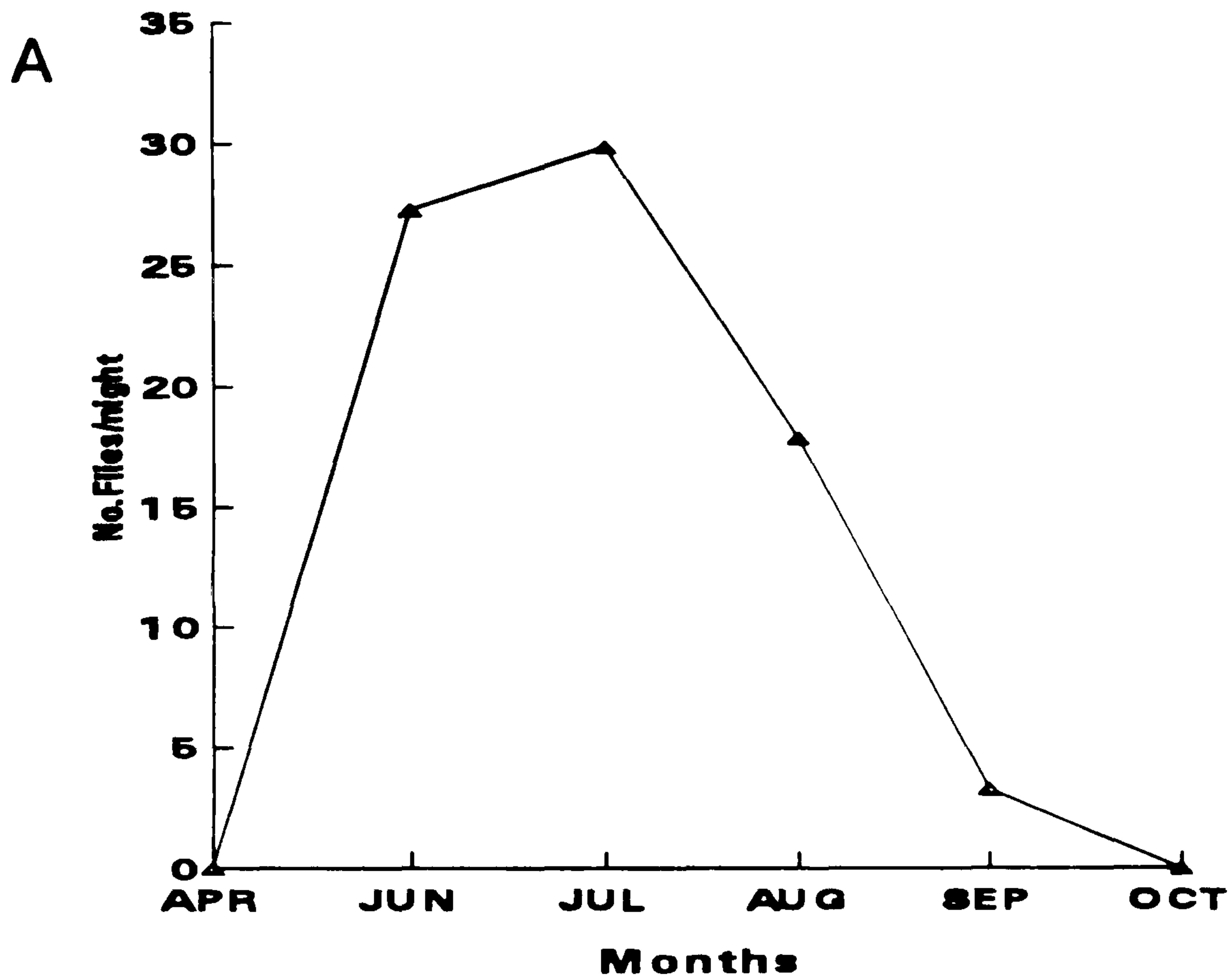
Month	SEX	P.P	P.S	P.A	P.SAL	P.K	P.KB	TOTAL
April	-	-	-	-	-	-	-	-
May	-	Unsampled						
June	M	-	-	1.3(4)	0.6(2)	6.3(19)	-	2.8(25)
	F	-	-	1.3(4)	1(3)	21(63)	-	7.8(70)
July	M	-	0.6(2)	-	0.3(1)	5.3(16)	-	2.1(19)
	F	-	0.3(1)	2(6)	0.6(2)	24.6(74)	-	9.2(83)
Aug.	M	-	1.5(3)	-	-	1(2)	-	0.6(5)
	F	-	5(10)	0.5(1)	0.5(1)	17(34)	-	7.8(48)
Sept.	M	-	-	-	-	-	-	-
	F	-	-	-	-	3.3(10)	0.6(2)	4(12)
Oct.	M	-	-	-	-	-	-	-
	F	-	-	-	-	-	-	-
Total			16	15	9	218	1	280

P.P=*P.papatasi*; P.S=*P.sergenti*; P.A=*P.alexandri*; P.SAL=*P.salangensis* P.K=*P.keshishiani*; P.KB=*P.kandelakii burneyi*

Note: 3 nights sampling done each month except August when sampling could be done only for 2 nights. Within Parenthesis are the total number of sandflies.

Figure 6.21

Seasonal variation in the densities of *P.keshishiani* (A) and *P.sergenti*, *P. alexandri* & *P. salangensis* (B) on dog baited sticky paper traps from Thor village, Northern Areas.



**Table 6.21. Mean number of *Phlebotomus* spp. collected on dog baited sticky papers per night in Rehra village, Azad Jammu and Kashmir (1992).**

MONTH	SEX	<i>P.hindustanicus</i>	<i>P.sergenti</i>	<i>P.major</i>	Total
April	-	-	-	-	
May	-	-	-	-	
June	M	17.6(53)	3.3(10)	3(6)	23(69)
	F	19.3(58)	1.7(5)	0.6(2)	21.6(65)
July	M	1.3(4)	-	-	1.3(4)
	F	3.3(10)	-	-	3.3(10)
Aug.	M	9.6(29)	1(3)	-	10.6(32)
	F	10.6(32)	1.3(4)	-	12(36)
Sept.	-	Unsampled			
Oct.	M	0.3(1)	1.7(5)	-	2(6)
	F	1.3(4)	0.3(1)	-	1.7(5)
<b>Total</b>		<b>63.6(191)</b>	<b>9.3(28)</b>	<b>2.6(8)</b>	<b>75.6(227)</b>

**Within Parantheses are the total number of sandflies.**

dog-baited traps over 15 nights sampling. These in order of descending abundance were: *P.hindustanicus* (191: 84%), *P. sergenti* (28:12.3%) and *P.major* (8:3.5%).

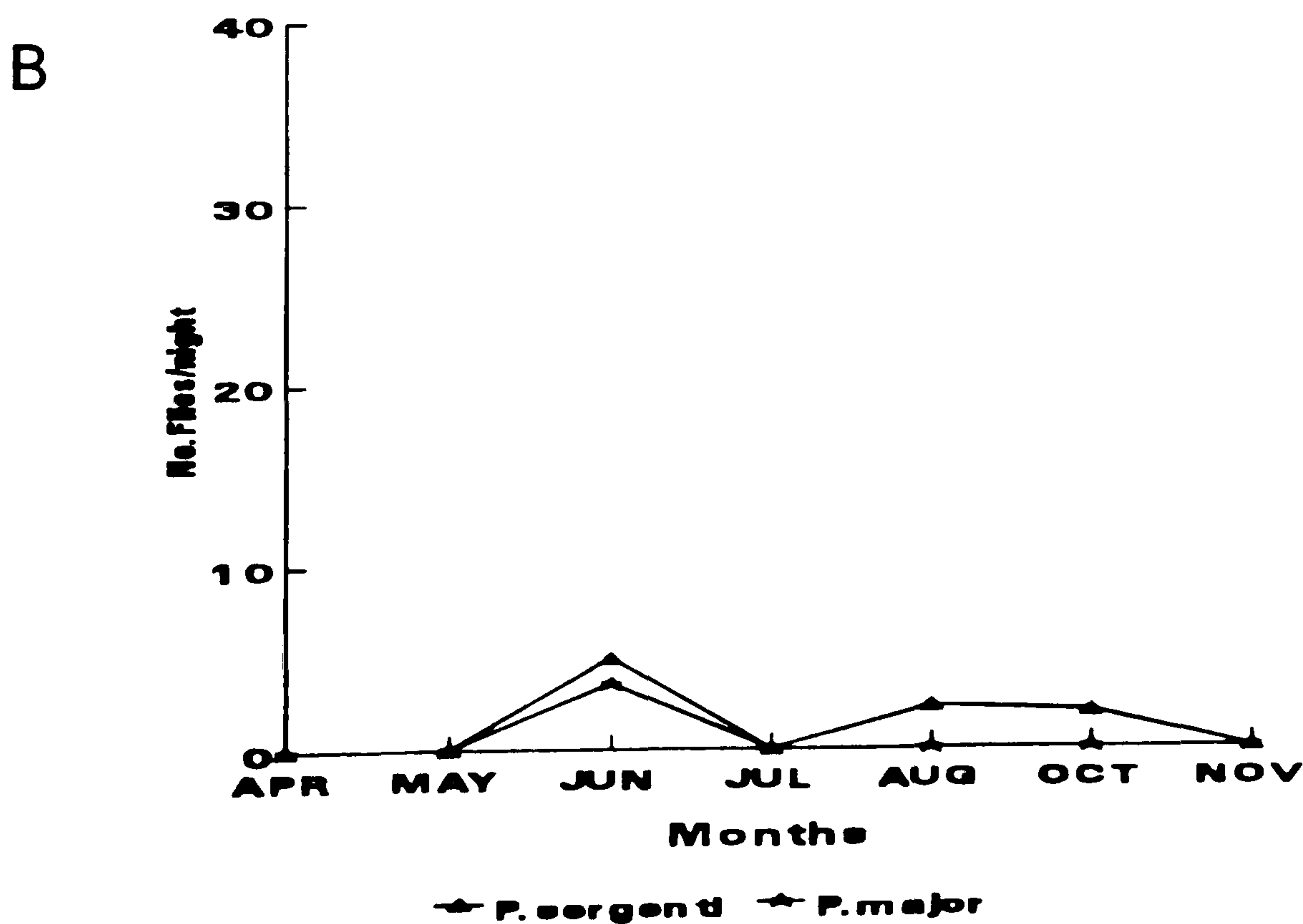
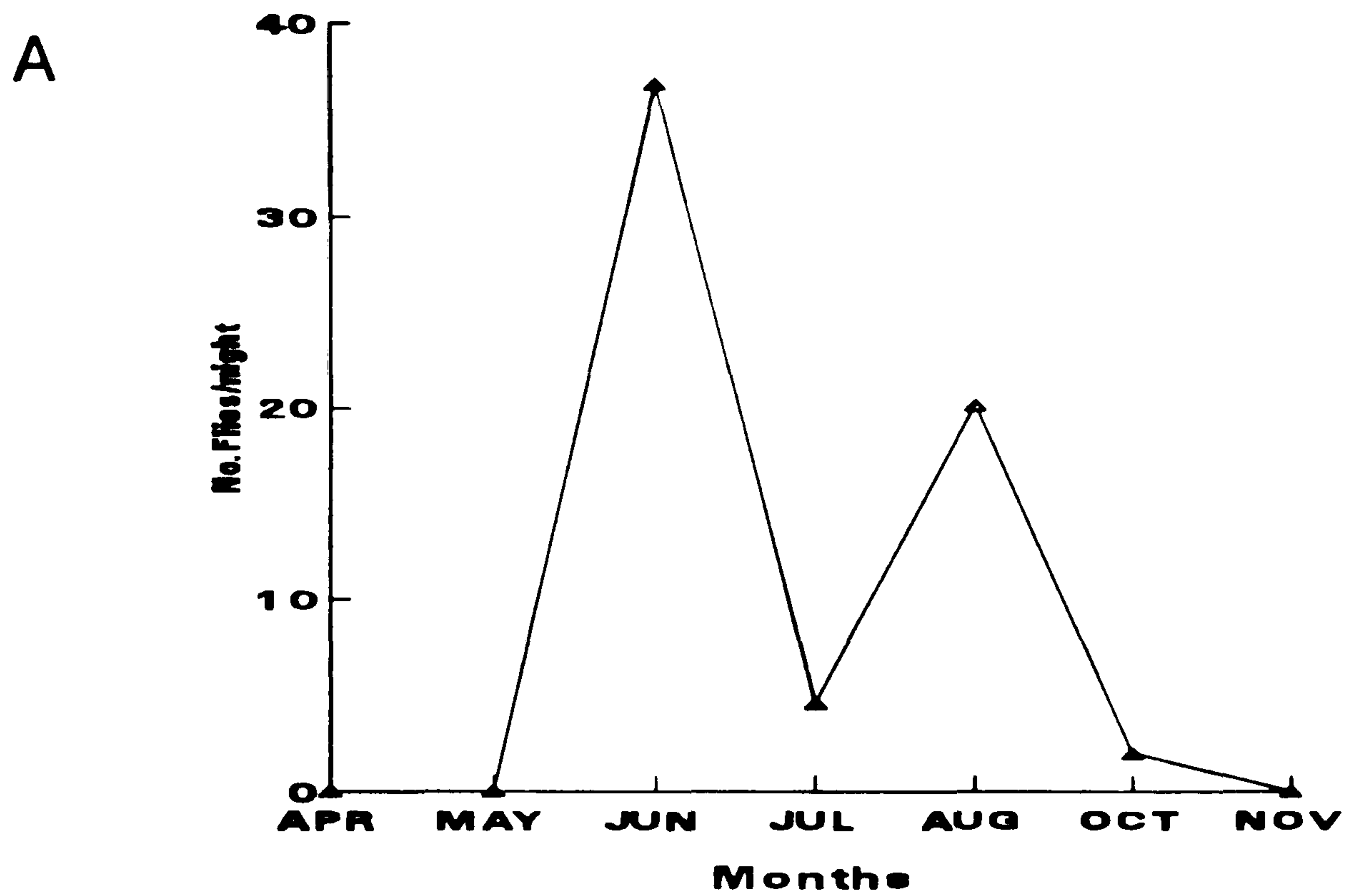
Figure 6.22 shows seasonal variation in the densities of these species on dog-baited traps. The most dominant species, *P.hindustanicus*, showed two peaks one in June and another in August, i.e just before and after the heavy rains. In contrast to these observations, the outdoor catches from CDC light-traps did not reveal a significant seasonal trend because of the low number of flies caught. However the trend is comparable to the over all density of sandflies in Azad Kashmir which also show peak activity in June. *Phlebotomus sergenti* and *P.major* on the other hand were too low in numbers and therefore meaningful interpretation of the seasonality cannot be made.

