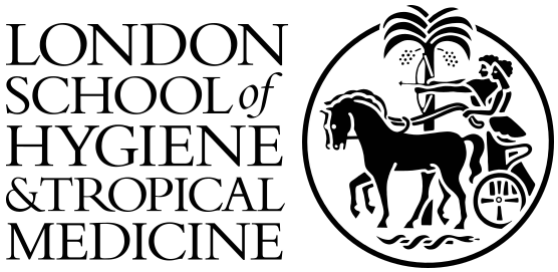

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STUDIES ON ONCHOCERCA GUTTUROSA (NEUMANN, 1910)

IN SUDANESE AND BRITISH CATTLE

by

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ABSTRACT

Onchocerca gutturosa is prevalent in Sudanese cattle, and aspects of its behaviour, transmission, and morphology were studied and where possible comparisons have been made with the same species in U.K. A vector, Culicoides kingi, was discovered and the complete development of O. gutturosa was studied.

The adult worms were found to inhabit the ligamentum nuchae and stifle joint. None were found in the gastro-splenic ligament. All adults and microfilariae found were O. gutturosa (Neumann, 1910). The microfilarial distributions in the skin around the hump region in Sudanese cattle contrasted sharply with those reported in English cattle, presumably an adaptation to the biting habits of C. kingi in the Sudan.

Pathological effects of O. gutturosa in the ligamentum nuchae of cows were minor. Distinct morphological differences were found between microfilariae in the uterus of adult worms and those in the dermis of cows. Those emerging from the uterus of adult females did not develop in the vector. There were also two forms of microfilariae in the uterus of adult females which appeared to represent two stages of maturity.

In the skin of Sudanese cattle microfilariae of O. gutturosa were found in the same region as microfilariae of O. armillata and of O. raillieti in donkeys. Microfilariae of O. gutturosa could be readily distinguished

from the other species by differences in size, number of nuclei anterior to the nerve ring and in arrangement of the caudal and cephalic nuclei.

C. kingi was discovered to be a vector at a dairy farm near Khartoum. Although the midge showed seasonal changes in abundance, there was no clear seasonal variation in number of microfilariae found in the dermis of Sudanese cattle. However, maximum numbers of microfilariae coincided with maximum activity of C. kingi. Biting activity of the midge showed 2 daily peaks. Ninety five per cent of the attacking C. kingi landed on the back of the cow and 25% were successful in obtaining a blood meal. The attractiveness of the hump surface was related to the lack of hair and less host disturbance.

Complete adult O. gutturosa were obtained from Sudanese cattle using a digestion technique. Living male and female worms were transplanted into the peritoneal cavity of different strains of mice; some survived up to 133 days.

The behaviour of O. gutturosa microfilariae in the Nile rat and in mice was studied for a screening model. Chemotherapy was also carried out in naturally infected cattle.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES AND FIGURES

CHAPTER I

INTRODUCTION

The genus Onchocerca Diesing, 1841 comprises long thin worms that live in subcutaneous or deep connective tissues, or in the skin of man and a wide range of ungulates. Most of the adult worms are enmeshed in fibrous connective tissues to the extent that it is very difficult to recover complete specimens, so that virtually all species descriptions have been made on portions of worms only.

In spite of some syndromes such as fistulous withers in cattle and periodic ophthalmia in horses which were believed to be caused by certain animal Onchocerca species (Steward, 1937; Eichler, 1970), animal onchocerciasis is usually considered to be of minor clinical importance. That might be one reason why it has, so far, attracted far less research interest than human onchocerciasis.

Human onchocerciasis (O. volvulus infection), on the other hand, is of serious consequence, resulting in disability and blindness (Nelson, 1970). However, research in this field, specially towards the development of safe chemotherapeutic agents, reliable immunodiagnostic tests and possibly the development of a vaccine, has been much hampered by the lack of a suitable laboratory host for O. volvulus.

Moreover, the need for the identification of infective larvae of animal Onchocerca species carried by the same Simulium species as transmit human disease, so as to determine the vector transmission potential in the onchocerciasis control programme (OPC) area of west Africa, has revived great interest in animal onchocerciasis.

Thirty-four species have so far been described in the genus Onchocerca and nine of these occur in cattle (Muller, 1979). Of cattle species, O. gutturosa is the most widespread both in temperate as well as in tropical zones. Moreover, it has been suggested that this parasite is transmitted by Culicoides species (Bain, , 1979), which are easier to maintain in the laboratory than the Simulium vectors of O. volvulus. Therefore, the cattle/O. gutturosa model seems to be of great potential as a laboratory model for human onchocerciasis.

In the present study, field and laboratory investigations have been undertaken in this model, both in the tropics (Sudan) and in a temperate country (Britain).

Onchocerca gutturosa, Neumann, 1910

(synonyms : O. bovis, Piettre, 1912

O. synceri, Sandground, 1938)

Onchocerca gutturosa was first described by Neumann in 1910 from Algerian and Tunisian cattle. It owes its name to the guttural dilatation of the body near the

anterior end. Its predilection site is the connective tissues surrounding the cervical ligament and it is found to a lesser extent in the tissues surrounding several joints (Johnston , 1921; Sandground, 1938; Ottley and Moorhouse, 1978a)

Another parasite of cattle, O. lienalis, occurs in the gastro-splenic ligament [illegible] though morphologically distinct from O. gutturosa (Bain,etal 1978; Bain,1979; Ottley and Moorhouse, 1978a) it has been synonymised with it by some workers (Steward, 1937; Eichler, 1973a)

O. gutturosa has a world-wide distribution and has been found in most countries where surveys have been conducted (Eichler and Nelson, 1971). In Africa it was reported from the Sudan and Egypt (Mohamed, 1931; Hussein et al., 1975; El Bihari and Hussein, 1978), Ethiopia (Fain et al., 1955), Kenya and Somalia (Clarkson, 1964), Uganda (Bwangomoi, 1970) and Tanzania (Mwaiko, 1979).

Bovine onchocerciasis was first reported in England by Steward (1937), who found worms in the connective tissues of the cervical ligament and the spleen of cattle in Cambridgeshire and Hertfordshire.

Regarding infection in Britain and Sudan, in a survey in an abattoir in the United Kingdom, Eichler and Nelson (1971) reported a 58% rate of infection in cattle and Hussein et al. (1975) found 68.5% of cattle slaughtered at

Omdurman central abattoir to be infected. A recent survey of skin snips in a farm in Khartoum has indicated an even higher rate of infection (El Bihari and Hussein, 1978) in Sudan.

Older animals are far more commonly infected than younger ones, and El Bihari and Hussein (1978) have demonstrated from skin snip evidence that the infection in cattle occurs from five years of age onwards, but the highest rate of infection occurs in animals of seven years of age and older. Younger animals might have contracted the infection even earlier, but the worms they are carrying may not have matured to produce microfilariae.

As with other Onchocerca species, O. gutturosa produces sheathless microfilariae that migrate to the skin of the host waiting to be ingested by an insect. These microfilariae congregate in the hump region of the host in Sudán and in west Africa (Bain et al., 1978; El Bihari and Hussein, 1978; Shastri, 1978), an adaptation which might be related to the favourite biting site of the vector. This feature might also be used to separate O. gutturosa from O. lienalis whose microfilariae concentrate in the skin of the umbilicus (Bain et al., 1978).

Cattle in most countries carry a mixed Onchocerca infection (Hussein et al., 1975; El Bihari and Hussein, 1978; Shastri, 1978; Chauhan & pande, 1978; Ladds, et al 1979; Ottley and Moorhouse, 1978a). In the case of Sudanese

cattle, the microfilariae of O. gutturosa and O. armillata occupy the same location, but both can easily be separated on morphological grounds (El Bihari and Hussein, 1976, 1978). However, this might not be the situation in other areas, making the accurate identification of the micro-filariae a necessity to separate each infection.

Nelson (1970) reviewed the literature on the pathology of onchocerciasis in man and animals and considered O. gutturosa to be an unobtrusive parasite that causes no ill-health in cattle and hence remains clinically obscure. Nevertheless, several skin conditions have been attributed to this parasite (Niimi and Kuono, 1954; Herin, Thienpont and Fain, 1955) with considerable damage to the hide and ulceration of the udder which might result in mastitis (Gnedina, 1959). Moreover, a condition concomitant with Brucella abortus and similar to fistulous withers has also been attributed to O. gutturosa infection in cattle (Le Roux, 1957; Al Zubaidy, 1973). Histologically an inflammatory reaction occurs around the worms with eosinophil infiltration, and in long-standing cases, caseation and calcification (Al Zubaidy, 1973; Hussein et al., 1975; Cheema and Ivoghli, 1978).

A number of cases of human onchocerciasis were reported to be due to animal Onchocerca species (Azarova et al., 1965; Siegenthaler and Gubler, 1965; Beaver et al., 1974; Khan, 1977) and although the exact identity

of the parasite was not determined beyond doubt, all these authors are of the opinion that these infections are most likely due to O. gutturosa.

CHAPTER 2

COMPARATIVE STUDIES OF O. GUTTUROSA INFECTION IN SUDANESE AND BRITISH CATTLE

A. Introduction :

In 1977 studies on animal onchocerciasis were initiated at the Faculty of Veterinary Science in Khartoum University as a result of collaboration between Khartoum University and the Department of Medical Helminthology at the London School of Hygiene and Tropical Medicine.

Onchocerca gutturosa (Neumann, 1910) is a common parasite of cattle which has an almost world-wide distribution, and it was intended to make comparative studies between Sudanese and British O. gutturosa. The species was initially described from the ligamentum nuchae of Algerian cattle by Neumann in 1910; Stiles (1892) had already reported a filarial parasite between the spleen and stomach of cattle as Filaria lienalis. Steward (1937) and Eichler and Nelson (1971) suggested that the worms from the ligamentum nuchae and those from the gastro-splenic ligament were taxonomically the same.

Adult worms of O. gutturosa are widely distributed in the animal body and it is very hard to define clearly the sites of adult worms in infected carcasses. Ottley and Moorhouse (1978a) reported O. gutturosa from the stifle

joint, scapular cartilage, hip, shoulder and elbow joints.

The prevalence of infection of the parasite varies from one place to another. Eichler and Nelson (1971) reported that 58% of cattle were found to be infected in the U.K.; Hussein et al. (1975) studied the infection in Sudanese cattle slaughtered in Omdurman central abattoir and found adult worms in 68.5% of cattle with worms located exclusively in the connective tissue of the ligamentum nuchae; infection rates of bovine onchocerciasis recorded by Ottley and Moorhouse (1978a) were up to 100% in cattle in Australia; Mwaiko (1979) studied the prevalence of O. gutturosa in eastern Tanzania and found 33% of animals were infected as determined by finding adult worms in the cervical ligament.

B. Material and methods :

To ensure a regular supply of parasites from naturally infected cattle surveys of infection on live cattle were performed at two Government farms in Khartoum area (Shambat and Kuku farms) besides a few animals from the Kosti area.

A regular supply of autopsy material was found at Omdurman slaughter house (5 miles from the laboratory), mainly of indigenous Zebu cattle. In this abattoir animals coming for slaughter originate from all districts of the Sudan and are mainly bulls with very few cows. In contrast, at Reading abattoir in the U.K. most animals were old cows.

About 280 animals are slaughtered daily in Omdurman abattoir for human consumption and so it was possible to take abundant autopsy material daily. Removal of the ligament from an animal does not involve an economic loss, and it was possible to examine gastro-splenic ligaments of the same animals, but it was not possible to examine the whole carcasses owing to the speed of slaughter-house procedure. Cattle slaughtered in Omdurman abattoir come from the whole of the Sudan and are Zebu and local breeds with high humps, especially in the male (Plate 1).

In Omdurman abattoir the animals are killed from 7.00 p.m. until 5.00 a.m. No skin snips could be taken from live animals in the slaughter-house.

The following examinations were carried out:

Skin snips from ears, umbilicus, udder, flank, back, chest, legs and hump regions were collected from animals of different ages. Care was taken that the snips from each region were as nearly as possible of the same size. In order to ensure that they were of about the same depth and surface area, a snipper was used to standardise size [illegible], this snipper punching out a round piece of skin of 10 mm diameter (Figure 5).

Gastro-splenic connective tissues wrer systematically examined when the viscera was removed in order to find O. lienalis while the spleen was still attached to the

Plate 1

Zebu cattle - this is a local breed in the Sudan with high hump and long horns

rumen (Plate 2). The ligamentum nuchae could often be removed from the carcass within ½ hour of the animal's slaughter. After they had been flayed and split into two halves, the ligament was dissected out as far back as the 8th or 9th thoracic vertebrae. Care was taken to keep together the two halves of the ligament, one half from each side of the cow.

The stifle joint was also examined, but it was impracticable to examine all joints from each animal as this would lead to mutiliation of the carcass.

It normally took 30 minutes to get the material to the laboratory and in practice it was usually 10 hours before the worms were extracted from the ligaments. However, adult worms of O. gutturosa are easily seen in the connective tissue covering the inner surface of the two halves of ligamentum nuchae (Plate 3) - unlike O. rail-lieti which penetrate between the bundles of yellow elastic fibres and burrow deeply into the funicular region of the ligament, and thus are extremely difficult to see (Saad el Din, personal communication).

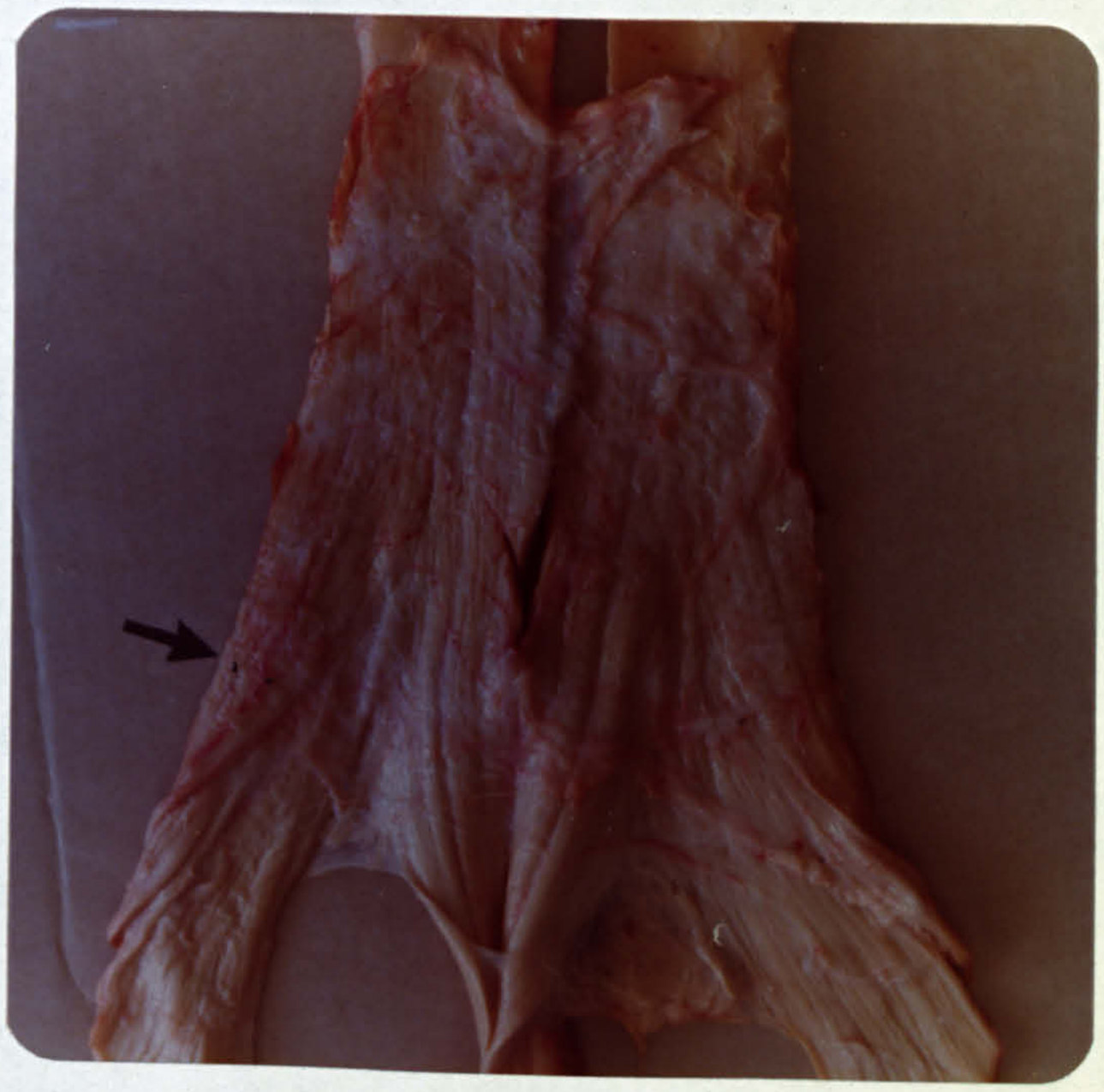
Each ligament and skin snips of the same animal were kept at $4^{o}C$ in plastic bags with a distinct number.

On the second day when adult worms were extracted, they were still active, especially the anterior part of the adult female; also the viability of uterine larvae, as judged by their activity on being released from the

Plate 2

Examination of the gastro-splenic ligament for O. lienalis

Plate 3



O. gutturosa on the covering connective tissue of the ligamentum nuchae can be easily seen (arrows)

broken uterus, was unimpaired.

In addition to the material from Omdurman abattoir, a few animals were examined in more detail in the autopsy room at the Faculty of Veterinary Science. Animals are killed every week there for other scientific purposes and this allows easy collection and inspection of autopsy material from individual animals.

The cattle examined were usually of different age groups randomly selected, ranging from two to ten years old. These animals were aged according to the state of their cropping teeth (Figure 4).

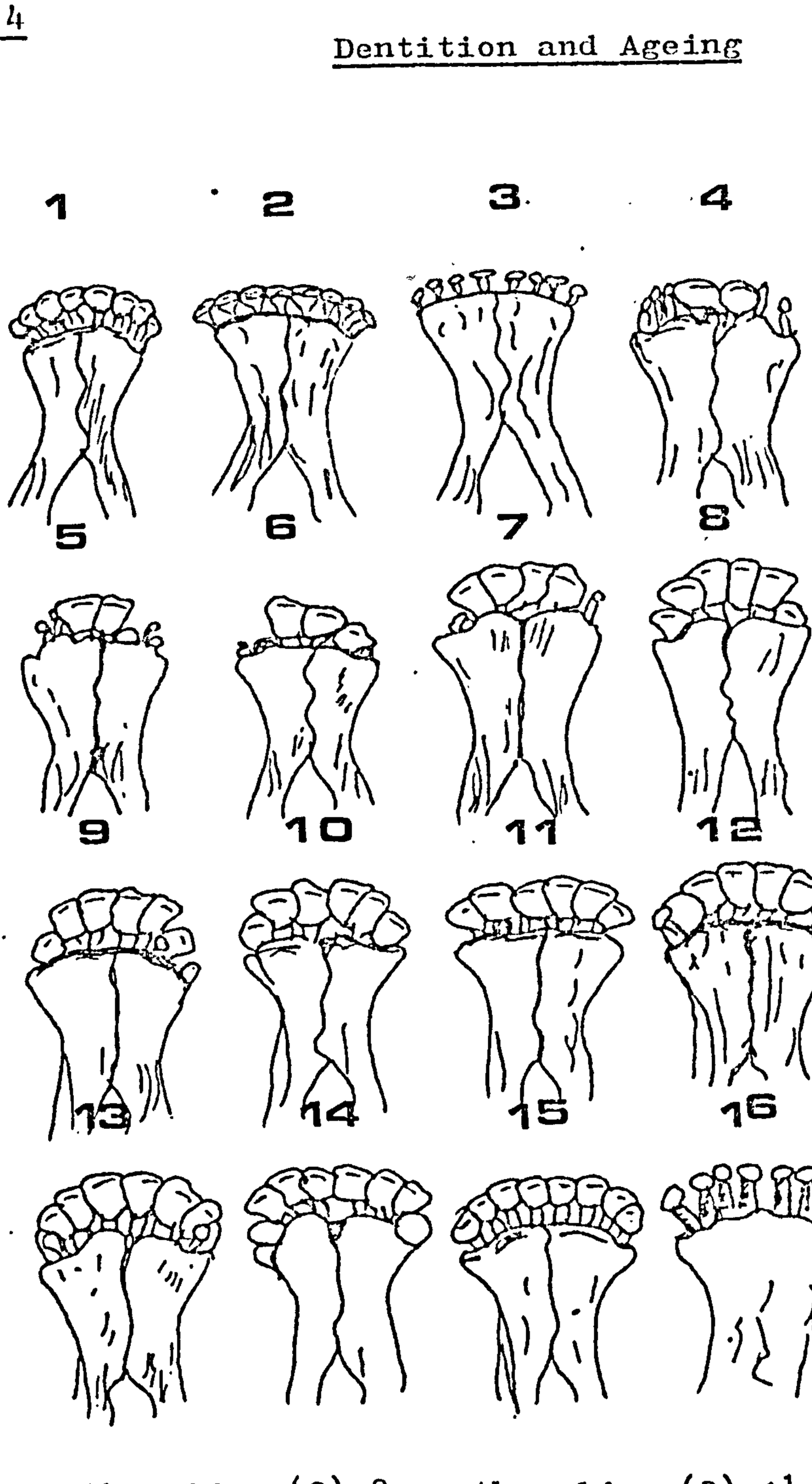
C. Collection of skin snips from live animals:

To study the relative prevalence of infection among different age groups, 541 animals were examined at different ages and microfilariae recovered from the skin of infected animals. Permission was given for a piece of skin from the centre of the hump to be removed from cattle in two Government farms. The aim was to find animals with known history and age, exposed to different degrees of transmission, ranging from nil to intense in the Khartoum area.

One farm was selected for examination at Shambat (the farm of the Faculty of Agriculture and Veterinary Science); half a mile from the College. The second farm was in the Kuku area (a farm of artificial insemination and fattening centre) 6 miles from the College.

Figure 4

Dentition and Ageing



(1) 4 months old (2) 8 months old (3) 1½ years old (4) 2 years old (5-6-7) stages in eruption of second pair of permanent incisors 3 years old (8-9-10) stages of eruption of third pair of permanent incisors 3½ years old (11) 4 years old (12) third stage well up in wear 4½ years (13-14) stages of eruption of fourth pair of permanent incisors (15) full mouth permanent teeth 5 years old (16) teeth of 9 years old cow.

All animals in Kuku and Shambat farms were born and raised on the farm with a history of never having left Khartoum. In addition 25 animals were examined which had been brought from Kosti for a schistosomiasis project.

The time of snipping of the vast majority was between 09.00 and 12.00 hours. The samples were taken from the hump only.

Following a study done by El Bihari and Hussein (1978) and repeated by the author, the microfilariae of O. gutturosa in Sudanese cattle were found to be concentrated mainly in the hump region.

Before a skin snip was taken the animal was held tightly in a standing or lying down position. The samples were taken from the centre of the hump using a snipper (Plate 5). A few drops of blood were present in the skin snip place. A piece of cottonwool soaked in antiseptic solution was placed in the lesion from which the skin snip had been removed. Each skin snip was kept in a clean screw-topped bottle labelled with the age and sex of the animal. A brief history was taken on the day of examination as well as the date of birth. If this was not available, an estimate was made by dentition (Figure 4).

All skin snips were examined on the same day and without any delay.

Plate 5

Skin snip from the hump of a live animal
using snipper technique

D. Treatment of infected material:

1. Cervical ligament

All autopsy material used to investigate the distribution of microfilariae in relation to adult worms was collected from Omdurman central abattoir which is the biggest abattoir in the Sudan situated in the west of Omdurman. The animals slaughtered in Omdurman are drawn from the whole Sudan.

Five hundred animals were examined, of which 410 had O. gutturosa in the cervical ligament (82%). From these, 25 animals were examined for microfilarial distribution in relation to adult worms. First a careful examination of the ligamentum nuchae was made under normal light to detect any obvious lesions and the intensity of infection of the worms on the surface of the ligament and the number and size of nodules (Plate 6). Then an examination was made under a dissecting microscope to help show if these had any worms penetrating into the bundles of elastic fibres of the cervical ligament.

The adult worms in the ligament were found in the connective tissue between the flat surfaces. The adult female worms could then be seen as white thread-like worms winding about in the connective tissue coiled together. Sometimes they were in a small area and sometimes spread along the whole connective tissue covering the ligament. But in most cases the worms were found just at

Plate 6

Infected ligament showing different size of nodules which scatter along the flat surface of the ligament at the point where the ligament is widest

the point where the ligament was widest (opposite the second to the fourth cervical vertebrae).

Male worms were very difficult to see on account of their extreme thinness, but they could be easily extracted whole by pulling them out of the connective tissue of the ligamentum nuchae. The best method for extraction of the worms was to remove the connective tissue from the cervical ligament. This was then placed in a petri dish containing Tyrode's solution and examined under a dissecting microscope.

The worms could be extracted by using fine forceps. The connective tissue was pierced above the worms, then they were drawn out carefully. A few males were extracted intact and very mobile, but complete females were never obtained by this method.

Intact adult females free from the host tissue were isolated by the use of enzymatic digestion; two types of enzymes were used, Collagenase and pepsin, by the same method used by Schulz - Key et al. (1977).

2. Method used for isolating complete adult females:

The connective tissues were removed from the infected ligament and immediately put in a fresh solution of Tyrode's solution in a petri dish. The loose tissue of the outer parts of the connective tissue containing adult worms were carefully removed with scissors under a dissecting

microscope - this must be done very carefully because the anterior parts of the worms are very fine and therefore easily cut. The connective tissue containing the worms was then transferred into fresh Tyrode's medium to which collagenase was added up to a final concentration of 1.5 mg/ml. The amount of digestion solution depends on the size of connective tissue which has to be covered with the fluid. Then the fluid containing the tissue was incubated in a warm bath at 37°C with constant gentle shaking. After 6 - 8 hours the tissues were examined under a dissecting microscope and loose host tissue was removed carefully using fine forceps. Loose worms were isolated with fine forceps and live ones transferred to fresh Tyrode's medium containing bovine serum in a petri dish. The dead worms were preserved in 70% glycerine alcohol.

The same technique was used in extracting adult worms on a large scale using pepsin digestion method in a concentration of 1.5 gm/100 ml distilled water + 0.01 ml concentrated HCl. All worms extracted by this method were dead and all internal organs were digested except in very rare cases.

The collagenase technique was thus very much better but is rather expensive to carry out because of the high cost of the reagent.

3. Treatment of infected skin snips

Microfilariae were detected by placing each snip in a separate container in Tyrode's solution in 20% serum (Nelson et al., 1966) and cutting it into small pieces (Eichler and Nelson, 1971); the teased skins were kept at room temperature of 27°C for 4 hours and then examined for microfilariae under a medium power (x 40) objective of the binocular microscope.

The technique for large scale extraction was modified from a method used by Nelson et al. (1966). The pieces of skin were shaved and separated from any subcutaneous tissue before being weighed. They were then teased into small pieces using scissors and then transferred to Tyrode's solution + 20% sheep serum to cover the whole snip in a small clean bottle with a screw-topped cover.

All bottles containing teased skin snips were left overnight at room temperature (approximately 27°C) to allow the microfilariae to migrate out of the tissue. On the following day the tissues were removed, then the suspension was filtered for the purpose of removing tissue fragments and the suspension of microfilariae was all used for counting the total microfilariae present, using a counting chamber under the medium power (x 40) microscope.