### 6.3.8. SANDFLY DISSECTIONS FOR NATURAL INFECTIONS WITH LEISHMANIA PROMASTIGOTES.

From the visceral leishmaniasis focus of Azad Jammu and Kashmir a total of 301 (265 from Rehra village and 36 from Banjosa & Gali Malot) females *P.hindustanicus* were dissected and guts of 85 flies (all from Rehra village) were also squashed onto the hybrid membrane for DNA probing. Only 1 female from Banjosa (1720m) was found naturally infected with *Leishmania* promastigotes. The sandfly was captured from inside the living room of a mud constructed house surrounded by dense vegetation. The promastigotes were found in the mid gut, head and proboscis. Parasites were inoculated into NNN media supplemented with suitable antibiotics but unfortunately the culture was lost due to contamination. However DNA hybridization (Fig 6.23) gave a positive signal with *Leishmania infantum* specific probe LUCA D2

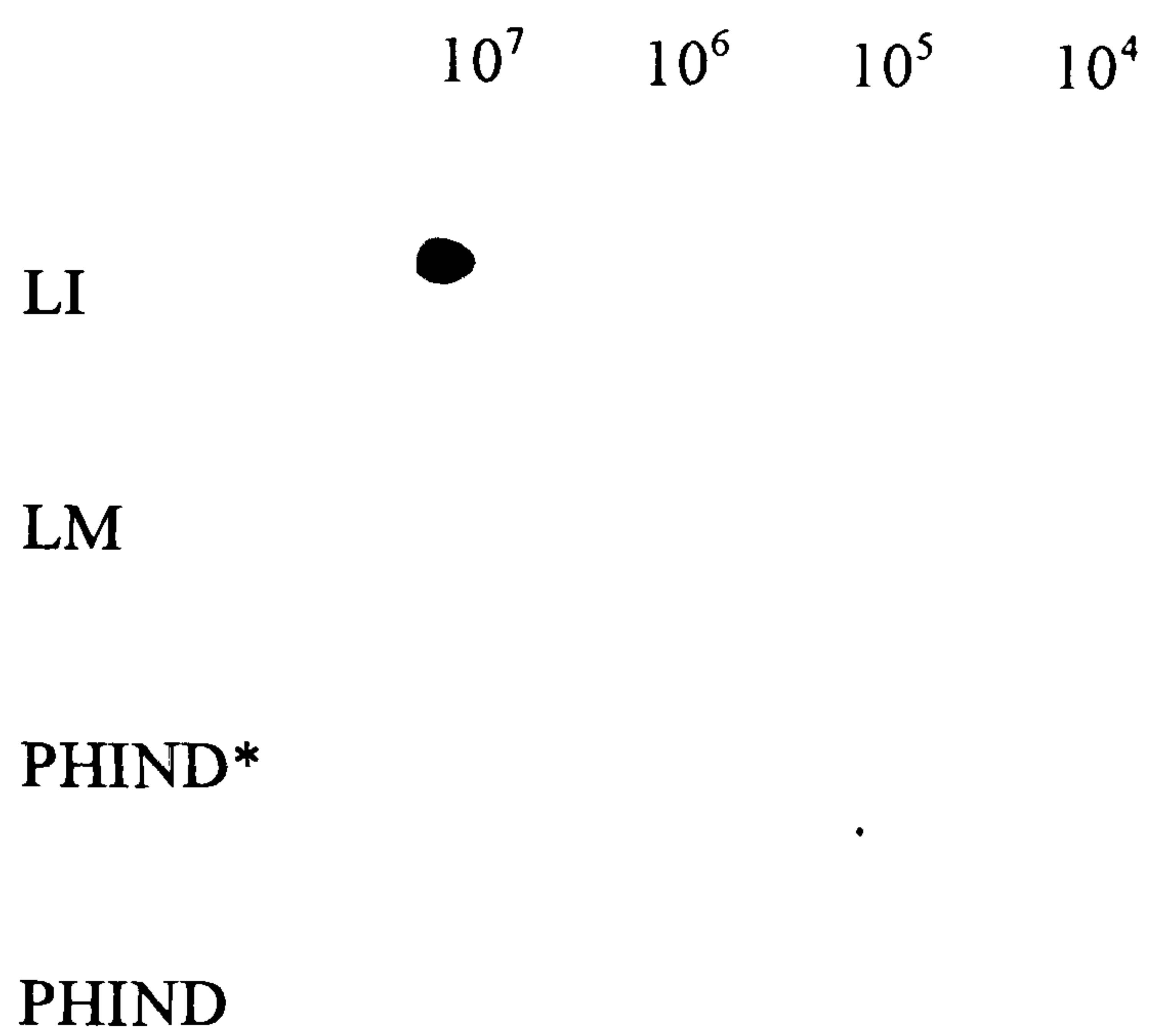
Figure 6.22

Seasonal variation in the densities of *P. hindustanicus*(A) and *P. major* & *P. sergenti*(B) on dog baited sticky paper traps from Rehra, Azad Jammu & Kashmir.





**FIGURE 6.23**



Hybridization signals using *Leishmania infantum* specific probe (LUCA D2 200 bp). LI, *Leishmania infantum* (positive control); LM, *Leishmania major*, PHIND\*, *Phlebotomus hindustanicus* naturally infected with *Leishmania* promastigotes, PHIND, *P.hindustanicus* (microscopically negative gut).

200BP thus confirming the identity of promastigotes. Out of the other 84 microscopically negative midguts probed, the probe hybridized (gave a positive signal) with one female (Fig. 6.23). Therefore a total of 2 (0.7%) females of *P.hindustanicus* were found positive out of 302 dissected.

From Northern Areas a total of 754 *P.keshishiani*, 36 *P.salangensis* and 15 *P.alexandri* were dissected for natural infections with *Leishmania* promastigotes and none were found positive. A total of 568 mid guts of *P.keshishiani*, 36 *P.salangensis* and 10 *P.alexandri* squashed on DNA Hybond membranes were probed with LUCA D2 200BP and none found positive.

## 6.4. DISCUSSION.

### 6.4.1 SPECIES COMPOSITION IN NORTHERN AREAS AND AJK.

The first step in vector incrimination is to determine the diversity and distribution of populations of sandflies in different habitats in the *Leishmania* endemic foci (Lewis & Ward 1987). This was achieved by sampling phlebotomine sandflies using variety of methods. From the faunal checklist thus obtained we can predict one or more species likely to be the vector(s) of disease.

Phlebotomine sandflies were sampled from two active foci of visceral leishmaniasis, viz Northern Areas & AJK, using variety of methods. The most startling result is that the sandfly fauna of two areas is different (Table, 6.1). During the present study 10 species belonging to the genus *Phlebotomus* representing four subgenera, *Phlebotomus (Phlebotomus)*, *P. (Paraphlebotomus)*, *P. (Larroussius)*, and *P. (Adlerius)* were recorded from the visceral leishmaniasis foci.

#### 6.4.1.1. Northern Areas.

Six species (*P.papatasi*, *P.sergenti*, *P.alexandri*, *P.keshishiani*, *P.kandelakii burneyi*, and *P.salangensis*) were collected from Northern Areas (Table, 6.1) at two different altitudes (1200m & 1700m). With the exception of *P.kandelakii burneyi* which was found only at the higher altitude, the rest of the fauna was similar in both villages sampled. The proportion of each species differed however. *P.papatasi* followed by *P.sergenti* was the dominant species in Hudur village (1200m), whereas *P.keshishiani* was dominant in Thor (1700m). All other species were found in low numbers.

#### 6.4.1.2. Azad Jammu & Kashmir.

In Azad Jammu & Kashmir a total of five species were found (*P.sergenti*, *P.hindustanicus*, *P.major*, *P.kandelakii burneyi* and *P.(L) Sp.A.*(Table 6.1). *Phlebotomus hindustanicus* was clearly dominant followed by *P.sergenti*. Comparison between the fauna from lower altitudes (longitudinal studies) in Rehra with that of the higher altitudes (general survey) revealed that *P.major* is restricted to low altitudes only and *P(L) sp A.* to high altitudes above 1500m, *P(A)hindustanicus* and *P.sergenti* were found at both altitudes. Only 2 male *P.kandelakii burneyi* were collected from AJK.

Comparison of the sandfly fauna between Northern Areas and AJK revealed that only two species *P.sergenti* and *P.kandelakii burneyi* were common to both the disease foci.

Most of the species collected during the present study were also found by Lewis (1967) with the exception of *P.salangensis* and *P.hindustanicus*. These two

species seemed to have been confused previously and misidentified as *P.chinensis longiductus* (= *P.longiductus*). During the time when Lewis studied the phlebotomine sandflies of Pakistan, the taxonomy of the subgenus *Phlebotomus (Adlerius)* was itself not clear and most of the valid species were treated as subspecies of *P.chinensis* (Theodor 1953, 1958; Theodor & Mesghali 1964). The situation became clear more recently when the subgenus was reviewed by Artemiev (1980) and most of the previously designated subspecies were recognized as valid species with the description of several new species. We identified *P.salangensis* and *P.hindustanicus* based mainly on the identification criteria of Artemiev (1980). It is of epidemiological interest to mention here that during the present study *P.hindustanicus* was recorded from Azad Jammu & Kashmir (1200-1900 m) and the Margella hills (1700 m) which represent the sub-Himalayan range whereas *P.salangensis* was only recorded from Northern Areas which are part of the western Himalayas and Karakorum range of mountains. The latter species was found at altitudes between 1200 m and 2500 m in Baltistan mainly above 2000 m.

*Phlebotomus kandelakii burneyi* was previously recorded only from Northern Areas at heights above 2000 m (Lewis 1967), whereas in the present study this subspecies was also collected from AJK at an altitude of 1200m and Thor village in Diamir district at an elevation of 1700m. The species seems to have a much wider distribution in Pakistan than previously thought and further studies in other areas will clarify the situation. The taxonomic characters on which this species differs from the nominate species *P.kandelakii* have been discussed in detail in the previous chapter on Systematics.

Phlebotomine sandflies from the focus in Azad Jammu & Kashmir have been studied for the first time. The fauna of this area is however very similar to the sandfly fauna described by Lewis (1967) from the Margella hills with the exception of *P(Paraphlebotomus) nuri*. During the present study *P.nuri* was found neither in AJK nor in the Margella hills (Saidpur). Failure to find this species particularly from the type locality (Saidpur) indicates that it might be sporadic in distribution. As we only surveyed this locality for a short time as a part of general survey, further long term studies will explain the situation.

The fauna of Azad Jammu & Kashmir is also comparable to that of the disputed territory of Jammu & Kashmir (Jacob & Kalra, 1951). Studies carried out in that part of the country reported four *Phlebotomus* species viz: *P.papatasi*, *P.sergenti*, *P.major* and *P.longiductus*. We did not find *P.papatasi* and *P.longiductus* from Azad Jammu & Kashmir although *P.papatasi* was collected from the Margella hills near Islamabad. The reason for the absence of *P.papatasi* from AJK is not immediately clear, but one reason could be the relatively high rainfall in Bagh district. The absence of *P.papatasi* even at optimal temperature in areas with heavy rains throughout the year or for 6-7 months without interruption has already been documented (Perfiliev, 1968). The area we studied receives constant rains throughout the year thus making the environment presumably inhospitable for this species to survive. I do not claim that *P.papatasi* is non-existent in the entire Azad Jammu & Kashmir as it may well be present in the areas which receive relatively less rainfall. The presence of *P.longiductus* in the disputed territory of Jammu & Kashmir needs to be re-examined as it may be a misidentification for the same reasons as described

above in connection with the subgenus *Phlebotomus (Adlerius)*. During the present study we also identified another species belonging to the subgenus *Phlebotomus (Larroussius)* from AJK at an altitudes above 1500 m. This species is closely related to the other species of *Phlebotomus major* group but shows significant variation in certain taxonomic characters and it has therefore been treated as a separate and new species. Detailed comparison of this species with other closely related species have been discussed in the previous chapter on Systematics. *Phlebotomus sp.A* has not been reported from the neighbouring Jammu & Kashmir.

The Zoogeography of Pakistan has been discussed by Lewis (1967). Briefly three faunal sub-regions are represented in Pakistan, these include the Mediterranean and the Siberian subregions of Palearctic region and the Indian subregion of the Oriental region. The *Phlebotomus* sandfly fauna of Northern Areas and Azad Kashmir is strictly the Palearctic with Mediterranean and central Asian affinities. Species with largely Mediterranean distribution are *P.papatasi*, *P.sergenti*, and *P.alexandri* and those with Central Asian affinities are *P.nuri*, *P.kandelakii burneyi*, and *P.keshishiani*. *Phlebotomus hindustanicus* and *P.salangensis* can also be included among species with central Asian affinities.