In a preliminary experiment it was found that 80%

of microfilariae of O. gutturosa in the skin of infected animals emerged from the skin after 4 hours. Accurate estimation of the number of microfilariae per gram of skin could therefore only be made after an emergence time of at least 24 hours.

Richard (1973) found some microfilariae of O. cervicalis remained in the skin of positive samples after soaking for 30 hours.

Uterine microfilariae were obtained directly from adult worms taken from the ligamentum nuchae of infected animals.

After the extraction of the adult worms with fine forceps, each piece of adult worm was carefully examined under a dissecting microscope and those sections which were seen to contain microfilariae were put into a bottle containing Tyrode's solution + 20% sheep serum and chopped into small pieces with fine forceps. After 30 minutes when most microfilariae had emerged from the cut ends of the uterus they were separated from the fragments of adult worms by sieving through very fine mesh sieves which permitted only uterine microfilariae to pass through.

E. Results

In the present study during the period November 1977 to August 1978, the prevalence of O. gutturosa infection in British cattle was made based on recoveries of adult worms from both halves of cervical ligaments of 239 cows examined from the slaughter house at Reading; 168 (70.3%) contained adult worms.

During the period September 1978 to August 1979 nuchal ligaments were collected from Omdurman (Sudan) abattoir. O. gutturosa was found to be very common in Sudanese animals, particularly in old cattle. This was confirmed by autopsy material collected from the slaughter house, Five hundred animals were examined and 410 (82%) contained worms in the cervical ligament. This was probably a true infection as it was based on the examination of both halves from each side of the same carcass. Twelve out of 20 animals examined were infected with adult worms in both ligamentum nuchae and stifle joint in Sudanese cattle (60%).

A total of 100 positive cervical ligaments from cows were examined carefully, 80 in Omdurman abattoir and 20 animals in the Veterinary College autopsy room, for adult worms in the gastro-splenic ligament. None of these animals revealed adult worms in the gastro-splenic omentum.

The prevalence of infection was based on the presence

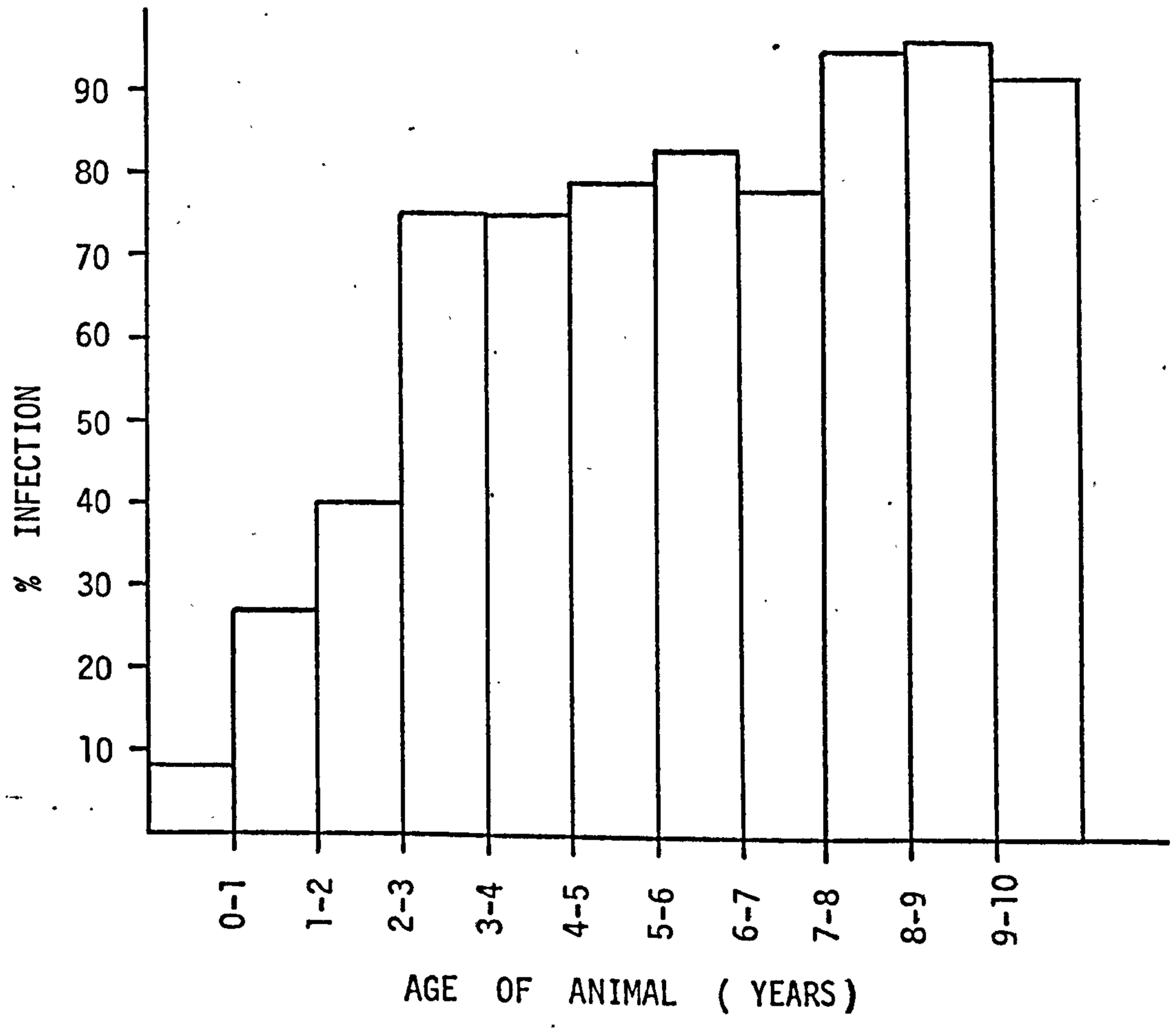
Table 1.

PREVALENCE OF O. GUTTUROSA IN 541 ANIMALS ACCORDING TO AGE

Age of animal (years)	No. of animals examined	No. of animals infected	Percentage infected
<1	22	2	9
1	40	11	27
2	50	20	40
3	40	30	75
4	80	60	75
5	59	44	79
6	60	50	83
7	80	63	78
8	40	38	95
9	30	29	96
10	40	37	92
Total	541	384	70

Figure 7.

Frequency distribution of infection rate among different age groups.
Based on recoveries of microfilariae from the skin (from table No.(1))



or absence of the microfilariae of O. gutturosa from infected hides. This survey was carried out at Omdurman slaughter house, Kuku farm, Shambat agriculture farm and a few animals from the Kosti area.

O. gutturosa microfilariae were recovered from 70% of these animals and as can be seen from Table 1, the probability of infection increased with age (Figure 7). A high prevalence rate of O. gutturosa infection was found among animals in the 8-9 year-old age group.

Since all animals in the Kuku and Shambat farms were born and reared in the area of high prevalence, they must have acquired the infection locally and a suitable vector is therefore in the area.

It is quite clear that infection increases with age and the youngest positive animal was nine months old - this calf was born at Shambat farm. The results are in agreement with earlier reports (El Bihari and Hussein, 1978; Hussein et al. 1975; Eichler and Nelson, 1971).

The lower prevalence in younger animals is possibly related to less exposure to intermediate hosts and consequently a decreased chance of infection. In addition several of the young animals may have been recently infected, resulting in skin concentrations of microfilariae being low enough to allow the infection to go unrecognized.

Where animals with a high number of adult worms in the cervical ligament still gave negative results for microfilariae,

more skin snips were taken from these animals. Buckley (1938) proved that microfilariae are irregularly distributed in the skin and have a tendency to clump together in certain regions of the skin, whilst in other spots not far distant there would be few or no microfilariae at all. This effect was confirmed by Mellor (1973) and by Anthony&Cello (1975). Because of this effect about the variation in the concentration of microfilariae in a single cow, multiple samples must be taken before final judgements are given. However this was not attempted in the present survey.

I. ADULT WORMS

(a) Location of English and Sudanese adult worms

1. Cervical ligament

The ligamentum nuchae (= nuchal ligament = cervical ligament ((Figure 8) consists mainly of two parts, the lamellar part and the funicular part. Both parts are composed almost entirely of bundles of yellow elastic fibres and extend medially from the occipital bone to the withers. It is a powerful elastic apparatus which assists the extensor muscles of the head and neck. The lamellar part is divided into two lateral halves of rounded cross-section at the occipital attachment. From the second cervical vertebrae the funicular part becomes wider and flatter posteriorally, and its widest part lies on either side of the neural spine of the thoracic vertebrae and gradually narrows and fades out in the lumbar region. The lamellar part consists of bundles of fibres passing from the funicular part to the neural spine of the cervical vertebrae.

Onchocerca gutturosa (Neumann, 1910) is most frequently found in the connective tissue surrounding the cervical ligament, when it is widest opposite the second to the fourth cervical vertebrae. The location of the adult worms within the body of the host has been an important criterion for specific differentiation particularly of those species found in cattle (Clarkson, 1964; Hussein et

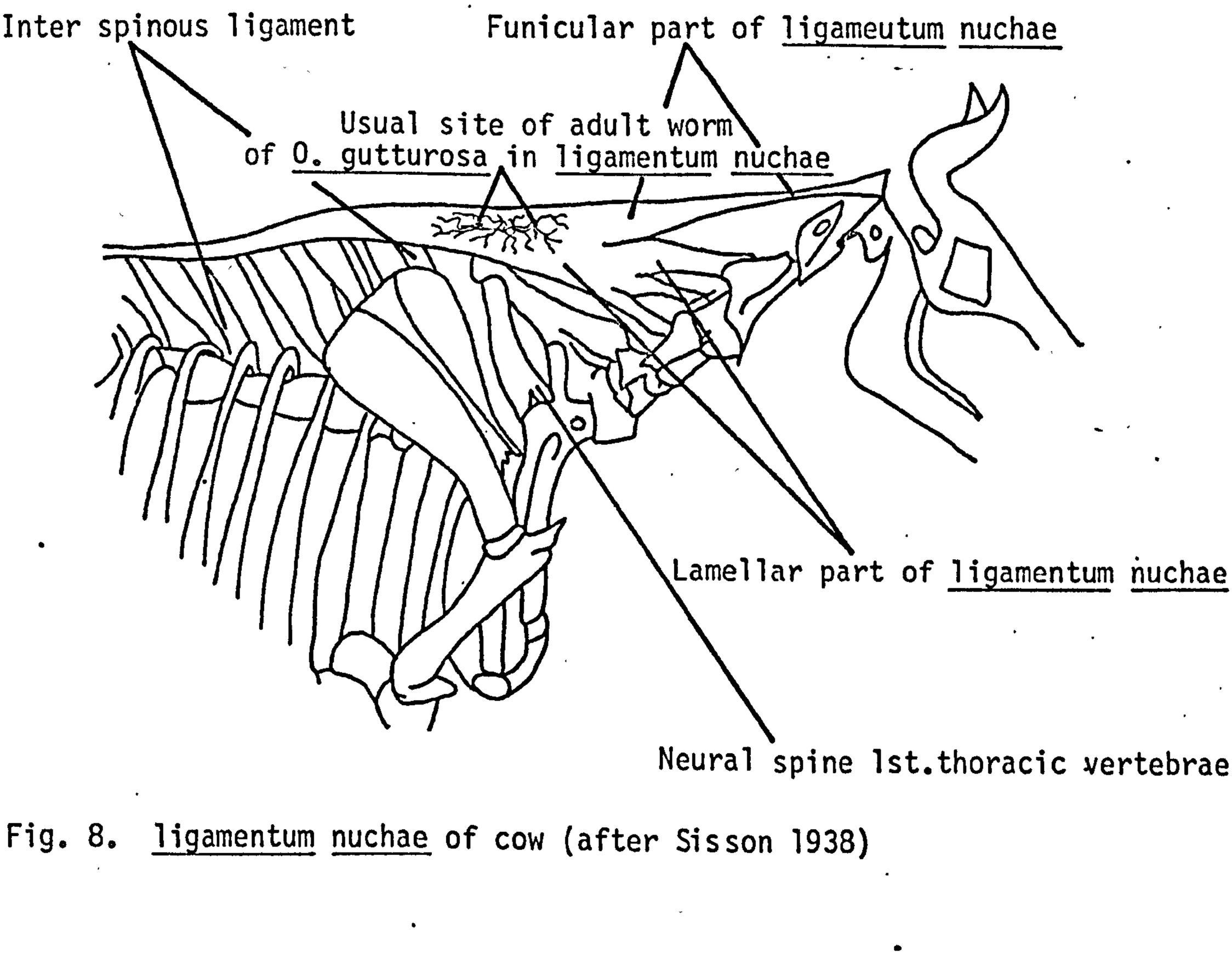


Fig. 8. ligamentum nuchae of cow (after Sisson 1938)

al., 1975; Bain et al., 1978; Muller, 1979). Some authors, however, reported O. gutturosa from different sites of the body (Ransom, 1920; Johnston, 1921; Steward, 1937; Eichler, 1970; Ottley and Moorhouse, 1978a).

The position of the adult worm in the neck of infected animals was usually at the level of the sixth cervical to fifth thoracic vertebrae, and the majority of adult worms were found in the inter-connective tissue on the internal face of the two lateral halves. Occasionally, with very heavy infections, parts of the worms slightly overlap the external face of the ligament. No penetration of the elastic tissue bundles of the ligament has been reported except by Bwangamoi (1970).

Autopsy material collected from cattle slaughtered at Omdurman central abattoir showed the characteristic position of adult worms of both sexes generally found when the ligament is still on the carcass. The worms were located mainly at the level of the anterior thoracic spine 2 - 5 cm posterior to the junction where the funicular part meets the lamellar part of the ligament. It is very difficult to locate O. gutturosa in the ligamentum nuchae, and unless one makes a careful search or knows the precise points where they frequently occur, one could miss them even if they were in a large mass. The worms are often hidden by the tall spines of the thoracic vertebrae and it is necessary to dissect out the ligamentum nuchae before the worms can be seen.

2. Gastro-splenic ligament

Bwangamoi (1970) found 82% of cattle were infected with O. gutturosa in the ligamentum nuchae, but found no cases of Onchocerca in the gastro-splenic omentum.

Hussein et al. (1975) examined 248 animals and in the 170 (68.5%) positive ones the worms were located exclusively in the connective tissue of the nuchal ligament.

In the present study, the gastro-splenic ligaments of 100 animals were examined during the period October 1978 to September 1979. At first the ligament was examined carefully while it was intact in the animal by stretching the ligament between the stomach and spleen towards the light (Plate 2) and looking at every angle for the worms under the connective tissue. Then the connective tissue capsule of the ligament was dissected free from the rumen and spleen and examined using a dissecting microscope for the presence of adult worms. Although 75 of these animals were positive for O. gutturosa by finding adult worms in the ligamentum nuchae, no worms were found in the gastro-splenic omentum; although the typical anatomical location for O. gutturosa is the sheath of the ligamentum nuchae

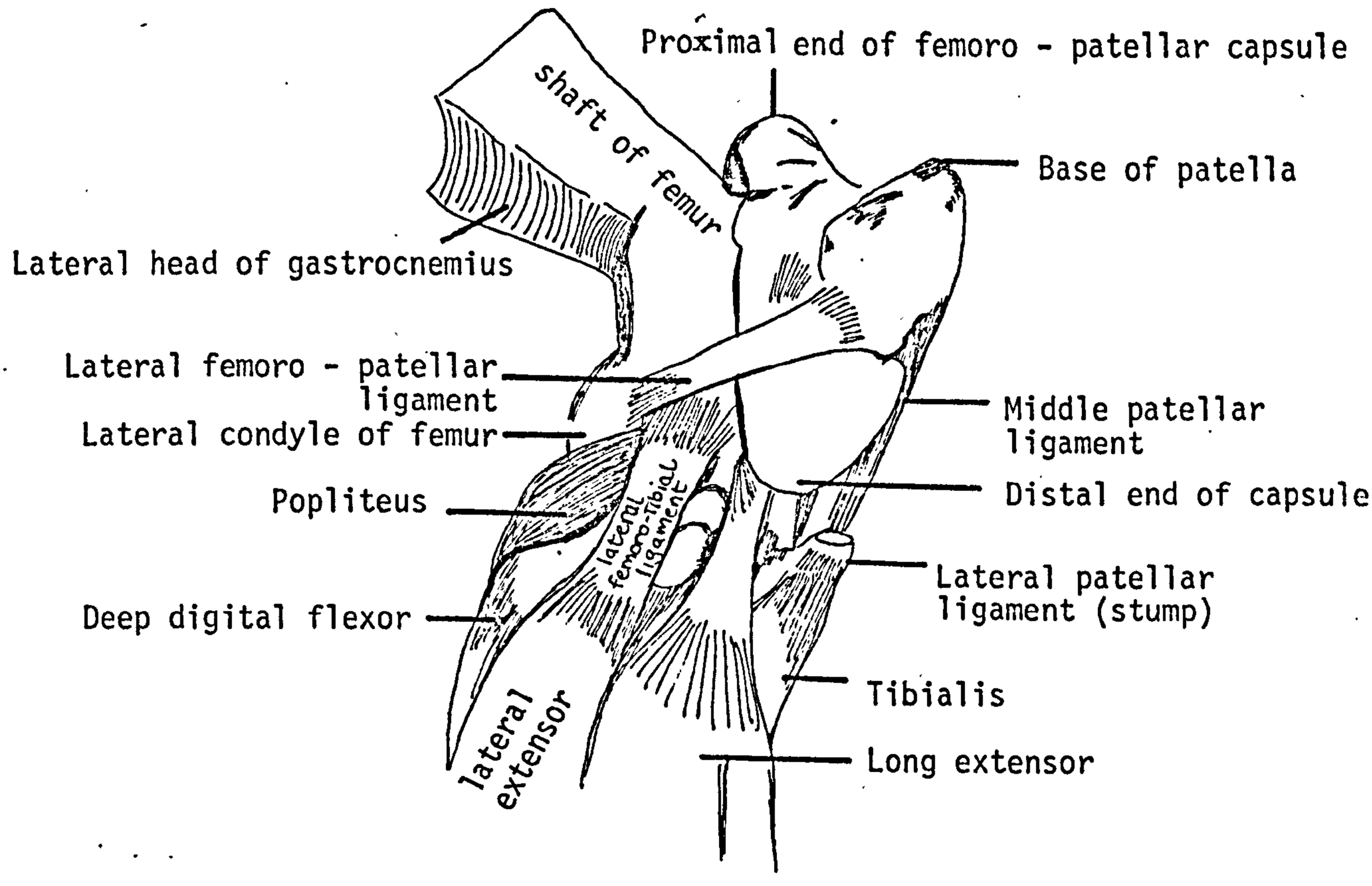


Fig. 9 Right stifle joint ; lateral view

and that of O. lienalis is the gastro-splenic ligament.

3. Adult worms in the stifle joint

This joint, which corresponds to the knee joint in man, is the largest and most elaborate of all articulations; in reality it consists of two joints. The femoro-tibial joint and femoro-patellar joint are surrounded by large groups of ligament as shown in Plate 9.

Some authors reported the presence of an Onchocerca species in the stifle joint without identification of the species (Ransom, 1920; Johnston, 1921). Other studies have examined specimens from the stifle joint and concluded that morphologically they were similar to the parasite from the cervical ligament (Webster et al 1977; Ottley and Moorhouse, 1978a). The present study tends to support this viewpoint because all the morphology agreed with the description of O. gutturosa given by Johnston (1921).

O. gutturosa from the cervical ligament of Sudanese cattle was first studied by Hussein et al. (1975) and El Bihari and Hussein (1978).

O. gutturosa was previously unknown in the stifle joint of animals in the Sudan, but was found in 12 of 20 animals (60%) in the present study and the parasite was widely distributed around the joint and was collected from the loose

connective tissue overlying dense elastic tissue of the ligament. The best way is to separate the joint to locate the adult worms in the periosteum of the tibial crest, the medial aspect of the tendon, the lateral patellar ligament and the popliteus muscles.

The identity of adults in the stifle joint in specimens from the knee joint of Sudanese cattle was diagnosed as O. gutturosa by comparison with specimens from the ligamentum nuchae. We concluded that only one species was involved. It may be that more detailed studies will reveal some adults of Sudanese O. gutturosa in other situations.

4. Other sites

It is difficult to make a true assessment of the number of sites of adult O. gutturosa in Sudanese cattle owing to the limited situations which were examined during this study. But O. gutturosa has been recovered from the trochanter major, and scapular cartilage in British cattle (Bianco, personal communication).

(b) Morphology of adult worms from Sudan

The adult female of O. gutturosa is tightly coiled, especially in the posterior part of the worm (Plate 10) and haphazardly intertwined, and encased in dense connective tissue preventing its recovery intact for study; thus many descriptions have been based on portions of the worms. In

addition the worms may have degenerated or even be calcified, thus having lost all specific features.

Nevertheless, detailed knowledge of the morphology of the worms parasitic in cattle usually enables a pathologist to make specific diagnosis.

O. gutturosa is by far the most common filaria in cattle on a world-wide basis and can be distinguished from other species found in cattle tissue (Muller, 1979) by the specific character of striation in the cuticle (Johnston, 1921).

The most complete specimens available for study were 20 adult female and 15 male of O. gutturosa extracted by the digestive method from cervical ligaments and stifle joints, besides a few fragment of females teased from the tissue, which were all fixed in glycerine alcohol. In all specimens the characteristic guttural dilation of the body at the nerve ring was evident (Plate 11).

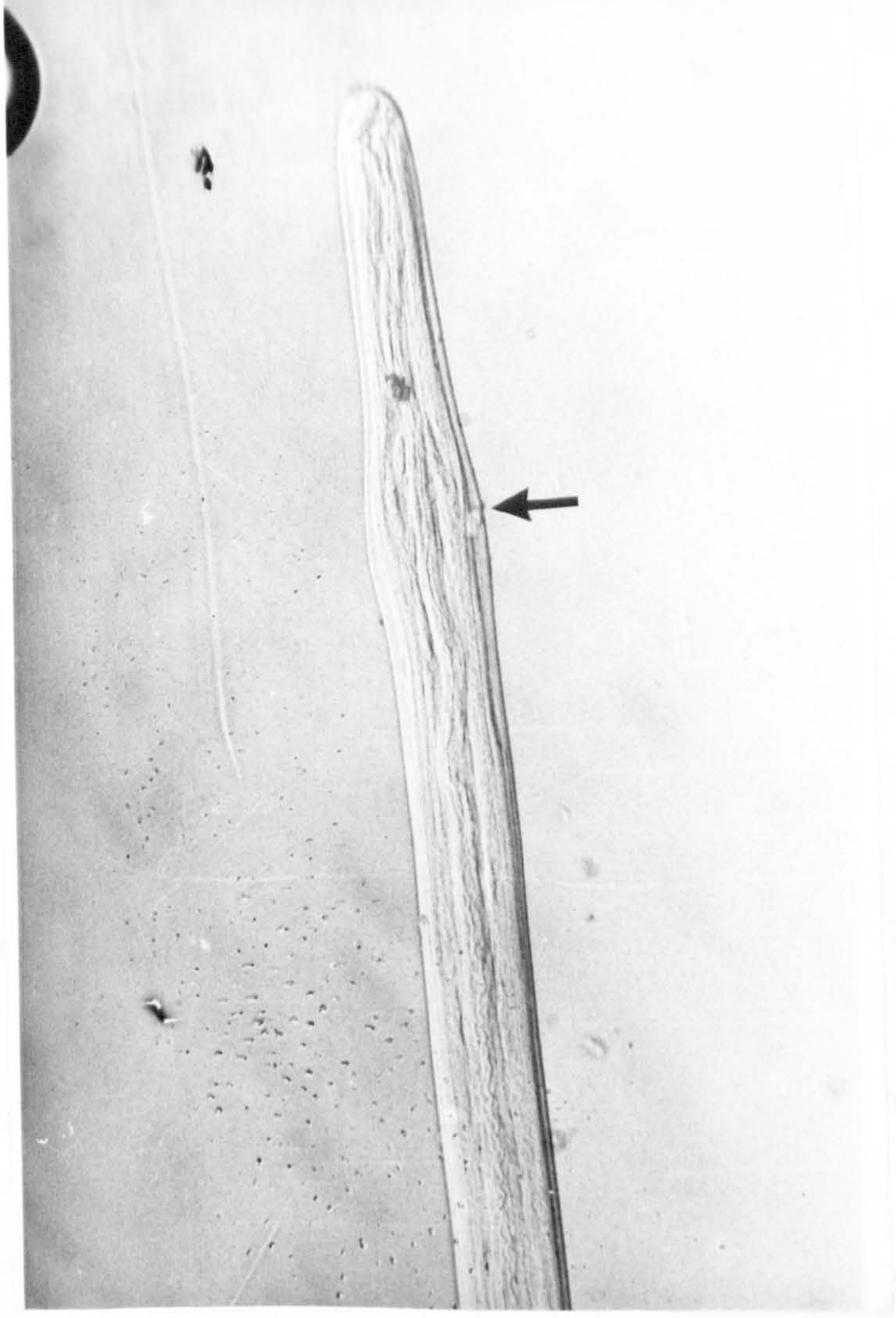
In most respects the worms examined from the stifle joints resembled those from the nuchal ligament; both were collected from the loose connective tissue overlapping dense elastic tissue.

The morphology of all worms examined from stifle joints and nuchal ligaments agreed with the description of O. gutturosa given by Johnston (1921), Steward (1937), Eichler (1973a), Beaver et al. (1974), Chauhan and Pande (1978), and Muller (1979).

Plate 10

The posterior region of the adult female of O. gutturosa.
It is highly coiled and very difficult to release from the connective tissue of the ligamentum nuchae.

Plate 11



Anterior guttural dilation (arrow) is evident in all specimens.