The *Phlebotomus* fauna of Northern Areas and Azad Kashmir share some common species with the neighbouring countries of Iran (Theodor & Mesghali, 1964); Afghanistan (Artemiev, 1978, 1980); China (Zahar, 1980), and the disputed territory of Jammu & Kashmir (Jacob & Kalra, 1951). The Oriental species *P.argentipes*, an important vector of kala azar (caused by *Leishmania donovani*) in India, was not found during the present study. Except for *P.papatasi* and

*P. sergenti* which do not transmit visceral leishmaniasis, all the other *Phlebotomus* species found in the two disease foci are epidemiologically important because they belong to subgenera with proven vectors of visceral leishmaniasis (*Leishmania infantum*) (Killick-Kendrick, 1990c). Global as well as the local distribution of these species has been discussed in the previous chapter on Systematics.

#### 6.4.2. SEX RATIOS:

The sex ratio (Table 6.11) in the field samples was unequal in most cases and was influenced by the method of collection. Thus, in Northern Areas and Azad Jammu & Kashmir in *Phlebotomus* species captured on sticky paper traps, males of all species dominated. In CDC light-traps the female to male ratio was significantly higher except *P. papatasi* and *P. sergenti* which showed significantly a higher proportion of males. In Azad Jammu & Kashmir, <sup>the</sup> proportion of males was always found to be significantly higher than females irrespective of the type of trap used. Higher proportions of males on sticky paper-traps have been reported in many other studies carried out elsewhere (Buttiker & Lewis, 1979; Lewis & Buttiker, 1980; Beier *et al.*, 1986; Lane *et al.*, 1990). Contrary to our findings, Chaniotis, *et al.*, (1971, 1974) and Christensen & Herrer (1980) in Panama, and Killick-Kendrick *et al.*, (1984) and Gibbs *et al.*, (1988) in southern France collected higher proportions of females than males.

Sticky traps are presumed non-attractive and simply intercept sandflies moving about. Given a normal sex ratio of 1, then a skewed ratio in favour of males indicate that they move about per unit time more than females, although female travel greater distances than males in search of blood feeding and oviposition sites. The presence of

more males than female of *P.papatasi* and *P.sergenti* in CDC light-traps is not immediately clear. This may either be due to the fact that the males of these species are equally attracted by light as females or another possibility could be that the traps were placed near to emergence sites, where males are generally abundant. Similar high male to female proportions have been observed by Lewis (1967) in the previous studies from Northern Pakistan.

#### **6.4.3. COMPARISON BETWEEN SANDFLY SAMPLING METHODS.**

The comparison between the catching methods, CDC light-traps and sticky paper traps (Table 6.12; 6.13) showed significant differences in the proportions of different species caught. Significantly higher proportions of *P.papatasi* and *P.keshishiani* were found in light-traps than *P.alexandri*, *P.sergenti* or *P.hindustanicus* which showed higher proportions on sticky traps. This difference in the catching methods could be because *P.papatasi* and *P.keshishiani* are more phototropic than *P.alexandri*, *P.sergenti*, and *P.hindustanicus*. The proportions of flies caught in light-traps when compared with human bait catches showed that *P.papatasi* and *P.keshishiani* were caught in significantly higher proportions on human baits than light-traps which clearly reflects their anthropophily.

#### **6.4.4. SEASONAL VARIATION.**

Studies on the seasonal fluctuations in sandfly densities are of vital importance in incriminating a vector by determining the possible and more likely period of transmission in humans, as well as to plan control measure (Killick-Kendrick,



1978; Lewis and Ward, 1987) and are therefore pertinent in the areas where vector control has to be undertaken. Such seasonal changes result from a complex interplay between the biotic potential, physical and biological environmental resistance which often operates against the early stages.

Many of these variables particularly the biotic ones are difficult to estimate to any reasonable approximation under field conditions. However, some of the physical variables, such as meteorological conditions, which control population density from season to season can be measured.

The seasonal fluctuations in population density of phlebotomine sandflies which result from the changing meteorological patterns have been widely studied in different geographical regions. Generally in temperate zones such changes are associated with temperature and sandfly adults are present only in the summer (Lewis, 1974). Here transmission may be most intense at the end of the season when the maximum number sandflies are parous (Guilvard *et al.*, 1980). In tropical regions, flies can flourish in the wet or dry season, or they may occur throughout the year (Lewis, 1974) but the seasonal changes have generally been associated with rainfall and topography (Ward *et al.*, 1973).

#### **6.4.4.1. Northern Areas.**

Based on the collections from CDC light traps (both indoors and outdoors) and sticky paper traps (outdoors) sandfly densities in Northern Areas are strictly seasonal, being associated with the hot dry summer months when the mean minimum temperature is in the range of 18°-27°C and mean maximum temperature is in the range of 29°-37°C with average humidity as 23-42% (Figure 6.4).

The most important factor influencing sandfly densities in Northern Areas was temperature or at least most highly correlated with sandfly density (Table 6.4). Peak sandfly activity was observed when the temperature was high and humidity low. Although this behaviour of sandflies seems strange as normally they are presumed to be more active at low temperatures and high humidity, the period during which the temperature is favourable for the activity of sandflies is only five months i.e. between May and September when the minimum temperature is in the range of 17.5-20.3°C and the maximum temperature is in the range of 29.6-31.1°C. This is a shorter period than that in many other countries and perhaps this could be one of the reasons that sandflies in this region have adapted to the prevailing conditions for survival, even at higher temperatures and lower humidity. Similar behaviour to that in Northern Areas has also been observed in Oman, where *Phlebotomus* species were found to be at their peak activity during the time when temperature was higher and humidity low (Roberts, 1994). However, studies carried out in other countries have led to different opinions e.g. Abul-Hab & Al-Hashmi (1988) considered that neither temperature nor humidity had any correlation with the activity of *P.papatasi* in Iraq. Mohsen, (1983) considered the preferred temperature for *P.papatasi* to be 23°C, while higher temperatures (up to 28°C) reduced activity. As sandfly activity varies in different geographical regions depending upon the local environmental conditions, it is hard to generalize from observations made in one region to another.

At the lower of two altitudes (Hudur village, 1200 m) adult sandflies (e.g. *P.papatasi* & *P.sergenti*) seem to emerge somewhere between the end of April and early May consistently increasing throughout the summer months and then declining in late August

or early September and completely diminishing in October (Figure 6.1). A similar trend was observed at higher altitudes (Thor village, 1700 m) where the dominant sandfly *P.keshishiani* emerges sometime in May gradually rising thereafter with peak activity in June, abruptly declining in September and completely vanishing in October (Figure 6.5).

As at lower altitudes, sandfly activity was found to be directly related to temperature. The temperature at the higher altitude was 2-3°C less (19.5-32°C) (Fig. 6.7) than at the lower altitudes and the average humidity was higher, the maximum being in the range of 42-59% (Fig 6.7). Other sandfly species recorded both from high and low altitudes were too scarce (Figure 6.2, 6.6) to show any meaningful seasonal trend in the population densities. The seasonal pattern observed in Northern Areas during the present study is more or less similar to that seen in neighbouring China. Studies carried out in east and north-east China revealed adult activity of *P.chinensis* beginning in mid or latter May reaching a peak in the mid or latter half of June and completely disappearing in the second half of August (Zahar, 1980), whereas in north-west China (most similar to northern Pakistan) the peak activity was in the mid or latter half of July (Hsiung *et al.*, 1979). Earlier studies in Kansu Province showed that the seasonal activity of *P.alexandri* in east China occurred from early June to the latter part of August or the middle of September with a single peak during early July (Guang-Hua *et al.*, 1963).

#### i). Indoor and outdoor densities.

The difference between sandfly abundance measured indoors and outdoors for the dominant species was significantly greater at high altitudes than at low altitude.

*P.papatasi* was found to be more abundant inside houses in May (Figure 6.1) whereas the maximum outdoor activity of this species was in June and July. *P.sergenti* also showed similar pattern except that an additional peak was observed in August (Figure 6.1) which is perhaps the result of some biotic rather than the physical factors (Chaniotis *et al.*, 1971) which are impossible to ascertain at present. Also the period of adult activity for *P.sergenti* extends till August i.e. a month longer than *P.papatasi*. At high altitudes, *P.keshishiani* (Figure 6.5) showed peak outdoor activity in June whereas there was no prominent peak indoors, although maximum numbers were caught in August.

The presence of sandflies indoors during the early part of the season in Hudur could be attributed to the lower outdoor temperature in May (18°C) and also the presence of many humans and domestic animals indoors. This is similar to that observed in the hilly region of Saudi Arabia (Al-Zahrani *et al.*, unpublished). The activity of flies outside houses during summer in Hudur could be related to their search for blood because most of the human and animal population migrate to higher pasture during the summer months returning back some where in October and as such most of the houses are empty. Part of the activity outdoors could also be related to their search for sugar sources, since at this time of year fruits are abundant and vegetation green. Lewis (1967) also noticed low indoor densities in Northern Areas in June and July.

#### 6.4.4.2. Azad Jammu & Kashmir.

In Azad Jammu and Kashmir, three *Phlebotomus* species (*P.hindustanicus*,

*P. sergenti* and *P. major*) were found in Bagh district. *Phlebotomus hindustanicus* was predominant. Based on the data obtained from the CDC light trap collections the seasonal fluctuations in these species showed that the flies were active from May till November. Although the sandfly season was found to be longer in Azad Kashmir compared to Northern Areas, the overall density was lower. Perhaps heavy rainfall in this area is the major factor affecting population size. The population fluctuations are strictly influenced by heavy rains and also temperature. The maximum densities were observed in the dry months when mean minimum and maximum temperatures are high (20-36°C) and average precipitation and humidity low (Figure 6.13, 6.14) in May and June. The monsoon starts in July and heavy rains continue till September, which sometimes results in heavy floods, as for example in September, 1992 when we could not get to the field. During the rainy season the minimum temperature sometimes goes below 16°C and nights are particularly cold and humid. Mean minimum and maximum relative humidity is in the range of 34% and 88% respectively. Population fluctuations in the sandfly fauna of this area strictly follow a similar pattern. The adult flies being dry season species emerge sometimes by the end of April or early May after the spring showers, reaching a peak in June when the season is comparatively dry and the temperature (20°-36°C) and humidity (34-62%) are favourable. A short time later in July with the onset of the monsoon, the flies suddenly disappear and are then found in extremely low numbers till the end of the monsoon. By the time the monsoon is over the winter months have already approached and the numbers diminish completely.

The seasonal changes observed in AJK during the present study are similar to those observed in the neighbouring disputed territory of Jammu & Kashmir. According

to Jacob & Kalra (1951), in the Jammu and Kashmir valley the season extended from April to October as observed by us in Azad Jammu & Kashmir. Their study was however of short duration mainly aimed at the identification of the sandfly fauna rather than understanding the population fluctuations. The authors therefore did not discuss weather conditions in relation to population changes. No other studies exist on the population fluctuations or any other aspects of sandfly behaviour from this part of the World.

i). Indoor and outdoor densities.

The densities of flies indoors compared to outdoors were significantly higher reflecting a marked degree of endophily. It seems that the flies prefer to remain indoors to escape adverse climatic conditions outdoors, as for example heavy rain and sudden fluctuations in the temperature and humidity. Sandflies in the neighbouring Kashmir valley were collected mainly from inside houses (Jacob & Kalra, 1951) but it is not clear whether much trapping took place outside. The density curve for all *Phlebotomus* species in Bagh district showed a single peak of abundance in the month of June preceding the heavy rains of the monsoon, and thereafter were found in low numbers till November. The overall density curve showed a unimodal peak thus indicating a single generation per season.

The seasonal abundance of the dominant *Phlebotomus* species both in Northern Areas and Azad Jammu & Kashmir showed a unimodal peak suggesting a single generation per season. The density peak is broader in Northern Areas where favourable warm dry conditions remain for longer whereas a sharp peak was observed in AJK

with the onset of the rains. Occurrence of a single generation of sandflies has been generally observed in temperate regions (Lewis 1978), particularly in the hilly areas and the areas where the summer season is short e.g. hilly region of Saudi Arabia (Al-Zahrani *et al.*, unpublished), and southern France (Rioux & Golvan, 1969).

Based on various studies carried out in different parts of the Old World it is evident that in southern latitudes or at low altitudes, where the weather is hot for most of the year, there is a marked bimodal distribution while in temperate areas there is usually only one summer peak.

In eastern Saudi Arabia, where the weather is hot most of the year, the abundance curve showed is bimodal (Büttiker and Lewis, 1983), whereas in the highland area of southwest Saudi Arabia where the temperature is cooler, sandflies showed a unimodal peak (Al-Zahrani *et al.*, Unpublished). Similar observations were recorded in Khuzistan, Iran (Nadim *et al.*, 1977) where phlebotomine sandflies in the littoral plain had two peaks of abundance, one in March and other in October. In the hilly parts of the province, the active season was short and the abundance curve had a unimodal peak in September. A similar phenomenon has been observed in many territories of the Mediterranean. For example, the annual fluctuation in the density of *Phlebotomus perniciosus* in Tunisia (Croset *et al.*, 1970), Algeria (Parrot *et al.*, 1933), USSR (Petrischeva, 1962) and Italy (Biocca *et al.*, 1977; Maroli & Bettini, 1977) shows bimodal peaks, one in June and another in September, suggesting the existence of two generations, whereas in southern France with a short warmer season *P. perniciosus* and *P. ariasi* showed a monophasic curve indicating a single generation (Rioux & Golvan, 1969). In Uzbekistan, phlebotomine sandflies usually have two

peaks of emergence, one in spring and other in autumn (Dolmatova & Dergacheva, 1961, 1963).