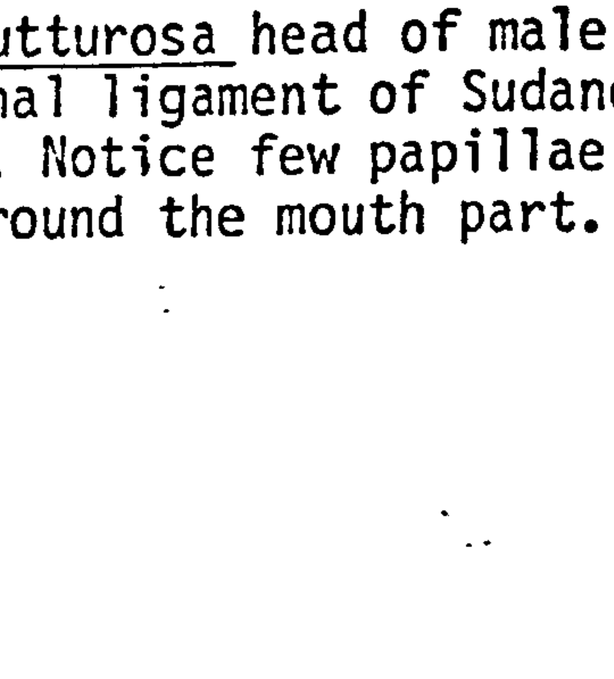
1. Description of male:

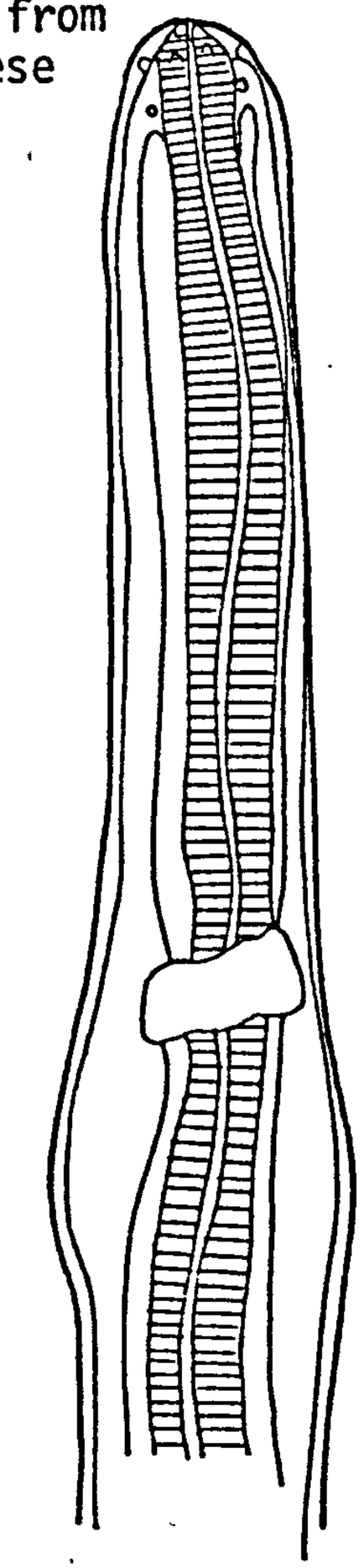
The males lie loosely coiled or tangled in spaces adjacent to the females or at a little distance, and can be easily removed intact. The male worm is smaller than the female, it is filiform in shape, the anterior end tapering with lack of striation. The mouth part is simple and terminal surrounded by minute papillae (Plate 12). The middle is thread-like and of uniform width, the posterior end curved ventrally and ending bluntly. The cuticle is thinner and less prominent, its thickness reaching the maximum in the middle (2.5 - 4 μm). The striae never become as prominent as they do in the female. The widest part occurs at the nerve ring and is expanded, behind which it narrows slightly. The digestive tube comprises a mouth, oesophagus, intestine, cloaca and anus. The mouth is simple and terminal, a few papillae appear to be present at a little distance behind the mouth; their number is difficult to count. The oesophagus is long and slender and in most cases ends in a bulb posteriorly. The nerve ring surrounds the anterior portion of the oesophagus, between the oesophagus and mouth.

The male worm has two copulatory spicules, unequal in length, the left one longer than the right (Plate 12) and both are slightly arched. The proximal end of the left spicule is just anterior to the beginning of the cloaca with fluted proximal and pointed distal extremities,

Fig. 12

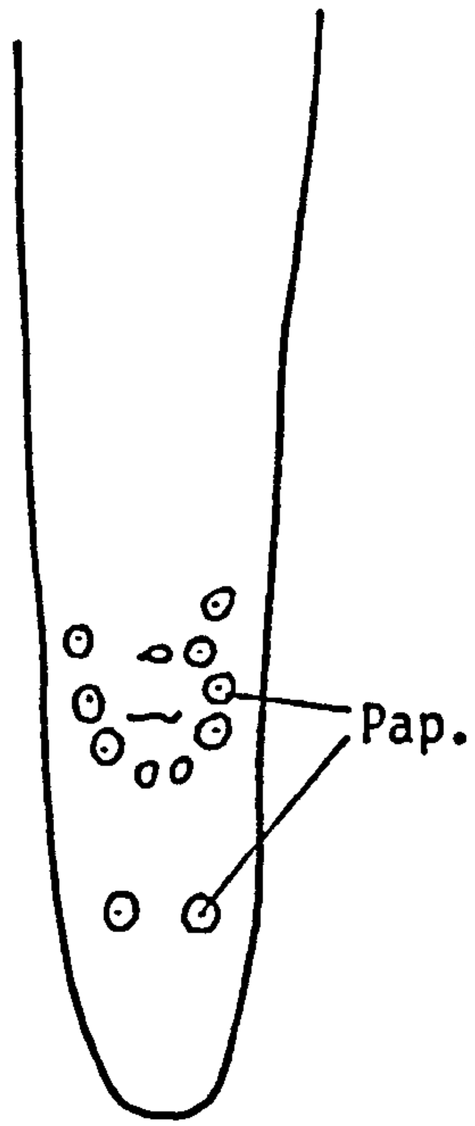
O.gutturosa head of male from nuchal ligament of Sudanese cow. Notice few papillae surround the mouth part.

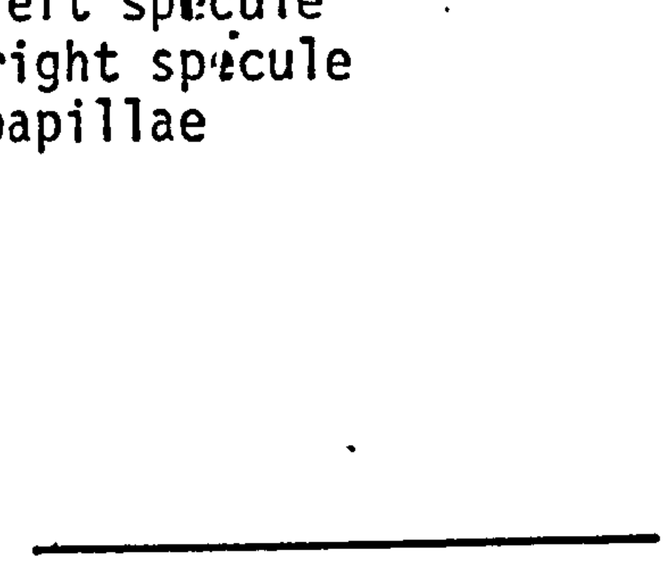




Posterior part of the male : Note unequal spécule

1. L.S. - left spécule
2. R.S. - right spécule
3. Pap. - papillae





while the proximal end of the right spicule is slightly posterior to the beginning of the cloaca, slightly broader and truncated proximally, terminating in a foot-like tip (Plate 12). There are 2-3 pairs of pre-anal, 2-4 pairs of adanal and 2-3 pairs of post-anal caudal papillae. These caudal papillae are arranged in a very haphazard fashion in most specimens. The number varies considerably, however, and there may be four on one side and three on the other. The anus was located venterally 81-95$^{\mu m}$ (88) from the posterior end.

2. Description of the female worm:

Adult female worms were obtained from the cervical ligament as well as from the stifle joint. Twenty complete females and other specimens which were more than 15 cm in length, but lacking either the head or the tail were examined. The average length of a complete female was 45:7 cm and the diameter of the body at the middle was up to 135^{um}.

The normal type of striae commence to appear in the anterior region of the worm (at about the level of the vulva) and become more prominent further back and disappear 95^{um} before the end of the worm (Plate 13).

The variation in the appearance of the cuticle in the different parts of the body is obviously a potential source of confusion for fragment study. The tail of the female ends bluntly and possesses a tiny rounded projection

at its termination and lack of striation (Figure 13).

Johnston (1921) detected a pair of very minute papillae in his specimens.

The body, except in the anterior and last part of the posterior regions, is marked by well-developed cuticular ridges which are more prominent along the middle part of the worm (Figure 14). The distance between the ridges is greatest in the middle of the worm, where they are generally from 4 - 6 microns in height, and less at both ends. In the middle, which has an almost uniform diameter, the ridges are a regular distance apart from each other, i.e. 95$^{\mu m}$. The underlying cuticle measures on an average 24$^{\mu m}$ thick along most of the body. In general there are 3 striae of the inner layer between each two adjacent ridges and one under each ridge, the posterior extremity being nearly free from striation.

The digestive system is the same as that of the male. The mouth is terminal and is followed by a long tubular oesophagus about 1.52 mm long. The anus lies about 2.5 mm from the termination of the worm.

The reproductive system is a tube-like system running almost the entire length of the worm and consists of paired ovaries, oviducts, seminal receptables and uteri, the two uteri uniting to form the vagina which terminates at the vulva.

Each uterus of a gravid female is divided into three

regions, based upon maturation of the embryos. There is a short proximal region, an extremely long middle zone, and a short distal region. In any one region of the uterus, a single stage of embryo predominates, but other stages are usually present.

The short proximal region contains numerous zygotes and many embryos in an early stage of development, with a thin membranous sheath. The embryos in the middle zone of the uterus have differentiated into elongated microfilariae, these microfilariae are coiled and remain enclosed within the thin membranous sheath. The shorter anterior region contains mature microfilariae in which the membranous sheath was not identified. The two uteri join near the anterior end of the worm to form the vagina, the lumen contains mature microfilariae. The termination of the vagina is the vulva which is located from 0.4 to 0.51 mm from the anterior end.

3. Comparison between O. gutturosa from Sudan and Britain

The first paper in which, to my knowledge, an attempt was made to demonstrate specific differences in the cuticlar marking of the female is that of Johnston (1921), who represents diagrams for differentiation of different species of bovine onchocerciasis. The sketches of this author show distinct differences in the striation relationship at

Figure 13.

Posterior end of adult female of O.gutturosa
Note the lake of striation in the last 95um of the worm

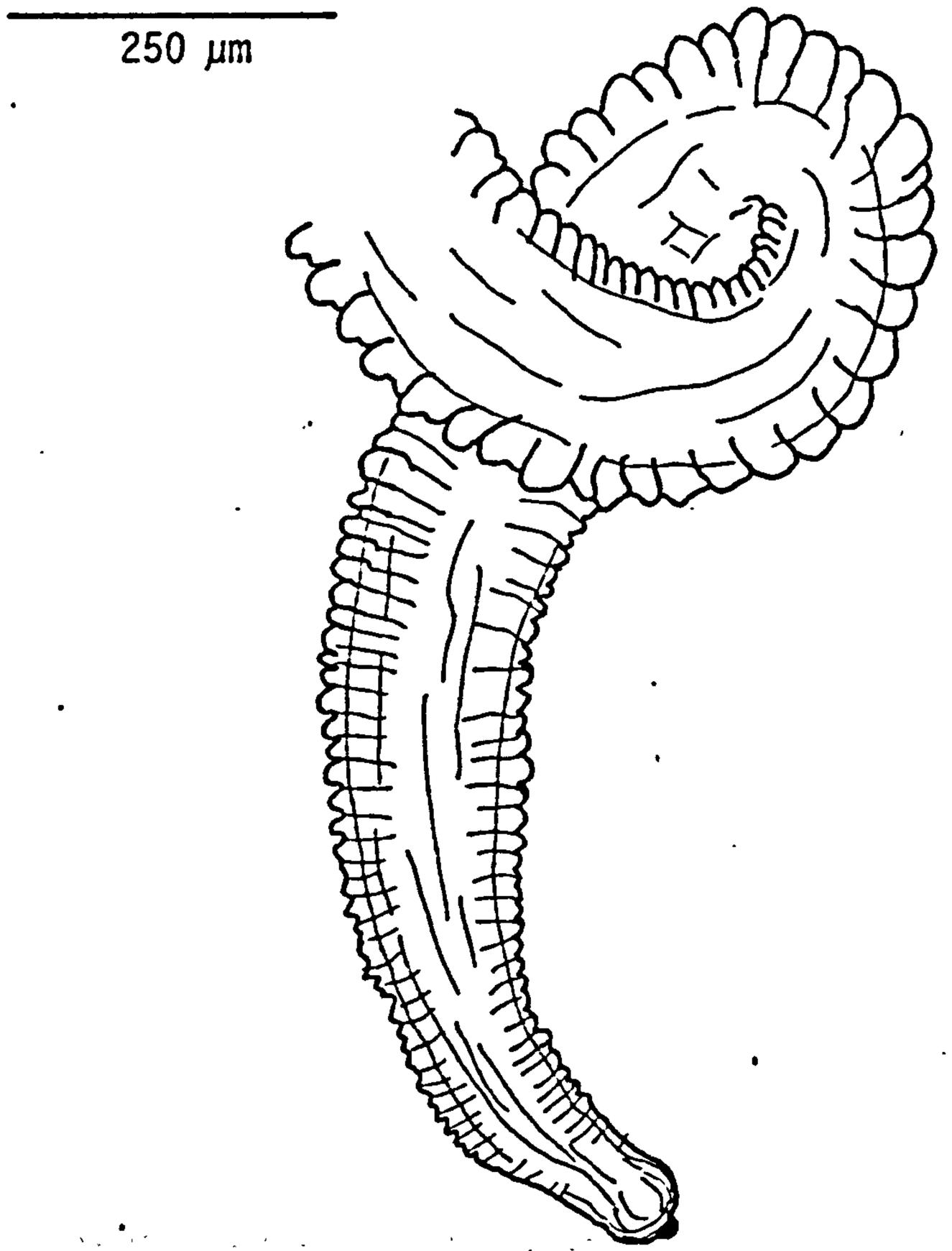
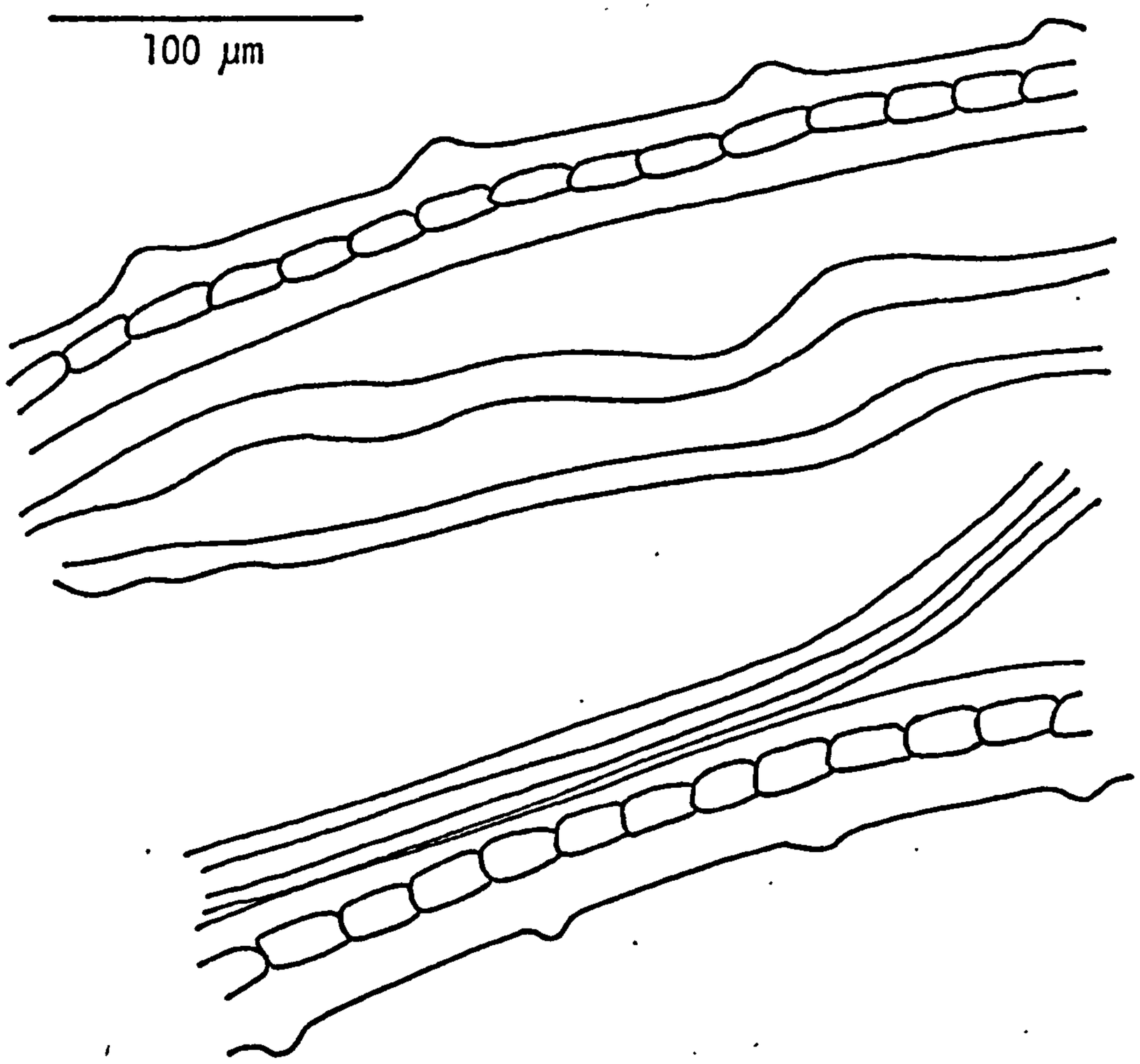