The present study suggests that in Northern Areas and Azad Kashmir, owing to the shorter summer season followed by extreme cold winters, it is most likely that the sandflies found here undergo diapause. The situation in Azad Kashmir seems to be even more complicated. Here, a sudden sandfly population explosion takes place during the early part of the season which then suddenly disappears a short time later, indicating that *Phlebotomus* species in AJK possibly undergo quiescence and diapause. In Northern Areas perhaps they undergo diapause only. Quiescence has been described as dormancy induced and terminated immediately by changes in temperature or moisture (Killick-Kendrick, 1978). Diapause involves a prolonged and predetermined period of arrested development of an insect, which is not immediately reversible and which is the result of intrinsic rather than extrinsic factors (Ready and Croset, 1980). Further studies under laboratory conditions will help to clarify the situation in Northern Pakistan.

#### **6.4.5. EXOPHILIC AND ENDOPHILIC BEHAVIOUR.**

Exophilic and endophilic behaviour of phlebotomine sandflies is important from the control point of view, in incriminating vectors and determining where transmission takes place. Endophily may be due to a combination of factors. The initial presence of flies in houses may be due to host preference, or the proximity of breeding places (Lewis, 1971). The tendency of a fly to remain in a house after a blood-meal may depend on the sites where people sleep, and the negative



phototropism of flies after biting.

Regular monthly hand capture of phlebotomine sandflies from inside houses was not possible during the present study because of the resistance of the local people. An attempt was however made to search a few houses during the peak sandfly season as part of the general survey.

#### 6.4.5.1. Northern Areas.

In Northern Areas, at low altitudes two species *P.papatasi* and *P.sergenti* were commonly found resting inside houses during the day time. In the main town of Chilas in cement constructed houses, *P.papatasi* was mainly found resting in bathrooms under the sinks where the humidity was high. In the Palaearctic region, *P.papatasi* and *P.sergenti* are well known endophilic species (Lewis, 1971). *P.papatasi* has been found to be endophilic in Saudi Arabia (Buttiker & Lewis, 1983), Sudan (Hoogstraal & Heynemann, 1969; Musa *et al.*, 1991),

parts of Russia (Perfil'ev 1969), India (Lewis, 1978), Iran (Nadim, 1978), Iraq (Abul-hab & Baghdadi, 1972) and Egypt (Beier *et al.*, 1986). *P.sergenti* has been commonly found resting indoors in Iraq (Abul-hab & Baghdadi, 1972), Afghanistan (Artemiev, 1978; Javadian *et al.*, 1982), Serbia (Zivkovic, 1980), Saudi Arabia (Buttiker & Lewis, 1983).

At higher altitudes, in Thor village (1700 metres), *P.keshishiani* was not found resting indoors during the day time as found in Afghanistan by Artemi'ev (1978). The same was also true in Baltistan (Mehdiabad) during the general survey, although immediately after sunset sandflies suddenly started appearing from some unknown

sites entering living rooms and verandahs and vigorously biting man throughout the night. The density of flies was so high that it was possible to catch 50-60 flies with the help of a mouth aspirator from a single room and adjacent verandah in about two hours. Most of the flies collected this way were *P.keshishiani* with a few *P.salangensis*, and females were more abundant than males.

#### 6.4.5.2. Azad Jammu & Kashmir.

In Azad Jammu & Kashmir it was difficult to find *P.hindustanicus* resting indoors, perhaps due to the low overall density of sandflies in this area. However, a few males and females were caught by mouth aspirator from indoors, especially animal stables. Inspection of chicken houses proved fruitless. In Afghanistan, Artemiev (Personal communication) however found this species mainly outside houses in rocks.

Based on the above observations and the results of the indoor and outdoor CDC light trap collections, it seems that *P.papatasi* and *P.sergenti* are peridomestic as well as endophilic at low altitudes in Chilas and *P.keshishiani* is peridomestic but exophilic at high altitudes. In Azad Jammu & Kashmir, on the other hand, *Phlebotomus hindustanicus* and *P.sergenti* are endophilic perhaps because of the adverse climatic conditions outdoors. Another strong reason to believe that *P.hindustanicus* is endophilic in AJK is that in 1992 immediately after the heavy floods I could still find this species indoors using CDC-light traps though weather conditions outside were appalling.

Caution however is required regarding the endophilic and exophilic status of *Phlebotomus* species as it has been observed that some wild species rest indoors if

they are abundant and have no alternative shelter, and endophilic species can some times be found outdoors (Lewis, 1971).

#### **6.4.6. HOST-VECTOR CONTACT.**

##### **6.4.6.1. Human biting catches.**

The human-biting rate is the classic method by which human biting populations of sandflies and other blood sucking arthropods have been measured (Hoogstraal and Heyneman, 1969; Gomez *et al.*, 1990). In practice, human biting catches are obtained by counting the number of sandflies biting the exposed skin of a volunteer per unit time. However, because of the risk of acquiring infections during biting catches, such studies are not generally undertaken.

Contrary to the feeding habits of sandflies in the New World forests where biting also takes place during the day time (e.g. *Lutzomyia wellcomei* in Brazil, Lewis & Ward, 1987), most phlebotomine sandflies in the Old World are nocturnal. The biting activity varies however according to local meteorological conditions (Dolmatova and Demina, 1971) and provides important information on host vector contact and the time when humans are at maximum risk of acquiring infection; e.g. the endophagic dawn and dusk biting activity of *P. papatasi* in parts of Iraq probably results in all members of a family being bitten (Lewis & Ward, 1987).

##### **i). Northern Areas.**

In the Northern Areas of Pakistan, *Phlebotomus keshishiani* was the only species biting humans at higher altitudes in Thor village (1700m). This species bit indoors from June till August with its peak activity at the end of the season.

a). Thor village(1700m).

All night biting catches on human volunteers showed that *P.keshishiani* generally bites throughout the night with peak activity between 22.00-24.00 hours. Biting activity was extended by two hours during July and August with another activity peak between 02.00-04.00 hours in August. The second peak in August perhaps represents parous flies. Ward *et al.*, (1977) were unable to detect any differences in the biting activity of parous and nulliparous *Lutzomyia flaviscutellata*.

There are no previous studies on the biting behaviour of *P.keshishiani* from Pakistan or for that matter from other parts of the Old World. Some work might have been done in the former USSR where this species has been considered the most likely vector of visceral leishmaniasis but, due to the language problem, it was not possible to follow all the Russian literature. *P.keshishiani* bites between the early evening and middle of the night, in contrast to other species of the subgenus *Larroussius* which have maximum man-biting activity soon after sunset e.g. *P.ariasi* in the south of France (Brooks, 1985), *P.orientalis* (Ashford, 1974) and *P.longipes* (Foster *et al.*, 1972) in Ethiopia, and *P.perniciosus* in the canine leishmaniasis focus of Central Italy (Maroli *et al.*, 1984). Peak biting densities during the early part of the night were also observed in species of the subgenus *Synphlebotomus* e.g *P.martini* and *P.celiae* the confirmed vectors of visceral leishmaniasis in Ethiopia (Gebre-Michael, 1992). As already mentioned, climatic factors such as temperature, humidity, rainfall and wind velocities are known to influence the nocturnal activities of most vectors (unfortunately not determined during the present study in relation to night biting catches) and this phenomenon may in part explain the biting pattern seen in Northern

Areas.

b). Hudur village (1200 m).

From low altitudes (Hudur village) in Northern Areas two species *P.papatasi* and *P.sergenti* were the only sandflies found biting man throughout the night.

Although *P.papatasi* is the proven vector of zoonotic cutaneous leishmaniasis and *P.sergenti* is the vector of anthroponotic cutaneous leishmaniasis in many parts of the Old World, these species have no epidemiological importance so far as the transmission of visceral leishmaniasis is concerned (Killick-Kendrick, 1990a) However, they transmit certain viruses to man, particularly by *P.papatasi* (Tesh, 1988) and therefore information on the biting behaviour of these species will perhaps have relevance to public health.

Both species bite man from June till August with peak indoor biting activity in June (Fig. 6.15). The all-night biting catches showed that *P.sergenti* bit man throughout night, but in very low numbers. This is a very curious behaviour because in other parts of the World this species has generally been found biting man in reasonable numbers in many countries. For example, in Baghdad, Iraq it is closely associated to humans being endophilic and endophagic (Abul-hab & Al-Baghdadi, 1972). Human biting has been observed as early as 19.00 hours with two major peaks one at 21.00 hours and other between 05.00-06.00 hours (Abul-hab & Mehdi, 1970, Sukkur, 1974). Similar behaviour has been observed in Afghanistan (Artemiev, 1978) and in Iran (Seyedi-Rashti *et al.*, 1984). The species seems to be mainly zoophilic in Pakistan but rests inside houses.

*P.papatasi* was found vigorously biting throughout the night starting from

20.00hrs till 06.00 hours. The biting rhythm of this species varied between months. The peak biting activity in May was between 24.00 and 02.00 hours and in June between 20.00 and 04.00 hours respectively. In July and August two biting peaks were observed one between 22.00-24.00 hours and other between 04.00-06.00 hours. The additional peaks seen during the last two months perhaps again represent the parous population at the end of the season as mentioned above for *P.keshishiani*. Variation within the months could be due to climatic factors. Previous studies on the biting behaviour of this species in Pakistan are lacking. Studies carried out in other countries showed more or less a similar biting pattern to that observed by us in Northern Areas e.g a 01.00 hour peak in Pondicherry, India (Srinivasan & Panicker, 1992b) and around midnight (between 23.00 and 24.00 hours) in Baghdad, Iraq (Mohsen, 1983). In El Agamy, Egypt, most activity was observed at midnight (El-said *et al.*, 1986).

From the above results it can be concluded that at high altitudes, (Thor villages) in Northern Areas, where *P.keshishiani* is the dominant sandfly biting humans, people can contract leishmaniasis any time at night after 20.00 hrs till 06.00 hours in the morning. They are however exposed to the maximum risk between 22.00-24.00 hours at the beginning of the sandfly season and between midnight (22.00-24.00hrs) and early morning (04.00-06.00 hrs) at the end of the sandfly biting season. It is important to note from the epidemiological point of view that almost all the people in Hudur village and Thor village sleep inside houses irrespective of the weather conditions.

**ii). Azad Jammu & Kashmir (1200 m).**

In Azad Jammu & Kashmir we could not find *P.hindustanicus* or *P.sergenti*

biting human volunteers indoors or outdoors. This could be due either to the low overall density of sandflies in this area or perhaps a preference for domestic animals (like buffalo, cattle) being kept in the rooms next to where families generally sleep.

#### 6.4.6.2. Blood-meal analysis.

Host blood-meal identification is an integral part of most arthropod transmitted disease investigations (Service *et al.*, 1986). Little is known about the feeding habits of phlebotomine sandflies because of their small size and obscure ecology (Lewis, 1974., Killick-Kendrick, 1978., Mutinga *et al.*, 1984, 1990; WHO, 1990). Knowledge of the feeding habits of sandflies in relation to transmission of leishmaniasis is essential to determine the degree of anthropophily and zoophily in the disease foci (Killick-Kendrick, 1987), and point to the possible reservoir hosts (Bray, 1982).

The results showed that the dominant *Phlebotomus* species: *P.keshishiani* (high altitude), *P.papatasi* and *P.sergenti* (low altitude) from Northern Areas and *P.hindustanicus* from Azad Jammu & Kashmir had all fed on human, dog and bovine blood. The proportion of feeding on a particular host however varied between species and also localities. *P.keshiahiani* fed both on man (32%) and dog (30%) in contrast to *P.papatasi* and *P.sergenti* which were found mainly fed on man (18 & 14% respectively) and to lesser extent on dogs (8.3 & 3.4%). *P.hindustanicus* on the other hand preferred bovines (43%) over dogs (22%) and humans (17.8%). The results thus strongly indicate that *P.keshishiani* and *P.hindustanicus* are both anthropophilic as well as zoophilic. This situation parallels earlier observations made on the feeding behaviour of certain Old World sandflies such as *P.argentipes* (Das *et al.*, 1976; Addy

*et al.*, 1983; Gosh *et al.*, 1990), *P. aiasi* in France (Guy *et al.*, 1984), *P. perfiliewi* in Emilia-Romagna, Italy (Killick-Kendrick *et al.*, 1977), *P. martini* (Mutinga *et al.*, 1990; Ngumbi *et al.*, 1992). In each of these examples, the sandfly vector has been observed to feed on a wide range of domestic animals, with varying degrees of anthropophily.

Recent studies have implicated dogs as the reservoir host of visceral leishmaniasis in Northern Areas and Azad Kashmir (Rab, 1994), although wild animals were not screened. The propensity of two potential vectors *P.(L)keshishiani* and *P.(A)hindustanicus*, to feed on humans and dogs is therefore of great epidemiological significance in relation to disease transmission in Northern Areas and AJK.