Figure 14.

Female of O.gutturosa
Middle part of the worm/notice the prominent cuticular striation ,4-5 between each pair of annulations



different levels of the body of O. gutturosa. Neumann, 1910, published the first formal description of O. gutturosa. He gave it the specific name because of the "guttural" dilation of the body at the level of the nerve ring.

The first descriptions of Onchocerca in the neck of British cattle was written by Steward (1937), who was also the first person to describe the life cycle of both O. gutturosa and O. cervicalis in the U.K. Many authors have described the morphology of O. gutturosa, e.g. Johnston, (1921); Strong et al., (1934); Steward, (1937); Eichler, (1973a); Beaver et al., (1974); Chauhan & Pande. (1978).

The next section does not consist of a formal re-description of O. gutturosa in British cattle because this would not add anything to what has been already said by Eichler (1973a). Nevertheless, an attempt has been made in the present study to assess whether the two strains of O. gutturosa are similar or there are some differences.

4. General remarks

The British and Sudanese complete adult worm strains were obtained from cervical ligaments of cattle by digestion technique (Schulz-Key et al., 1977) and were found to be morphologically similar to those identified as O. gutturosa. Neumann, 1910, and the above authors agree in the measurement of the thickness of the cuticle and prominence of cuticular ridges. In the Sudanese strain the ridges are

Table 2.

COMPARISON OF MEAN MEASUREMENT OF ADULT WORMS OF O. GUTTUROSA (SUDANESE AND BRITISH STRAIN) BOTH FROM THE CERVICAL LIGAMENT

Measurements	Sudanese		British	
	Male	Female	Male	Female
Length (cm)	28.8 (27-30.6)	45.7 (20-59.5)	34.7 (28-45)	42.4 (27.8-54)
Maximum width	90.4 (80-108)	117.9 (103-135)	100 (85-160)	289 (216-313)
Mouth to nerve ring	251 (213-266.5)	235 (217-247.8)	311 (241-332)	317 (273-340)
Oesophagus	950 (800-1026)	1122 (1013-1168)	877 (745-1165)	1183 (1070-1389)
Vulva from anterior part		460.8 (380-575)		612 (510-604)
Right spicule	70 (60-80)		76 (69-92)	
Left spicule	233.3 (210-280)		224 (185-254)	
Tail	85 (81-90)	210 (190-230)	101 (89-113)	198 (152-257)

All measurements given in microns except where stated.

more prominent than in the British strain, being 6 to 4.5 microns in height respectively.

The number of striae between two adjacent ridges was 4 - 5 in both strains.

If measurement information related to this Sudanese strain and O. gutturosa from the U.K. (Eichler, 1973a) contained in the accompanying Table (Table 2) are compared, it will be noted that in regard to the males practically all the measurements are slightly different, except the length of the right spicule which are nearly similar and the structure of the caudal region of the male of both species shows no constant morphological difference.

In the case of the female, the agreement in the length of the worm is practically complete, although other measurements are markedly different in regard to the length of the oesophagus, genital opening and situation of the nerve ring, also the British species is much broader than the Sudanese strain.

(c) Relationship between age of the animals and adult worm burden

O. gutturosa is a widely-distributed parasite from the nuchal ligament of cattle in Europe, Asia, Australia, Africa and America (Eichler and Nelson, 1971). A detailed knowledge of the distribution of the worms within the tissue of the host is often essential for an understanding of the

pathological effect of the parasite and the relationship between worm burden in the host and the number of microfilariae in the skin of the same animal.

To extract the whole worms from the connective tissue of infected animals is not only laborious but it is also very difficult. It is also very difficult to locate the adult male in the tissue due to its thickness and it can be easily missed between the bundles of fibres. Steward (1937) attempted to extract adult worms from cervical ligaments using different digestion method without success. Gibson (1952) and Eberhard (1977) both used a digestion method and obtained intact adult worms successfully.

Eichler and Nelson (1971) tried to study worm burden in British cattle by extracting the worms and found the majority of infection to consist of one male to three females associated with one lateral half of the ligament, six being the maximum. Their count was based on the number of anterior halves and they encountered difficulties in counting the number of males and suggested that they were usually paired.

For studying the worm burdens in Sudanese and British cattle we used the technique used by Schulz-Key et al. (1977) for studying the worm burden in human onchocerciasis. We used the pepsin enzyme instead of collagenase because collagenase was found to be very expensive used on a large scale.

1. Materials and methods

Most autopsy material was collected from Khartoum veterinary college autopsy room (13 animals) plus 10 animals from Omdurman central abattoir. Different ages of cattle were examined.

The ligamentum nuchae of infected animals was cut from the carcass very carefully, most attention being given to removing the whole ligament with both halves with connective tissue attached. It is probable, however, that some small pieces were left in situ in most cases. At the same time a piece of skin was cut from the hump region as big as possible (10 mm square). Sometimes the whole skin of the hump was removed. Other sites were examined for the presence of adult worms, e.g. gastro-splenic ligament and stifle joint. All animals were negative for both the latter sites.

The worms were concentrated mainly in the nuchal ligament. The whole connective tissue covering both halves of the ligament was removed very carefully under the dissecting microscope because often the anterior part of the worm is very easily missed. Then the whole tissue was removed, immediately placed into a big flask containing the digesting solution of pepsin enzyme in concentration of 1.5 gm/100 ml distilled water, and 3 ml of concentrated HCl for each 500 ml of the solution added. In the cases of a huge amount of connective tissue, each half was digested separately.

The digestion solution should cover the whole amount of connective tissue to be digested.

The solution containing the tissue was incubated in a water bath at 37°C with constant gentle shaking to accelerate digestion. After 8 - 16 hours the tissue was examined to see whether the whole tissue was digested, and if not it was re-incubated again.

After the whole tissue was digested, the whole solution was filtered through the sieve, the container used for digestion washed with warm water to ensure no worms remained, and also the undigestable tissue was washed several times with warm water to dissolve the fat around the worms. Then the filtrate was transferred to fresh distilled water in a petri dish and examined under a dissecting microscope using fine forceps to isolate each loose complete worm and transfer it to glycerine alcohol.

Then each anterior and posterior part of the female worms were kept separately in glycerine alcohol and attention was paid to not confusing between anterior portions of females and males (Cameron, 1928). It was found that the posterior part of the female worms were coiled tightly and were always the most difficult part to release from the tissues, so care must be taken in extracting this portion if a complete worm was required. Then all the other fragments of the worms were discarded. All males were isolated and kept separately in glycerine alcohol.

After the digested solution had been exhaustively

examined of one animal, the total number of male and female worms was counted. First the number of the complete adult females and males was counted, then the number of anterior and posterior parts. If equal it meant no worms had been missed, but normally the anterior parts were less in number due to their thin size.

In the meantime the assessment of the number of microfilariae in the skin of the same animal was detected using the technique modified from Nelson et al.(1966) by taking 3 skin snips from each animal to minimize the effect of irregular microfilarial distribution (Buckley, 1938; Mellor, 1973; Anthony & Cello 1975) using snippers to standardise the depth and surface, then shaved, freed from any substance and subcutaneous tissue, before being weighed and placed in 80% Tyrode's plus 20% sheep serum. The snip was then teased into small pieces and left over-night at room temperature ($27^{o}C$) for 24 hours. Then the suspension was filtered and all microfilariae in the fluid were counted using counting chambers. Then the mean of the three readings was calculated.

The microfilarial concentrations were expressed as the number of microfilariae per mg of skin for each animal.

The age distribution of 23 animals which were examined during this study is recorded in Table 3.

Table 3.

RELATIONSHIP BETWEEN THE AGE OF THE ANIMAL AND NUMBER OF ADULT WORMS

No. of animals	Age (years)	No. of adult females		No. of adult males		Number of adults	
		Mean	Range	Mean	Range	Mean	Range
4	10	47	37-54	70	58-72	117	95 -126
2	8	45	35-55	60	50-70	90	85-125
2	7	29	28-30	36	35-37	66	65-67
3	6	18	5-37	19	7-32	37	12-69
5	5	12	9-20	12	7-17	24	16-37
3	4	10	5-15	13	7-20	23	12-35
2	3	6	3-10	8	5-13	11	8-13
2	2	3	3-3	5	4-7	9	7-10

2. Discussion

Schulz-Key et al. (1977) studied worm burdens in human onchocerciasis using a digestion technique. In the present study of worm burdens in Sudanese and British cattle, I used the same technique, but instead of expensive collagenase enzyme, pepsin enzyme was used.

The pepsin was found highly successful in digesting the connective tissue of ligamentum nuchae. Complete digestion was achieved within 6 hours for connective tissue obtained from 2 year old cows. In the case of a large amount of connective tissue, the digestion was completed within 50 hours. The only disadvantage of this enzyme was that all recovered worms were dead and most internal organs were digested,especially in those worms obtained after long periods of digestion.

The assessment of the total worm burdens in the animal body is rendered difficult as Johnston (1921), Ottley and Moorhouse (1978) and Bianco - personal communication - showed that O. gutturosa can be found in so many different sites. It was not possible to search for all adult worms in all the tendons and connective tissue of all bodies, but observation suggested most, if not all, adult worms of O. gutturosa in British cattle are in the nuchal ligament only and in Sudanese cattle will be found in the nuchal ligament and stifle joint.

In all these animals we can exclude two sites, the

stifle joint and gastro-splenic ligament, because these two sites were carefully examined and found free of worm burdens.

Extracting all worms from the cervical ligament can give an indication of the degree of the infection at that site. From Table 4 it is apparent that there is an increase in the total number of adult worms and skin microfilariae with increasing age of the definitive host.

This supports the observation of Anthony and Cello(1975) in equine onchocerciasis, and Schulz-Key *et al* (1975) in human onchocerciasis, but is in disagreement with Eichler & Nelson (1971) who stated that age had no effect on the number of adult worms recovered.

The present study revealed that the Sudanese animals carry more worms of *O. gutturosa* in their *ligamentum nuchae* than British cattle, the maximum number of adult female and male worms recovered from both halves of the *ligamentum nuchae* from Sudanese and British cattle were 47 females to 70 males and 15 females to 14 males respectively.

Regarding the sex ratio, Charles (1965) in *Wehrdikmansia cervipedis* found that female adult worms were in a majority. Schulz-Key (1975) reported in *O. flexuosa* that the adult male worms are in the majority.

In the present study there was a distinct predominance of males in all Sudanese animals examined by the digestive method, but support was given to the observation of Eichler

Table 4.

WORM BURDEN IN BRITISH CATTLE (ALL SPECIMENS COLLECTED FROM DAIRY COWS FROM READING ABATTOIR AND EXTRACTED BY DIGESTION TECHNIQUE)

No. of animals	Age (years)	Male worms	Female worms
1	8	14	15
1	5	9	8
1	Unknown	7	9
1	Unknown	9	9
1	Unknown	9	12
1	8	10	12
1	Unknown	8	10

and Nelson (1971) that numbers of O. gutturosa in British cattle are nearly equal.

The writer is in complete agreement with Steward (1937) and Eichler and Nelson (1971) in their statements that it is impossible to be certain of the number of male worms present in the tissue by extracting them manually because they are relatively very small and can be easily confused with muscle fibre of the ligament.

But the digestion method is more reliable and easy and gives a nearly accurate assessment of the numbers of adult worms.

(d) Histopathological studies on the nuchal ligament

Irrespective of the strain of parasite, the most severe clinical manifestation of onchocerciasis is due to the presence of microfilariae. The adult worms are unobtrusive and usually of secondary importance (Nelson, 1970). Although bovine onchocerciasis is very common; the pathology of this disease has not received much attention.

The pathology of O. gutturosa infection in cattle has been described by some authors, e.g. Mohamed (1931); Eichler (1970); Al Zubaidy (1973); Cheema&Ivoghli (1978); Hussein et al. (1975). Bovine onchocerciasis due to O. gutturosa is very common in Sudanese cattle. Hussein et al. (1975) and El Bihari and Hussein (1978), drew attention

to the high incidence of the disease among adult cattle amounting to approximately 95%. As far as I am aware, the only reference to pathological lesions caused by O. gutturosa in Sudan is in the report of Hussein et al. (1975). They claimed there is no defined clinical manifestation associated with the presence of adult worms of O. gutturosa in the nuchal ligament, despite the severity of the local tissue reaction.

No pathological lesion was attributable to O. gutturosa microfilariae, as was reported from Japan (Niimi and Kuno, 1954), Australia (Herin et al., 1955), and from Russia (Gnedina, 1959).

1. Materials and methods

Five hundred affected bovine ligaments were collected in Omdurman central abattoir and thoroughly examined. From this material samples from the infected ligaments were fixed in 10% formal saline, serial sections were cut at 5 µm after processing through paraffin wax and staining with haematoxylin and eosin.

2. Observations

A. Pathogenicity and pathology:

The pathology of O. gutturosa infection takes two forms:

1. The lesions caused by adult worms
2. The cutaneous lesions due to microfilariae

which are discussed in pages 88 - 97

3. Gross Pathology

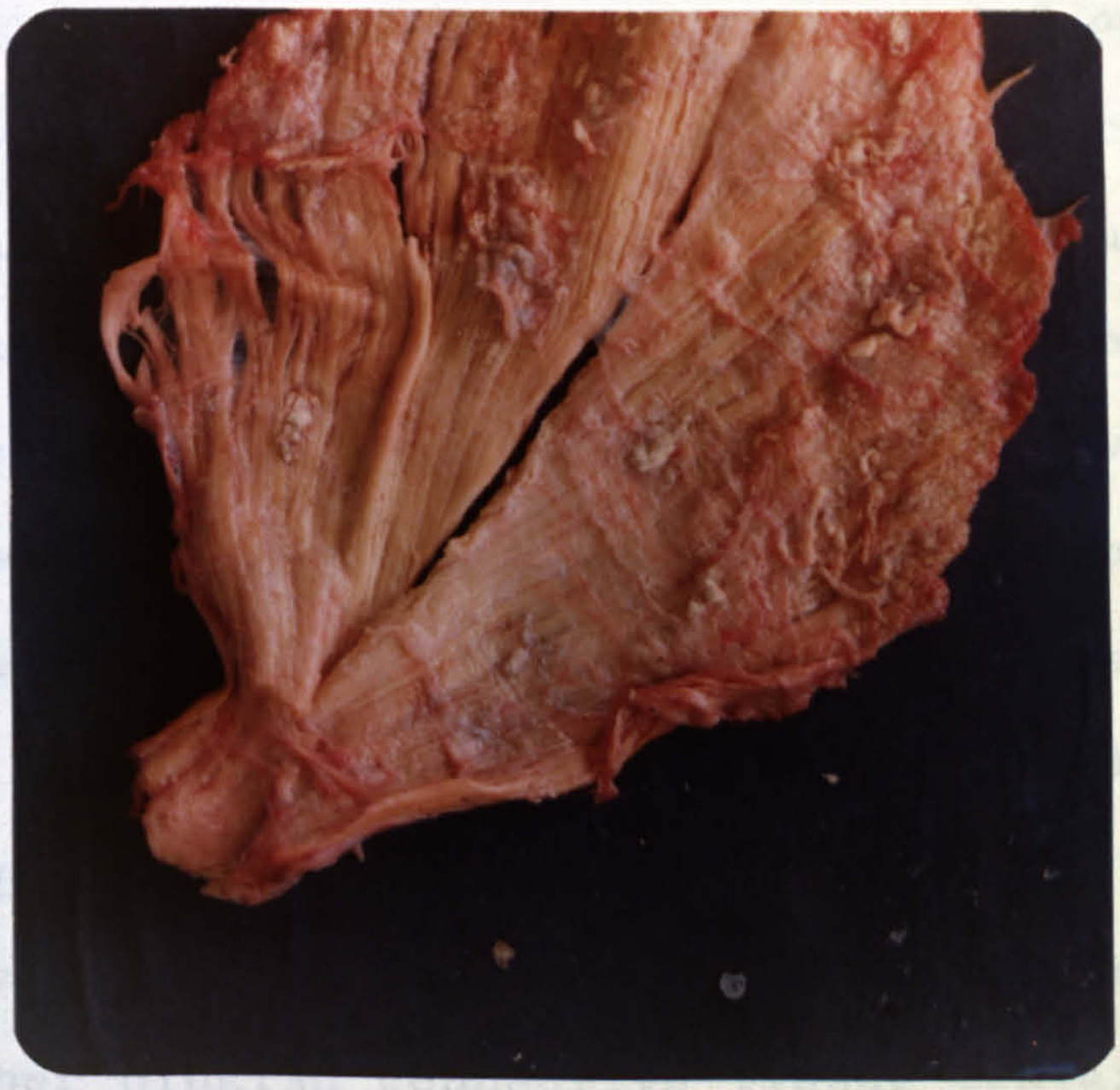
Gross examination of nuchal ligaments infected with O. gutturosa showed that in young animals (i.e. less than three years old) the affected ligaments were not significantly different to those in healthy animals, except that in the former very fine congested capillaries were sometimes seen in the immediate vicinity of the worms.

The amount and thickness of the connective tissue surrounding the worms in young animals was also relatively small and the tissue appeared pinkish in colour and rather homogeneous in consistency. In contrast to uninfected animals, old infected animals, especially heavily parasitized cases, showed a markedly increased thickness of the tissue in places where worms were embedded. On the other hand, in such heavily infected cases, the overall appearance of the ligament was that of atrophy, the degree depending on the intensity and possibly the duration of infection.

Calcified worms were seen in long-standing cases and these were readily recognized by their whitish calcareous appearance (Figure 15). The calcification of adult female worms usually started from the posterior region, while the rest of the worm appeared normal and still contained microfilariae.

In the absence of recognizable calcification, however, worms could still be detected by close inspection and soon

Plate 15.



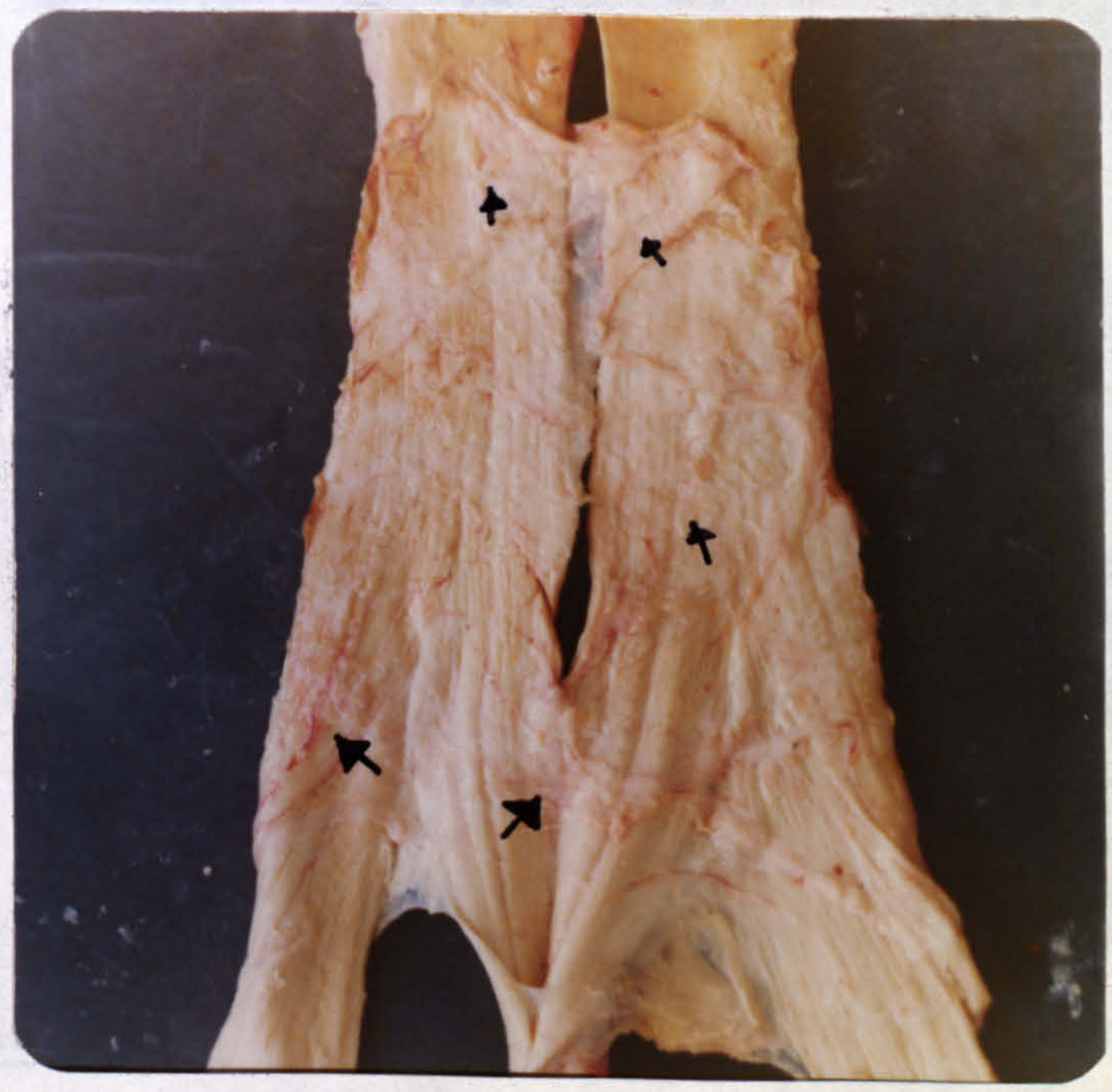
Ligamentum nuchae of cattle containing calcified adult worms - notice the white colouration of calcified worms.

as thin thread-like objects embedded within the connective tissue (Figure 16).

Apart from this, greyish nodules containing worms were often seen by the naked eye and these were always surrounded by a network of anastomosing blood vessels. The size of such nodules was variable, ranging from 15 to 25 mm in width, and they were often raised from the surface of the ligament by about 4 - 11 mm (Figure 17). They were usually hard in consistency and when cut were mostly found to contain dead calcified worms embedded within a caseous core and surrounded by connective tissue.

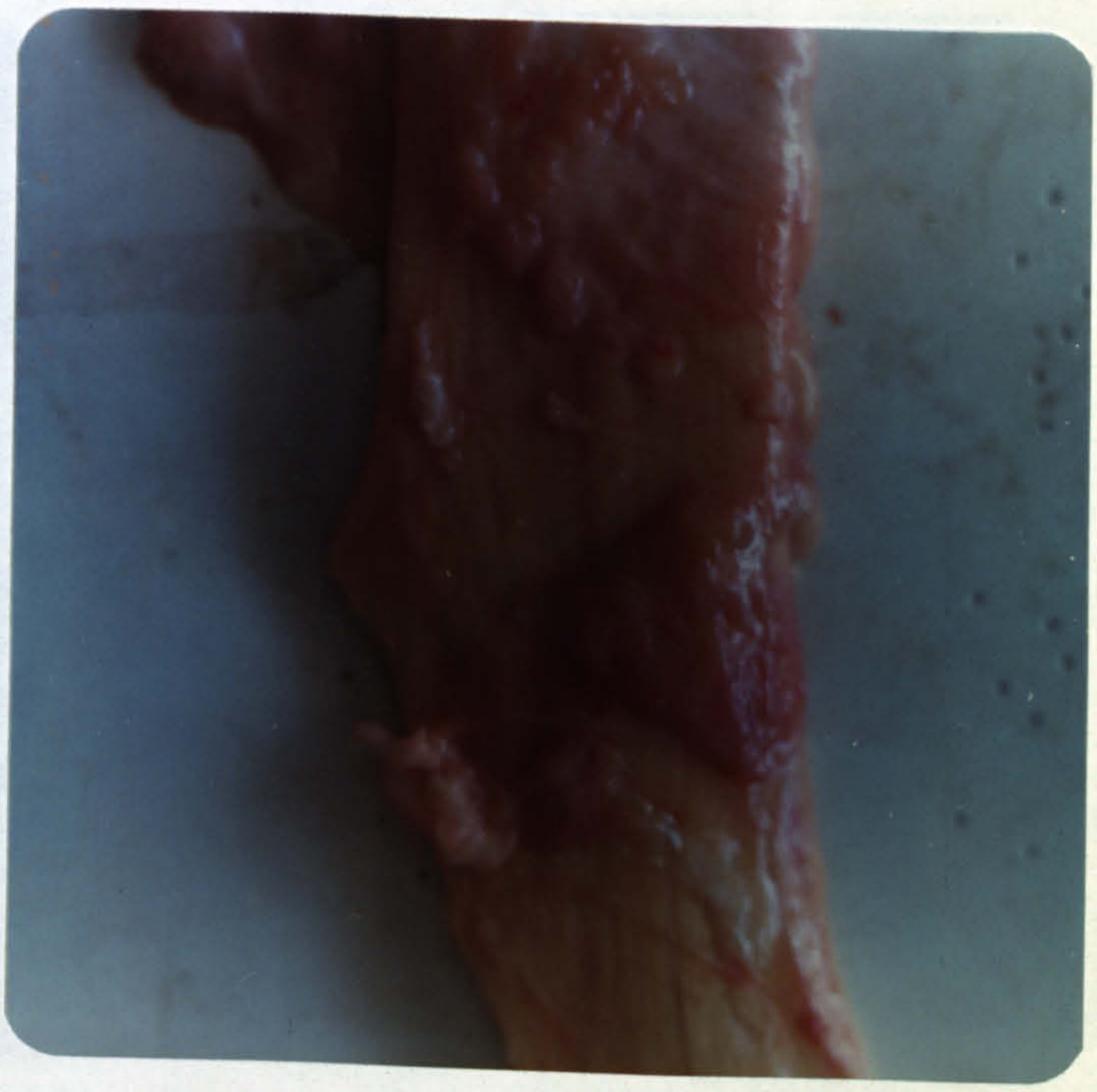
An extremely interesting case was recorded of a 10 year old cow examined at Omdurman slaughter house. In this animal a large mass was found in the funicular side of the ligamentum nuchae at the same location where O. gutturosa worms were usually found. This mass was surrounded by thick and rather pinkish connective tissue containing calcified worm nodules. The dimension of the whole lesion was about 11 x 8 cm and it was raised from the surface of the ligament by about 4 cm (Figure 18). When the superficial connective tissue covering this huge mass was removed, the mass was in fact two nodules, the larger one measuring 5 x 5 cm and the smaller one 3.5 x 3.5 cm. Both nodules were raised by 3 cm from the surface of the ligament. On palpation the larger nodule was hard and the smaller one softer and more yellowish; both lesions were

Plate 16.