Three sandfly species, *P. keshishiani*, *P. hindustanicus* and *P. papatasi* gave evidence of mixed feeding. Multiple blood meals have previously been detected in *P. papatasi* (Namita *et al.*, 1991; El-Sawaf *et al.*, 1989), *P. aiasi* (Guy *et al.*, 1984), and *P. martini* (Ngumbi *et al.*, 1992). Since most of the sandflies are gonotrophically concordant (Killick-Kendrick, 1979) this behaviour may be simply the result of a sandfly being disturbed during the act of feeding (Killick-Kendrick, 1984) or perhaps when it discovers that the animal it has bitten is not its favoured host (Weitz *et al.*, 1960). However, *P. papatasi* frequently feeds again before the first meal is digested (Killick-Kendrick, 1979) and this may explain the presence of multiple blood meals in *P. papatasi* during the present investigations. Whether *P. keshishiani* and *P. hindustanicus* do this is not known.

Failure to detect blood-meals in some *Phlebotomus* specimens during the present study could be due to several factors; the blood was too well digested, or the



flies had fed on a vertebrate host other than those tested for. Old blood-meals have reduced concentration of proteins for detection in the ELISA (Beier, *et al.*, 1988) and best reactions occur with blood-meals tested within 24 hours of ingestion. In sandflies the best results were obtained when blood meals were analyzed within a few hours of feeding (Ngumbi *et al.*, 1992), but in the system we used blood-meals could be detected accurately up to 36 hours post feeding (Lane, unpublished).

The fact that many of the blood fed sandflies captured inside houses had fed on dogs which, unlike bovines, are usually kept outside houses implies that females of exophilic species e.g. *P.keshishiani* sometimes enter houses to rest or to digest their blood-meals after feeding.

#### 6.4.7. DOG BAITED CATCHES.

Apart from the blood-meal analysis we used dog baited traps to determine the attractiveness of the principal reservoir to potential vectors. In contrast to the unbaited sticky traps, the baited traps caught more females than males particularly *P.keshishiani* and *P.hindustanicus* (Fig, 6.19 Table 6.17) This strongly suggest their attraction to the potential reservoir host in the disease foci. These trapping results confirm results from the blood meal analysis. The presence of males on the dog baited traps is not unusual as males are generally found near the preferred vertebrate host waiting to mate with blood seeking females (Chaniotis *et al.*, 1967; Lane *et al.*, 1990). Unfortunately due to the lack of a proper control empty cage, it is not possible to compare the absolute densities of various *Phlebotomus* species caught on the baited traps to that of the unbaited traps. Relative abundance of the various species was similar in the baited and

unbaited traps: *Phlebotomus keshishiani* was dominant on dog-baited traps from high altitude (Thor village) and *P.papatasi* with *P.sergenti* sub-dominant from the low altitude (Hudur village) in Northern Areas, and *P.hindustanicus* was predominant in Azad Jammu & Kashmir. Seasonal changes on the dog-baited traps paralleled that of sticky traps and CDC light traps. Based on the present results it is thus concluded that *P.keshishiani* from Northern Areas and *P.hindustanicus* in Azad Jammu & Kashmir show a marked degree of attraction to the reservoir host (dogs).

Host preference studies using animal baited traps have been successfully carried out by several workers (Johnson, *et al.*, 1993; Quinnell, *et al.*, 1992; Mutinga *et al.*, 1986; Christensen & Herrer (1980), Killick-Kendrick, *et al.*, 1977; Rioux and Golvan, 1969; Shaw and Lainson, 1968; Quate, 1964) to determine preferred mammalian hosts of the known and suspected vectors both in the Old and New World. In these studies *Phlebotomus* species were presented with a choice of different hosts, whereas in contrast the objective of our study was to find out which sandfly species in the two disease foci are attracted to dogs.

#### **6.4.8. VECTORS OF VISCERAL LEISHMANIASIS IN AJK AND NORTHERN AREAS.**

Visceral leishmaniasis is prevalent in northern Pakistan, mainly in Northern Areas and Azad Jammu & Kashmir. The disease has been established to be a zoonosis, and parasites isolated from humans and dogs have been typed as *Leishmania infantum* (LON49, MON1) (Rab, 1994).

The sandfly fauna of both the disease foci includes species belonging to the subgenera *Phlebotomus*, *Paraphlebotomus*, *Larrousius* and *Adlerius*. The last two

subgenera (*Larroussius* and *Adlerius*) are of epidemiological significance as the Old World vectors of visceral leishmaniasis (caused by *Leishmania infantum*) have so far been found to be generally restricted to these subgenera. Of the 27 species of phlebotomine sandflies of the Old World subgenus *Larroussius* at least twelve are proven or probable vectors of leishmaniasis (Killick-Kendrick, 1990). Among the proven vectors which fulfil all the five criteria of vector incrimination are *P. ariasi*, *P. perfiliewi*, and *P. perniciosus*. In the subgenus *Adlerius* only six species have been listed as suspect vectors; *P. longiductus*, *P. chinensis*, *P. brevis*, *P. halepensis*, *P. kyreniae* and *P. simici*. None of the species in this subgenus has so far been incriminated as proven vectors based on the five essential criteria.

Essential criteria for vector incrimination have been discussed in detail by Killick-Kendrick (1990a) and include: demonstration that the fly is anthropophilic and the repeated identification and isolation of the same species of *Leishmania* from the sandfly as found in patients. Additional criteria include observations that the sandfly commonly feeds on the reservoir host(s), that it is present in the places where the *Leishmania* and the disease it causes are found, and that it supports the flourishing development of parasites and can transmit the parasite by bite. Although not impossible, some of the criteria such as repeated isolation of parasites from the vector and experimental transmission are difficult to attain and require long term observations.

#### **6.4.8.1. Vectors of visceral leishmaniasis in Azad Jammu & Kashmir (AJK).**

The results of the present study revealed that in AJK *P. (A) hindustanicus* is the vector of visceral leishmaniasis caused by *Leishmania infantum* s.s. Out of a total of

301 sandflies dissected one was found naturally infected with *Leishmania infantum* promastigotes. The infected sandfly was captured in CDC light-trap, from the living room of a mud constructed house, in Banjosa village, at an altitude of 1700 metres above sea level. Parasites were found in the midgut, pharynx and proboscis, and there was no evidence of a blood-meal in the midgut. This is an important observation because the site and intensity of infection in a wild caught sandfly gives, an indication firstly, of the subgenus of the parasite, and secondly of the ability of the fly to support the growth of the parasite and possibly, to transmit it by bite (Killick-Kendrick, 1990a).

Isoenzyme characterization of the isolated parasites could not be done because the culture became contaminated and was lost but the positive midgut squashed on Hybond Nylon membrane did however give a positive signal when probed with *Leishmania infantum* specific probe Luca D2 200 BP. Furthermore out of the 85 microscopically negative midguts squashed on hybond membrane, DNA hybridization detected *Leishmania infantum* in specimen of the same species (*P.hindustanicus*). A similar conflict between DNA probe and dissection results was seen in *P.pedifer* (Laskay *et al.*, 1991) and *P.celiae* (Gebre-Michael, 1992) from Ethiopia where additional specimens which were microscopically negative were detected with a DNA probe. The second fly which gave the positive signal was also captured in a CDC light-trap from inside a patient house in Rehra village at an altitude of 1200 metres above sea level.

Overall, 2 out of 301 (0.7%) *P.hindustanicus* were found positive in AJK which is a reasonable infection rate to consider this species as vector involved in the

transmission of *Leishmania infantum*. Except in a few countries where a higher infection rate has been reported in various *Phlebotomus* species e.g. *P. ariasi* (2/41:4.9%) and *P. perniciosus* (29/579:5%) in Algrave, Portugal (Schrey *et al.*, 1989), *P. perniciosus* (3/149:2%) in Arrabida, Portugal (Pires, 1984); in *P. alexandri* (13/643:2%) from China (Guan *et al.*, 1986a), in *P. ariasi* (3/187:1.6%) in France (Rioux *et al.*, 1984) and in *P. orientalis* (263/10,411:2.5%) from Sudan (Hoogstraal and Heyneman, 1969) most other reports showed either similar infection rate as found in *P. hindustanicus* from AJK or less e.g. in *P. ariasi* (7/1088:0.6%) and *P. perniciosus* (6/1415:0.4%) in Spain (Rioux *et al.*, 1989); in *P. martini* (6/2930:0.3%) in Baringo, Kenya (Perkins *et al.*, 1988); in *P. martini* (16/2326:0.7%) and *P. celiae* (3/1044:0.3%) in Ethiopia (Gebre-Michael, 1992);

For *P. hindustanicus* four of the essential criteria of vector incrimination were met as follows:

1. Blood meal analysis showed that *P. hindustanicus* feeds on humans and was mainly found inside houses.
- 2). The distribution of this species is well in accord with the distribution of human & canine visceral leishmaniasis cases caused by *Leishmania infantum*. The species was captured from lower as well as the higher altitudes in Azad Jammu & Kashmir and sporadic cases of visceral leishmaniasis have been reported to occur throughout the area (Rab, 1994).
- 3). As evident from blood meal analysis and dog baited traps *P. hindustanicus* feeds on the reservoir host.
- 4). In the present study, naturally infected *P. hindustanicus* was found which

harboured a mature infection of *Leishmania infantum* (as detected by microscope and DNA probing) including promastigotes observed in the head and proboscis which indicate that the parasites can survive in the fly throughout its complete extrinsic life cycle. Although parasites could not be typed which is important for determining if the promastigotes are of the human or animal reservoir origin, this does not seem to be so essential for northern Pakistan as the parasite isolated both from dogs and humans in this area has been found to be the same i.e. LON49, MON1. The only other criterion remaining to be fulfilled is the experimental transmission of *Leishmania infantum*.

On the basis of the above criteria, *P.hindustanicus* is considered to be the proven vector of visceral leishmaniasis in Azad Kashmir. The possibility that other *Phlebotomus* species e.g. *P.(L) sp.A* are secondary vectors can not be ruled out as this species was always found close to human habitations.

#### 6.4.8.2. Vectors of visceral leishmaniasis in Northern Areas.

In Northern Areas the situation regarding the vector of visceral leishmaniasis is slightly more complicated compared to AJK because dissection of sandflies as well as DNA hybridization failed to detect any infected flies. Other aspects of the biology of local sandflies therefore need to be taken into account, so that some comments can be made on potential vectors.

The sandfly fauna of this area includes sandfly species which are proven or suspected vectors of visceral leishmaniasis in other countries. Leaving aside *P.papatasi* and *P.sergenti* which are unable to transmit visceral leishmaniasis, the other important species include, *P.(L) keshishiani*, *P(L) kandelakii burneyi*, *P(A) salangensis* and

*P(Par) alexandri*.

*Phlebotomus keshishiani* was the only dominant species in Northern Areas at high altitudes (Thor, village 1700m). Other *Phlebotomus* species were sporadic. Variation in the densities of sandflies in a given area has been associated with the deficiency in sampling methods or differences between species in longevity or biting frequency (Killick-Kendrick, 1990a). In Northern Areas low numbers of *P.alexandri*, *P.kandelakii burneyi* and *P.salangensis* captured during the present investigations do not seem to be due to the deficiency in sampling methods, because the same species were captured from Baltistan in high numbers using the same methods used for sampling sandflies in Hudur and Thor villages. Differences between the longevity of these species are yet to be determined and require further long term studies. Regarding biting frequencies *P.keshishiani* was again the dominant species biting humans and reservoir (dogs) compared to *P.alexandri*, *P.salangensis* and *P. kandelakii burneyi*.

Where there is a lack of evidence of natural infection in wild caught sandflies with *Leishmania* promastigotes, it becomes difficult to decide with certainty which species is/are involved in the disease transmission. However, a preliminary idea can be formed taking into consideration other aspects of sandfly biology. These aspects are discussed below for each species captured in Northern Areas.

Among the species representing sandfly fauna of Northern Areas, *P(Par) alexandri* is the only proven vector of visceral leishmaniasis caused by *Leishmania donovani*, in the Xinjiang Autonomous Province, of the Peoples Republic of China

(Guan Li-Ren *et al.*, 1986) However the question remains whether this species can support the growth and transmit *Leishmania infantum*. During the present study *P.alexandri* was found in the Northern Areas both at lower and higher altitudes. None was captured on human bait whereas a few were found on dog-baited traps. Blood-meal analysis however revealed that out of the two engorged females *P.alexandri* captured in an outdoor CDC light-trap one had dog blood.

*P.kandelakii kandelakii* has been considered as the suspected vector transmitting visceral leishmaniasis caused by *Leishmania infantum* in Azerbaidjan and Georgia (USSR) and has been listed as the suspected vector by Killick-Kendrick, (1990a) Only 11 specimens of *P.kandelakii burneyi* were found in Hudur village and Thor village, in Northern Areas. Four females were found on human bait but none in the dog trap.

*Phlebotomus(A) salangensis* was also among the rare species captured from Northern Areas except in Baltistan where this species is abundant at altitudes above 2500 metres. This species has been recently described from Afghanistan (Artemiev, 1980) but nothing is known of its medical importance. During the present study two females were caught on human bait and six in dog-baited traps. Blood-meal analysis revealed one female positive with dog blood.

*Phlebotomus keshishiani* has been considered as a suspected vector of visceral leishmaniasis, by Artemiev (1978), however, it was not included in the list of suspected vectors of VL by Killick-Kendrick (1990a). During the present study this species was found at both lower and the higher altitudes in Northern Areas, and was the dominant species at higher altitudes (1700 metres in Thor and 2300 metres in



Mehdiabad, Baltistan). The females were highly phototropic, and were caught in good numbers both indoors and outdoors. Higher proportion of this species was found on dog-traps and human bait catches than *P.alexandri*, *P.kandelakii burneyi* and *P.salangensis*. Blood-meal analysis also showed preference of this species for human and dog blood.

Although apparent abundance of a sandfly species is not a sufficient criterion alone to incriminate it as a vector, the present studies carried out in Northern Areas strongly suggest *Phlebotomus keshishiani* is a suspect vector involved in the transmission of visceral leishmaniasis caused by *Leishmania infantum* for the following reasons:

- 1) *P.keshishiani* is abundant in the area.
- 2) It feeds on humans in significantly high numbers as shown by human bait catches and blood meal analysis.
- 3) It commonly feeds on the reservoir host (dogs).
- 4) Its distribution coincides with human and canine leishmaniasis.

*Phlebotomus alexandri*, could be considered as a secondary vector but this species needs to be studied further.

#### 6.4.9. TRANSMISSION OF DISEASE.

Based on the results of this study and the complementary epidemiological investigations in the same disease foci by Rab (1994), the transmission of visceral leishmaniasis in Northern Areas seems to occur between the altitudes of 1500 to 1800 metres, where the population of the valleys usually migrate during summer.

*Phlebotomus keshishiani* is the dominant *Phlebotomus* species at the higher pastures. The seasonal changes in the sandfly population suggest that the disease is transmitted between the middle August and early September when the sandfly population is declining and the number of maximum parous flies are expected. Since *P. keshishiani* is anthropophilic and zoolphilic, both as well as exophagic and endophagic, humans are at risk both indoors and outdoors. As most of the people in these villages sleep inside houses it is most likely that transmission takes place indoors.

In Azad Jammu & Kashmir, out of the two positive sandflies, *P. hindustanicus* one female (promastigotes observed microscopically) was from a higher altitude (1900 m) and other was from a lower altitude (1200 m). This species is mainly endophilic, both exo- and endophagic as well as anthropophilic and zoophilic. Transmission seems to occur between the altitudes of 1100 to 2000 metres, as most villages are sited at these altitudes (Rab, 1994). Observations on the seasonal changes of *P. hindustanicus* in AJK suggests that it most probably transmits disease to man between the middle of June and early July with possibly low transmission in October also.

#### 6.4.10. FEASIBILITY OF VECTOR CONTROL

Regarding vector control in the two disease foci in northern Pakistan, its premature to be dogmatic because of the lack of appropriate data from pilot control studies, but on the basis of vector behaviour in the field some preliminary comments can be made.

In the light of the results obtained during the present investigations in northern Pakistan, *Phlebotomus hindustanicus*, the vector in Azad Jammu & Kashmir is mainly

endophilic, whereas *P.keshishiani* the highly suspected vector of visceral leishmaniasis in Northern Areas is exophilic, but enters houses at night to feed on humans and animals. In this situation it is reasonable to presume that indoor spraying with residual insecticides in both the disease foci will be helpful in suppressing sandfly densities and in providing partial protection to humans against sandfly bites. Based on observations of a detoxification enzymes in sandflies, including some on Pakistani sandflies, it appears that sandflies are susceptible to insecticides in northern Pakistan (see appendix 1). Health education of the community to use of repellants could be helpful and perhaps cost-effective as well.

It is however, strongly emphasized that before such a decision is implemented it is essential, initially to run a pilot control study to evaluate the effectiveness of insecticidal spraying against the vector species.

## SUMMARY

1. Visceral leishmaniasis is mainly prevalent in Northern Areas of Pakistan and Azad Jammu & Kashmir (AJK). The disease mainly affects young children and occasionally adults. Dog is the domestic reservoir host. The parasite isolated both from humans and dogs has been identified as *Leishmania infantum* (LON49; MON1). The sandfly fauna of Pakistan as studied by Lewis (1967) includes 10 *Phlebotomus* species belonging to six subgenera and 10 *Sergentomyia* species. There are no previous studies regarding vector incrimination, population dynamics and sandfly behaviour in the field.
2. The aim of the present study was to incriminate the sandfly vector(s) of visceral leishmaniasis in Northern Pakistan. Two contrasting areas were studied: Northern Areas (Chilas) and AJK (Bagh) through monthly sampling. In addition, a general survey was also undertaken in several sites representing different climatic and vegetational zones and the distribution of each species mapped.
3. Studies on sandfly systematics defined morphologically variable species of the subgenera *Phlebotomus* (*Larroussius*) and *P.* (*Adlerius*). The species belonging to the subgenus *Larroussius* were identified as *P.major*, *P.keshishiani*, *P.kandelakii burneyi* and *P.Sp.A* and those belonging to subgenus *Adlerius* as *P.hindustanicus* and *P.salangensis*. Morphological characters of all these species were intensively studied and their distribution recorded. For *Larroussius* species several female specimens were also dissected to reveal the morphology of the base of spermathecal duct as an additional aid for identification. Other *Phlebotomus* species; *Phlebotomus* (*Phlebotomus*) *papatasi*, *P.* (*Paraphlebotomus*) *sergenti*, *P.* (*Paraphlebotomus*) *alexandri* found during the present investigations were easily identified based on the taxonomic characters given by Lewis (1967, 1982) and therefore morphometric analysis was not necessary.
4. The presently recognised *P.major* was previously described by Lewis (1967) as a subspecies but detailed morphological measurements of this subspecies as well as the paralectotype of *P.major* from India, did not reveal any significant differences in almost all the important characters measured. This species differs from *P.neglectus* and *P.syriacus* in having a short base of spermathecal ducts. *P.major* was mainly found at altitudes between 1200-1500 metres in AJK and the Margella hills (near Islamaad).

5. Two allopatric forms of male *P.keshishiani* with different antennal formulae collected from Northern Areas are indistinguishable by other morphological characters. Comparison of the specimens from Pakistan with the type specimen and also with the early descriptions, showed that males and females of the Pakistani form have shorter antennal segments (A3, A4, A5) than to Russian specimens. The genital filaments and sperm pump are shorter in male specimens from Pakistan and the number of hairs on the coxite also varies (29 in Pakistani specimens and 20 in Russian specimens). *Phlebotomus keshishiani* can however be distinguished easily from all other *Larroussius* species by the shape of the male aedeagus (tip of the aedeagus being extremely narrow and rounded), pharyngeal armature and long length of the spermathecal ducts. In Pakistan this species occurs at altitudes between 1200-2500 metres in Northern Areas and the Margella hills, (Islamabad) being dominant above 1500metres .

6. *Phlebotomus kandelakii burneyi* was described as a subspecies of *P.kandelakii* by Lewis (1967). Detailed morphological measurements of the paralectotype, as well as wild caught specimens of *P.kandelakii burneyi* and also of *P.kandelakii kandelakii* from Iran and Azerbaizhan in the former Soviet Union (FSU) revealed that both males and females of the two taxa can be distinguished morphologically. In male *P.kandelakii burneyi*, the width of the distal end of the paramere is significantly greater than *P.kandelakii kandelakii*. The antennal formula (1/3-5, 2/6-15 *P.kandelakii burneyi* and 1/3-7, 2/8-15 *P.kandelakii kandelakii*) is also different in both subspecies. Females are separable on the length of palpal segment 5 which is significantly longer in *P.kandelakii burneyi*.

7. Regarding specific and subspecific status of *P.kandelakii burneyi* from Pakistan, we agree with Lewis (1967) and retain the subspecific status till further long term studies are carried out on the biology of this sandfly from Northern Areas and also from other regions. During the present investigations this species was mainly found at altitudes above 2400 metres in Baltistan. A few specimens were however also collected from lower altitudes in Northern Areas (Thor village, 1700m) and AJK (Rehra village, 1200m).

8. Another *Larroussius* species collected during the present study from AJK at altitudes between 1500-1890 metres is identified as a new species (*P.Sp.A*). This

species is close to *P.major* but measurements of various morphological characters of both sexes show that most parts of *P.Sp.A* are significantly bigger than *P.major*.

9. Females of the two species can be separated on the number of spermathecal rings (12-15 in *P.major*; 16-21 in *P.Sp.A*), length of the terminal process of spermatheca (significantly longer in *P.Sp.A*), arrangement of pharyngeal armature and size of the pharynx. Morphology of the base of the spermathecal duct however do not show significant difference between *P.major* and *P.Sp.A*. The male genitalia except the aedeagus are significantly bigger in *P.Sp.A* than *P.major*. The distance between the terminal, middle and proximal spines also differs significantly as do the number of hairs on the male coxite.

10. *Phlebotomus salangensis* is being described for the first time from Northern Areas of Pakistan. This species has so far been recorded from Afghanistan only (Artemiev, 1980). *P.salangensis* is close to *P.angustus* morphologically and is mainly abundant at high altitudes (2300-2500 metres) in Baltistan and sporadic at low altitudes (1200-1700 metres) in Northern Areas. This species was not found any in AJK and the Margella hills.

11. *Phlebotomus hindustanicus* in Pakistan was previously misidentified as *P.chinensis longiductus* from which the males differ in the antennal formula (2/3-7, 1/9-15), length of the genital filaments, sperm pump and F/P ratio, number of hairs on the coxite, position of the hairy patch as well as the distance of the aedeagus notch from its tip. Females can be distinguished on the basis of A3/L ratio which is higher in *P.hindustanicus* than *P.longiductus*. Other characters, like pharyngeal armature and number of irregular spermathecal segments as described by Artemiev (1980), are difficult characters for distinguishing between females. *Phlebotomus hindustanicus* was found only in the sub-Himalayan hill ranges in AJK and also the Margella hills at altitudes between 1200 metres and 1890 metres above sea level.

12. During field entomological studies in Northern Areas and AJK, 9656 sandflies belonging to the genus *Phlebotomus* were collected and identified (8797 during longitudinal studies and 859 during general survey). Four subgenera were represented: *Phlebotomus*, *Paraphlebotomus*, *Larroussius* and *Adlerius*. Species belonging to the subgenus *Phlebotomus* were not found at altitudes above 1800 metres.

13. Longitudinal entomological studies were carried out between April and

November 1991 and in the same months in 1992 using a variety of sampling methods. Of the 8797 *Phlebotomus* collected, 6920 were from Northern Areas, Chilas district (Hudur village, 1200m and Thor village, 1700m) and 1877 from AJK, Bagh district (Rehra village). Six species were captured from Northern Areas (*P.papatasi*, *P.sergenti*, *P.alexandri*, *P.keshishiani*, *P.kandelakii burneyi* and *P.salangensis*) and four from AJK (*P.hindustanicus*, *P.sergenti*, *P.major* and *P.(L) Sp.A.*). The species composition and relative abundance varied within and between the two areas. In Northern Areas, *P.papatasi* (35.75%) and *P.sergenti* (19.0%) dominated the collections from lower altitudes (Hudur village, 1200 m) and *P.keshishiani* (29.66%) was most abundant at higher altitude (Thor village, 1700 m), whereas in AJK *P.hindustanicus* (64.62%) was dominant.

14. Species differed in their seasonal abundance. In Northern Areas, village Hudur, the dominant species *P.papatasi* showed peak indoor activity in May but outdoor activity in July, whereas in Thor village, the dominant *P.keshishiani* was abundant both indoors and outdoors in July and August. In AJK, peak numbers of the dominant *P.hindustanicus* were found in June. In both Northern Areas and AJK the peak sandfly activity was positively correlated with temperature. In AJK peak sandfly activity preceded the monsoon. *Phlebotomus papatasi* and *P.sergenti* were endophilic and *P.keshishiani* was exophilic in Northern Areas whereas *P.hindustanicus* was endophilic in AJK.

15. Collection from human baits revealed *P.papatasi* (92%) and *P.keshishiani* (83%) as the only man biting species in Northern Areas. Peak biting density of *P.papatasi* was in June and that of *P.keshishiani* in August. Both species showed peak activity between 22-2400 hours with additional peaks between 0400-0600 hours at the end of the season. In contrast to the Northern Areas results, none of the *Phlebotomus* species in AJK was caught biting human volunteers although blood meal analysis clearly indicates that *P.hindustanicus* feeds on man.

16. Blood-meal analysis revealed that the dominant *Phlebotomus* species: *P.keshishiani* (higher altitudes), *P.papatasi* and *P.sergenti* (lower altitudes) from Northern Areas and *P.hindustanicus* from AJK all feed on humans, dogs and bovines. The proportion of feeding on a particular host however varied between species. *P.keshishiani* feeds mainly on humans (32%) and dogs (30%) in contrast to *P.papatasi*

and *P. sergenti* which mainly feed on humans (18 and 14%) and to lesser extent on dogs (8.3 and 3.4%). *Phlebotomus hindustanicus* on the other hand preferred bovines (41%) over dogs (22%) and humans (18%). These results clearly indicate that the two vector species *P. keshishiani* and *P. hindustanicus* are both anthropophilic and zoophilic which is important from the disease transmission point of view.

17. Apart from blood-meal analysis, dog baited traps were also used to determine the attractiveness to the principal reservoir. *P. keshishiani* is the dominant species in these traps from higher altitudes (Thor village) and *P. papatasi* with *P. sergenti* as sub-dominant from lower altitudes (Hudur village) in Northern Areas, and *P. hindustanicus* dominant in AJK. The most striking observation was that compared to unbaited sticky traps, the baited traps caught higher proportions of females than males, particularly *P. keshishiani* and *P. hindustanicus* which strongly suggests their attraction to the potential reservoir host. These trapping results confirm results from the blood-meal analysis.

18. Dissections of 301 *P. hindustanicus* from AJK revealed the presence of *Leishmania* promastigotes in one specimen only. Parasites were identified by DNA hybridization (using *Leishmania infantum* specific probe LUCA D2 200 BP) as *Leishmania infantum*. Of 85 microscopically-negative midguts squashed on hybrid membrane, DNA hybridization detected *Leishmania infantum* in specimen of the same species (*P. hindustanicus*) giving an overall infection rate of 0.7%. On parasitological and ecological grounds this species is incriminated as the principal vector of visceral leishmaniasis in AJK.

19. In Northern Areas dissections of 754 *P. keshishiani*, 36 *P. salangensis* and 15 *P. alexandri* failed to reveal a naturally infected sandfly. Also, DNA probing of midguts (568 *P. keshishiani*, 36 *P. salangensis* and 10 *P. alexandri*) using *Leishmania infantum* specific DNA probe (LUCA-D2 200 BP) failed to detect any parasites. Circumstantial evidence suggests *P. keshishiani* is the most likely vector involved in the transmission of visceral leishmaniasis in Northern Areas; and *P. alexandri* may serve as a secondary vector.



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**APPENDIX 1****SUSCEPTIBILITY OF SANDFLIES TO INSECTICIDES: A PRELIMINARY EVALUATION BASED ON THE ACTIVITIES OF DETOXIFYING ENZYMES.****INTRODUCTION:**

Continuous monitoring of changing levels of susceptibilities in insects to various synthetic insecticides is inevitable in planning a successful vector control strategy. Studies on the changing susceptibilities to insecticides in various insects of medical and veterinary importance have mainly relied on WHO bioassay kits for last many years. Although these bioassays have been the most widely used technology to determine insecticide resistance, there are several inherent limitations: only one insecticide can be tested per insect and without a known discriminating dosage large number of insects are needed to generate probit lines, also false positive may occur due to deteriorating filter papers or procedural variables, such as temperature or humidity, and finally the bioassays are ineffective in detecting resistance phenotypes at low frequencies. In view of these limitations a WHO Expert Committee on insecticide resistance and vector control (1970) recommended basic research on the genetics and biochemistry of resistance. As a result in-depth studies were undertaken, which led to better understanding of prototypes of new biochemical methods of detecting resistance (WHO/VBC/1988).

These new methods have been a subject of many excellent reviews (WHO/VBC, 1988; Brogdon, 1984, 1989) and have been successfully applied to monitor resistance in mosquitoes and many other insects. The assays are quick for detecting resistance in individual insects and identifying the type of resistance mechanism involved (Brogdon, 1989) and are particularly important in situations where limited amount of field material is available.

Microtitre plate assays have been successfully used for determining insecticide resistance in many insects including mosquitoes. The assays are based on

measuring the activities of certain enzymes related to insecticide detoxification which is recognized as one of the most important mechanisms by which insects become resistant to chemical insecticides (Brattsten, *et al.*, 1986).

Three enzyme systems are involved in the metabolism and degradation of toxic compounds: mixed function oxidases (especially Cytochrome P450) which degrade all groups of insecticides (Hodgson, 1983); Glutathione s-transferase (GSTs) important in the metabolism of organophosphate insecticides (OPs) and DDT (Motoyama & Dautermann, 1980); and, esterases which degrade carbamates, OPs, pyrethroids, and juvenile hormone analogues (Brogdon, 1987). Elevated activity of these enzymes in the resistant strains of insects have been demonstrated in several key studies. High level of GSTs have been reported in OP resistant *Musca domestica* (Motoyama & Dautermann, 1974) and in DDT resistant populations of various *Anopheles* species (Herath *et al.*, 1988; Hemingway *et al.*, 1985; Mouryia *et al.*, 1993). Similarly, significantly raised activity of one or more esterases have been strongly associated with OP resistance in many insect species including mosquitoes (Matsumura & Brown, 1961; Villani & Hemingway, 1987; Peiris & Hemingway, 1990; Devendra *et al.*, 1993).

The ultimate objective of measuring enzyme activities in various species is the early detection of resistance at low frequencies and possible prediction of cross-resistance in a single insect. This is clearly important in the rational use of insecticides in vector control programmes. At present the principal method for the control of sandflies remains unequivocally insecticides (Lane, 1991). There are numerous susceptibility studies on sandflies based on bioassays but only El Sayed *et al.*, (1990) have investigated insecticide degradation (DDT) in sandflies. So far resistance has been reported in one species *Phlebotomus papatasi*, from Bihar, India, which is resistant to 4% DDT (Dhanda *et al.*, 1983).

During the present study susceptibility of sandflies to insecticides has been measured using microtitre plate enzyme assays. Since there were no previous studies available regarding level of insecticide detoxification enzymes in sandflies it was necessary first to establish a base line enzyme activity. For this purpose baseline

activity levels in three enzyme systems (GST, esterases and G6PD) were measured in susceptible *Phlebotomus papatasi* from seven different geographical locations to determine the level of natural variation found in field populations of sandflies. The base line thus obtained was used to compare the enzyme titre measured in sandflies from visceral leishmaniasis foci of Pakistan to determine their susceptibilities to insecticides.

Unlike bioassays, it has been suggested that enzyme assays are independent of the physiological age of insects being tested (Brown & Brogdon, 1987). However, there are reports of changes in levels of some insecticide detoxifying enzymes with age in mosquitoes (Rowland & Hemingway, 1987, Mourya et al., 1993). If GST (the principal detoxifying enzyme in DDT resistance) declines with age in sandflies, it would not be possible to distinguish between a young susceptible and an older resistant insect. Therefore an enzyme which also declines with age but not associated to resistance was required to calibrate the field results. Such an enzyme is G6PD. To determine whether enzyme titre changed with age in sandflies two enzyme systems GST and G6PD were analyzed in insects of known age.

## MATERIALS AND METHODS.

**a) Materials:** The following colonized stocks were used:

*Phlebotomus papatasi* flies originating from Saudi Arabia (Al Hasa), Afghanistan (Kabul), Tunisia, Iraq (Baghdad), Cyprus, Spain and India (Pune) were obtained from the colonies of Professor R. Killick-Kendrick. *Phlebotomus papatasi* and *Phlebotomus keshishiani* were collected from Chilas and Thor, Pakistan for comparison with the baseline studies. Flies were stored in liquid nitrogen prior to use.

For age studies 1-6 days post emergence *Lutzomyia longipalpis* originally from Ile de Marajo, Brazil and 1, 3, 5, 7, 10, 15 and 30 days post emergence *Anopheles stephensi* generation 10 originating from USSR were collected and pooled into separated batches of 25 each and kept at -70°C till analysis. Sandflies were maintained on sucrose solution.

**b) Methods.**

i) Homogenate: Individual insects were homogenised in ice-cold distilled water (mosquitoes in 225 ul. and sandflies in 150ul.) and centrifuged at 10,000g at 4°C for 3-5 minutes and the supernatant used in enzyme assays. All the homogenates were on ice during the experiments.

ii) Protein assays: the protein content of all individual flies was determined by the bicinchoninic acid method (BCA) (Pierce Chemical Co.) so that all enzyme activities could be standardised. Protein assay reagents were incubated with 20ul. of homogenate for 30 minutes at 37°C, the absorbance was measured at 570nm on a UVmax microtitre plate reader (Molecular Devices) and converted to protein concentration against a bovine serum albumin standard curve.

iii) Glutathione s transferase: Enzyme activity was assayed by the method of Booth et al., (1961), using a 25ul. aliquot of supernatant incubated with a 80ul. of reduced glutathione (GSH) and chloro-2-4-dinitrobenzene (CDNB) in a microtitre plate (30mg GSH in 5ml. phosphate buffer and 40ul. of 3mg/ml. methanol solution, CDNB). The rate reaction was measured for 5 minutes on a UVmax microtitre plate reader at 340nm. Rate data was corrected for soluble protein content and expressed in absolute units using the extinction coefficient of 9.5mMol cm.

iv) Esterase: total non-specific esterase activity was assayed by Van Aspern's method (Van Aspern, 1962) using 25ul of supernatant in microtitre plates. Either 200ul of 1-naphthyl acetate or 2-naphthyl acetate were used as substrate. After 10 minutes the reaction was stopped with 50ul. fast blue in 2.5% sodium lauryl sulphate and absorbance read at 570nm. The optical density readings were expressed as nmoles of 1 or 2 naphthol/min/mg protein calibrated by means of 1- or 2-naphthyl standard curve.

Glucose 6 phosphate dehydrogenase: The method of Devendra et al., 1993) was employed using 25ul of supernatant in a microtitre plate with 100ul of substrate. The rate reaction was read over 5 minutes at 540nm. with a Vmax micro titre plate reader. Results were expressed as mOD/min/mg protein.

Data were subjected to analysis of variance and the significance of differences



between group means were determined by Sheffe's 't' test for multiple comparison.

## RESULTS.

### I) Effect of age on enzyme activity.

In *Anopheles stephensi* there was significantly more G6PD activity in one day old mosquitoes than in all other age groups whereas no significant changes were observed in GST activity ( $P < 0.05$ ) (Fig. 1A & B). In *Lutzomyia longipalpis* there was no systematic change in either GST or G6PD activity with time ( $P < 0.05$ ) (Fig. 1 C & D).

### II) Enzyme activities in *P.papatasi* populations from various countries for determining baseline levels.

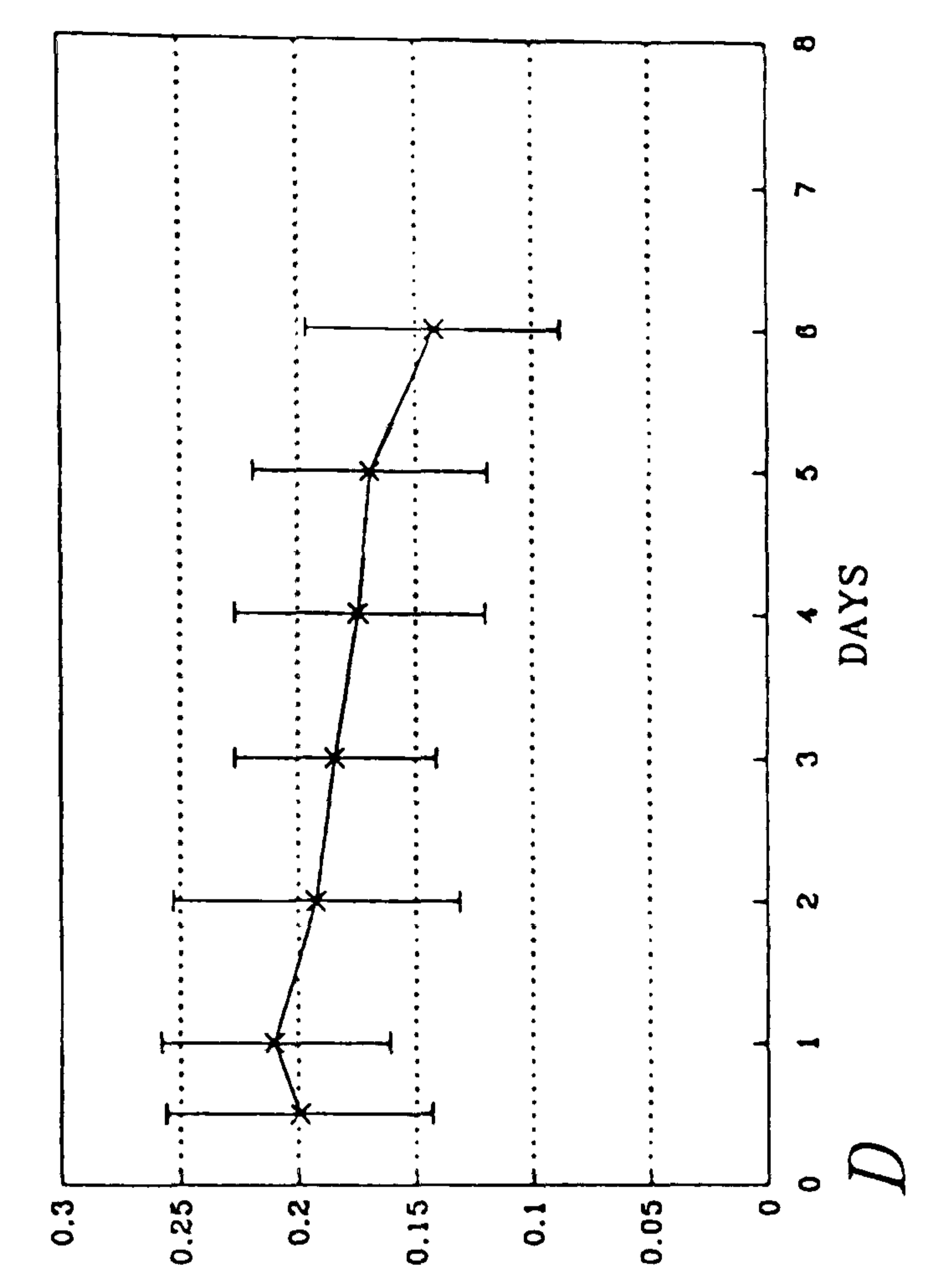
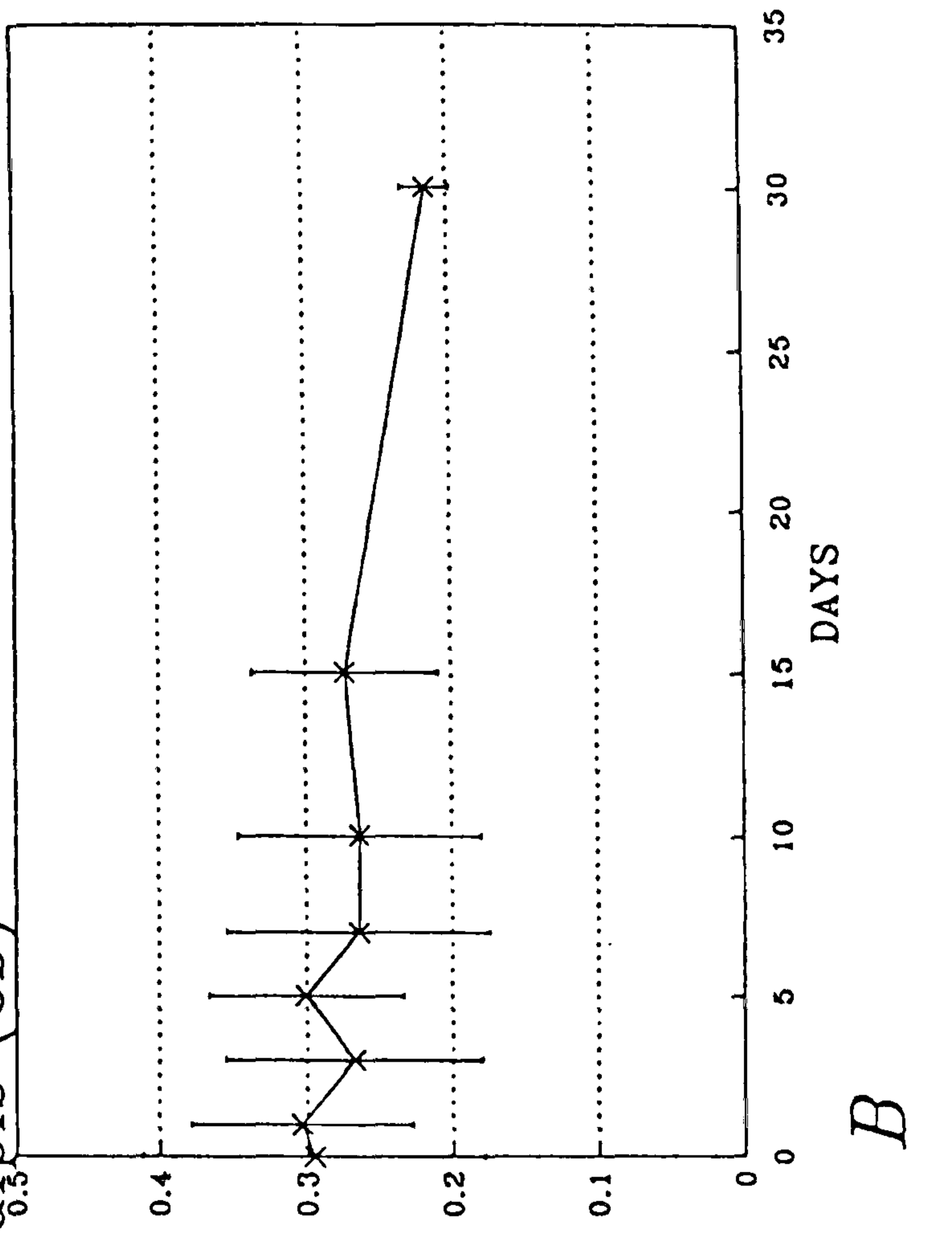
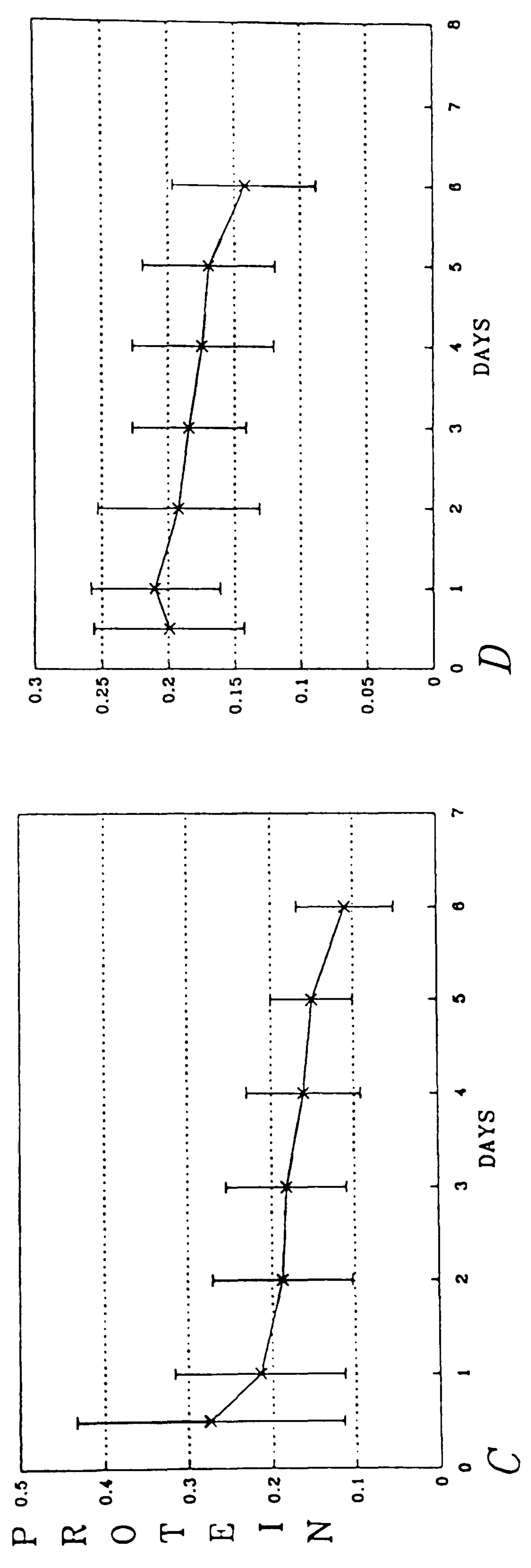
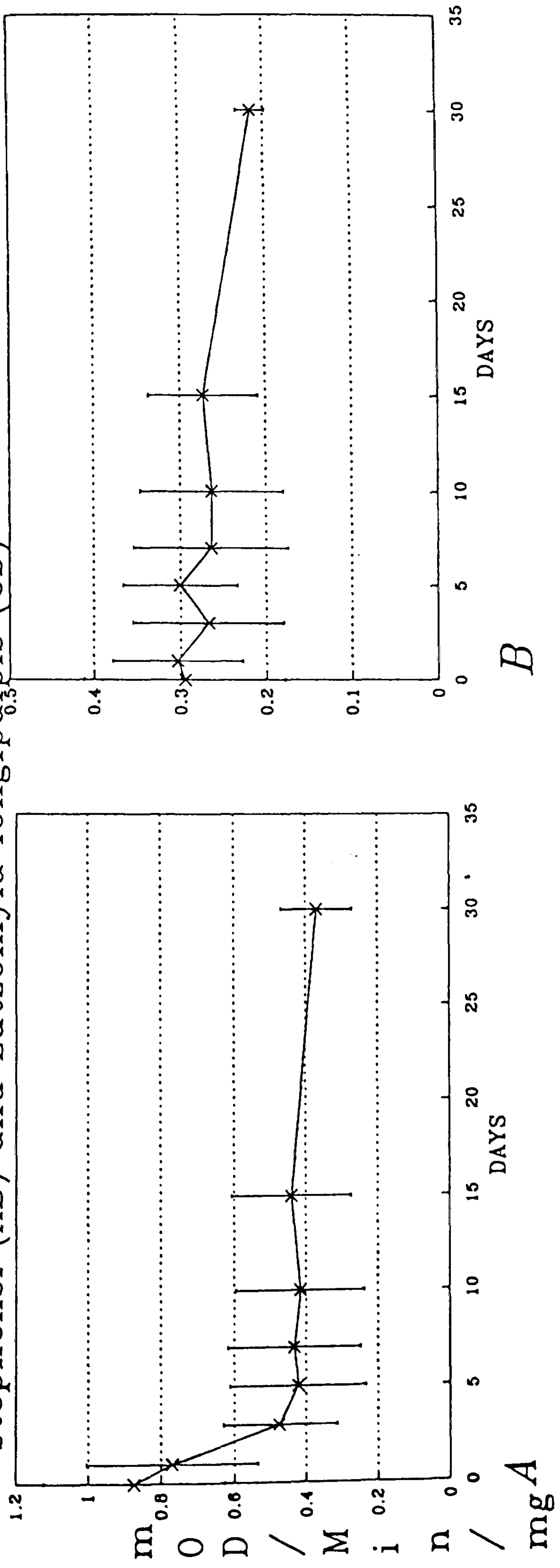
Table 1 shows the mean enzyme activity between seven populations of *Phlebotomus papatasi*. A significant difference in the means is evident. Some populations such as those originating in Spain, had consistently lower enzyme levels (corrected for protein content and therefore size) than other populations. Conversely, the sandflies from India (Pune) had higher enzyme activity in all four enzymes examined. There was a five fold difference between the sandflies from Spain and those from India.

We propose to use a meanbase line activity for *P.papatasi*, as 0.0063-0.33 nmoles/mg protein for GST, 0.05-0.3 nmoles/mg protein for esterase with 1 naphthyl acetate as a substrate and 0.03-0.42 nmoles/mg protein for esterase with 2 naphthyl acetate as substrate.

### iii) Activity of Glutathione S- transferase and esterase in *Phlebotomus* species from Pakistan.

Table. 2 shows the activity of glutathione s-transferase, Esterase 1 & 2 in *P.papatasi* and *P.keshishiani* from Northern Pakistan. Compared with the baseline enzyme activity determined above these values fall within the susceptibility limits although GST is on high side, slightly higher than in *P.papatasi* from India.

Fig.1 Glucose 6-phosphate and Glutathione S transferase in *Anopheles stephensi* (AB) and *Lutzomyia longipalpis* (CD)



**Table 1. Mean Gluthathione S-transferase (GST), Esterase-1 (EST1), Esterase-2 (EST2) and Glucose 6-phosphate (G-6P) in *Phlebotomus papatasi* from different countries.**

Country	GST (nmoles/mg protein)	EST-1 (nMoles/mg protein)	EST-2 (nMoles/mg protein)	G-6P mOD
S.Arabia	0.0063±0.0058 (0.001-.03)	0.1±0.05 (0.05-0.2)	0.04±0.025 (0.05-0.2)	0.18±0.12 (0.1-0.62)
Tunisia	0.0054±0.0047 (0.006-0.03)	0.12±0.13 (0.05-0.9)	0.09±0.08 (0.05-0.55)	0.17±0.19 (0.02-0.4)
Spain	0.0064±0.0053 (0.002-0.03)	0.05±0.07 (0.05-0.5)	0.03±0.03 (0.05-0.15)	0.05±0.06 (0.02-0.35)
Iraq	0.0089±0.0054 (0.002-0.0.02)	0.25±0.11 (0.1-0.7)	0.21±0.08 (0.1-0.55)	0.19±0.18 (0.1-0.4)
India	0.013±0.009 (0.002-0.035)	0.28±0.13 (0.15-0.1)	0.22±0.1 (0.1-0.8)	0.2±0.1 (0.05-0.6)
Cyprus	0.011±0.006 (0.002-0.03)	0.2±0.008 (0.1-0.45)	0.15±0.07 (0.05-0.4)	0.2±0.1 (0.05-0.45)
Afghanistan	0.01±0.008 (0.002-0.03)	0.13±0.12 (0.015-0.7)	0.1±0.09 (0.015-0.55)	0.03±0.02 (0.014-0.02)

**Table 2. Mean Glutathione s-transferase and Esterases (EST-1, EST-2) in *Phlebotomus papatasi* and *P.keshishiani* from northern Pakistan.**

Species	GST (nmoles/mg/protein)	EST-1 (nmoles/mg protein)	EST-2 (nmoles/mg protein)
<i>P.papatasi</i>	0.057±0.003 (0.005-0.14)	0.45±0.02 (0.09-0.9)	0.28±0.1 (0.1-0.48)
<i>P.keshishiani</i>	0.059±0.02 (0.03-0.12)	0.46±0.2 (0.1-0.9)	0.33±0.2 (0.12-1.08)

±=Standard deviation

Within paranthesis are lower and upper limits.

## DISCUSSION.

In contrast to *A.stephensi*, there were no systematic changes with age in the activity of either GST or G6PD in sandflies. Therefore sandflies of any age were used in subsequent enzyme assays. The results also show that measuring enzyme levels as means of age-grading sandflies is not practicable.

Resistance involving esterase falls into two broad categories. High levels of resistance can be obtained where increases in insecticide hydrolysis occur. This can be accompanied by no or low level changes in activity with the general substrate such as 1- and 2-naphthyl acetate (e.g *Simulium damnosum* from West Africa, Hemingway et al., 1989). Alternatively, lower levels of resistance occur when there is an increased quantity of an esterase produced which is able to sequester rather than metabolise insecticide (e.g. in *Myzus persicae* and *Culex quinquefasciatus*, Field et al., 1989). This type of mechanism produces an increase in activity with the naphthyl acetate substrate of between 4 and 100-fold depending on the resistance level (Peiris & Hemingway, 1990).

The variation in total esterase activity between the *P.papatasi* colonies measured was not great enough to suggest the presence of the latter type of mechanism and there are no reports of OP resistance in these areas to suggest the former type of mechanism is present.

We were unable to correlate this variation with either an east-west or north-south cline in any one enzyme. One possible explanation for the observed differences in enzyme activity in sandflies from different countries is that they represent differences to exposure in insecticides in the country of origin of the flies or that different esterase isozymes un-associated with resistance are present. Higher levels of GST in *P.papatasi* and *P.keshishiani* from northern Pakistan mainly seems to be the elevation of certain isozymes not associated to a resistance mechanism, since in the areas from where these flies were collected routine insecticidal spraying is seldom carried out. However, the elevated enzymes have to be determined further to clarify the situation.