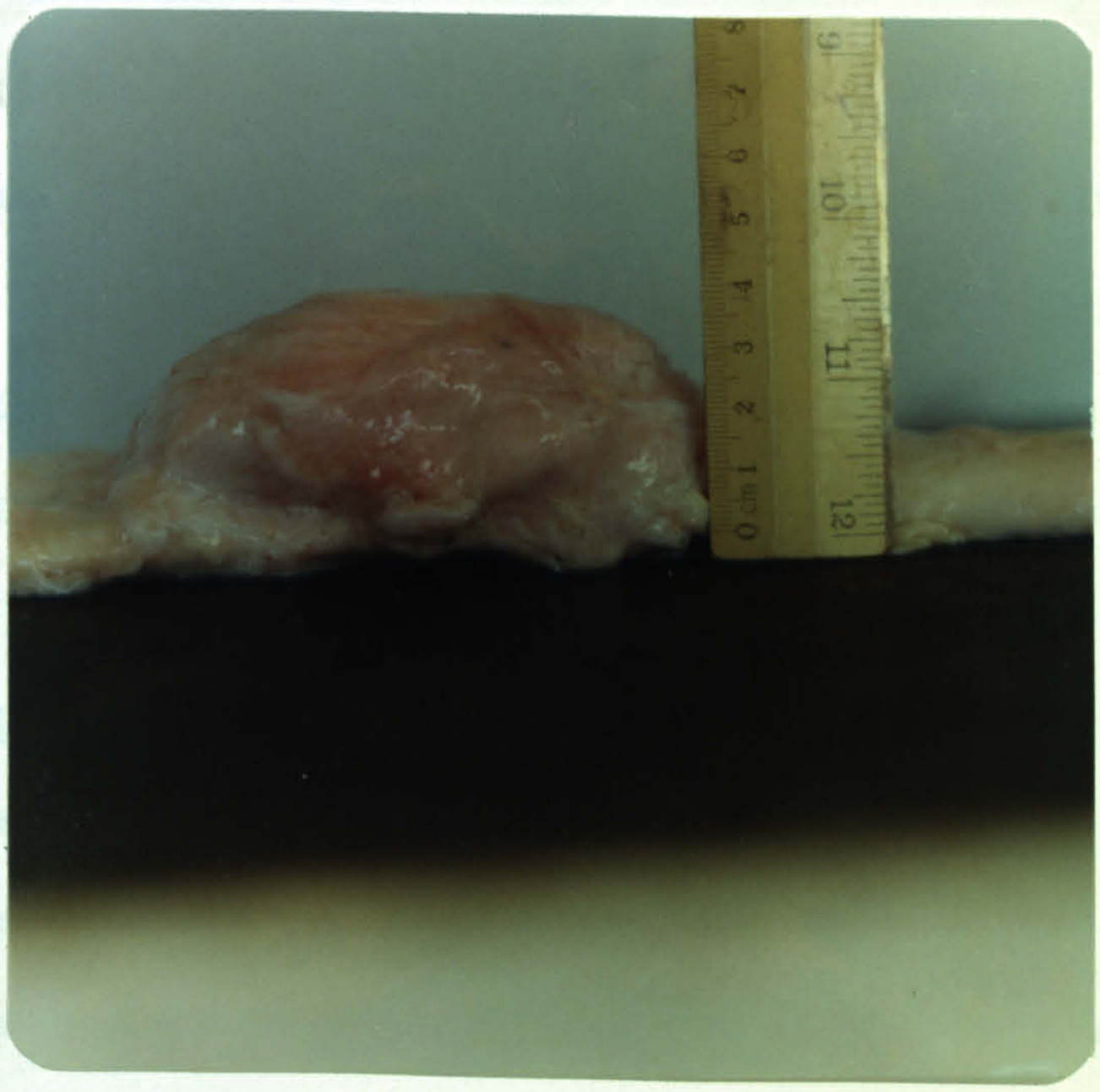
Heavily infected ligament with O. gutturosa adult worms looking like thin threadlike objects embedded within the connective tissue

Figure 17.



Ligamentum nuchae containing different size of nodules. Notice the network of anastomosing blood vessels which are highly congested with blood.

Figure 18.



Large nodule size in the ligamentum nuchae due to O. gutturosa

surrounded by congested blood vessels and haemorrhagic spots. When incised, each mass was found to consist of a centre partly soft and partly calcified. The caseous core was enclosed within a dense connective tissue capsule and transected by thick fibrous cords in which worms were also found embedded.

The contents of the lesion were negative by Gram and Ziehl-Neelsen stains, as well as negative to culture in blood agar and McConkey's medium for micro-organisms.

This is the first time in which a lesion of this type and size has been recorded in O. gutturosa infected cattle.

4. Histopathology

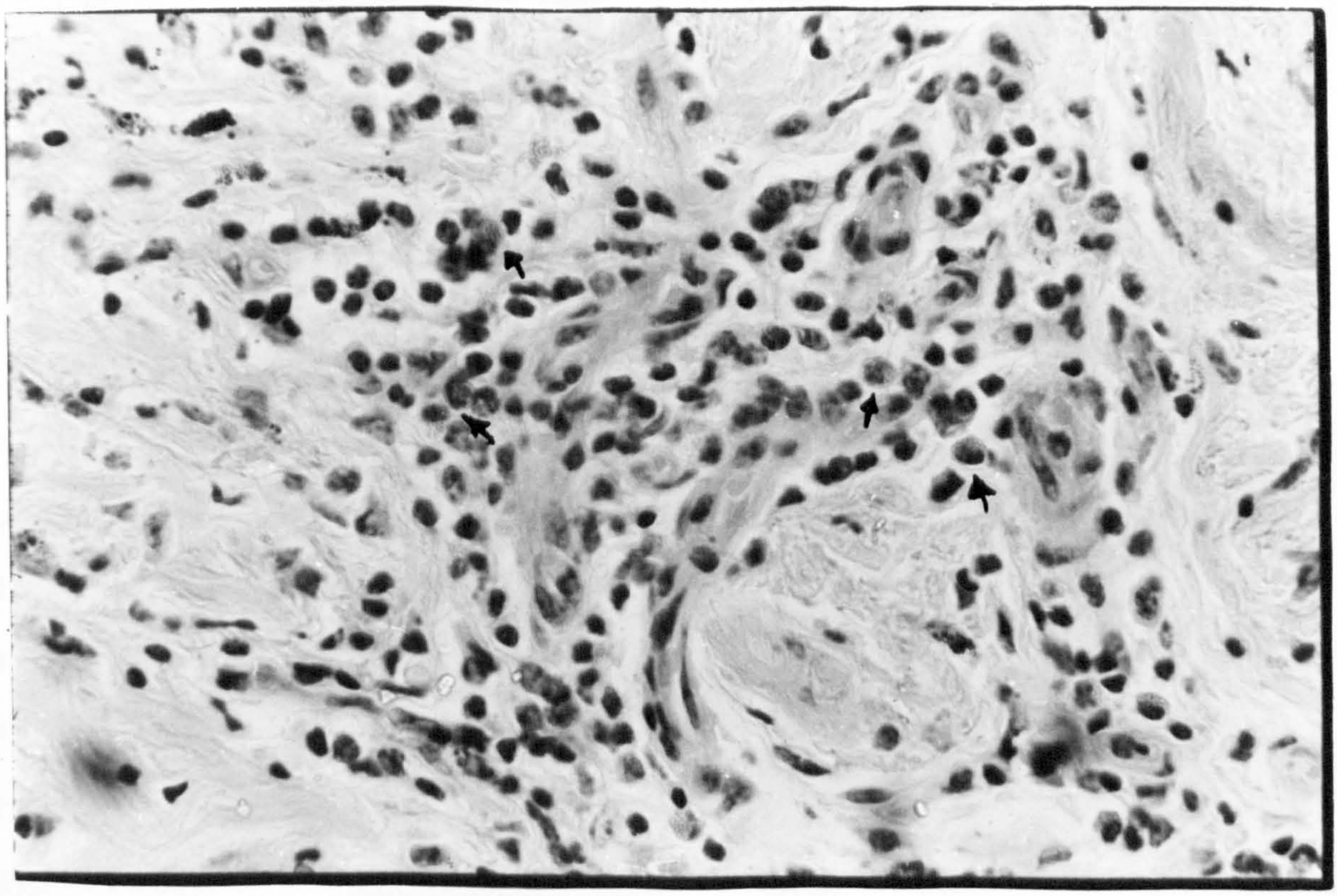
Microscopic examination of a section of infected ligament revealed calcified and/or uncalcified parasite fragments surrounded by inflammatory cells and fibrous connective tissue of variable intensity. These lesions were mainly located in the superficial layer of the ligament, although deeper substance of the latter sometimes also showed local inflammatory infiltrations, particularly around small blood vessels. In that case, the reaction consisted mainly and sometimes exclusively of lymphatic cells surrounding venules and arterioles. Occasionally, however, other cells including polymorphonuclear eosinophils and macrophages were found in small numbers.

Quite often the parasites were shrunken, apparently

due to fixation, and were thus separated from the surrounding reaction leaving an empty crescentic space. Healthy-looking parts of the female worms often showed embryonated eggs occupying most of the cross-section of the worms. The substance of the ligament at the infected site was loose and somewhat oedematous and more acidophilic than normal; in such affected places the dense elastic fibres of the ligament were clearly separated. The affected parts also showed mucoid degeneration with a typically hyaline appearance. In some sections the parasites were surrounded by a thick sheath along their course.

The inflammatory response induced by the presence of the parasite in the connective tissue in some of the sections was quite extensive, consisting of predominantly a massive number of eosinophils accumulating around the worms and also extending within different connective tissue bundles into the substance of the ligament (Figure 19). Such reactions were sometimes in the form of acidophilic granulomas around parasitic fragments and in such cases, the appearance suggested that the worms were undergoing degeneration.

Plate 19.



Section of connective tissue of nuchal ligament showing massive number of eosinophils (arrow) accumulating around the worms.

II. MICROFILARIAE

(a) Introduction

In this section, observations on the behaviour and distribution of microfilariae of O. gutturosa were made. The microfilariae of O. gutturosa are unsheathed and occur mostly in the superficial layers of the skin so that they are available for ingestion by the appropriate blood-sucking vector.

So far nothing is known about the trigger mechanisms which activate them, or how far they travel in the skin. Duke (1968) reported that the passage of O. volvulus microfilariae from the uterus of the adult female to their location in the skin may take as long as 4 to 8 weeks, and the life span for these microfilariae in the skin of a natural host is up to one year. Nelson et al. (1966) recovered microfilariae of O. gutturosa from mice after 120 days. It would appear that the microfilariae have directional mechanisms which ensure that they migrate to a specific site, regardless of the site of the adult female, to ensure their transmission.

Buckley (1938) found a concentration of O. gibsoni microfilariae in the pectoral region of cattle in Malaysia which represented the preferred biting site of the local vector, Culicoides. Eichler and Nelson (1971) in the U.K. found the microfilariae were always concentrated around the umbilicus, and that represented the preferred biting site

of the local Simulum vector.

The observations of the present study are in agreement. Microfilariae of O. gutturosa in Sudanese cattle were found concentrated in the back of the infected animals (El Bihari and Hussein, 1978), and were thus most likely to be taken up by the vector C. kingi, which usually prefers biting in that region.

The distribution of the microfilariae in the infected skin even in a very small area, is irregular and they have a tendency to nest in groups, as noticed by Buckley (1938), Mellor (1973) and Anthony and Cello (1975) In some countries animals are infected with more than one species of Onchocerca, such as Sudan (Hussein et al., 1975); India (Shastri, 1978); Australia (Ottley and Moorhouse, 1978a); U.K. (present study). This means that a method of identifying the microfilariae of each species is essential for any meaningful studies on the effects of distribution in the skin on their transmission. Differentiation studies were carried out by Gibson (1952), Bremner (1955) and Ottley and Moorhouse (in press) in Australia. Irrespective of the strain of the parasite, the most severe clinical manifestations of animal onchocerciasis are caused by the presence of micro-filariae in the skin, as reported by Datta (1939), Dikmans (1948), Niimi and Kouno (1954), Herin et al. (1955) and Thomas (1958); as well as pathological lesions in the eye of the horse caused by microfilariae of O. cervicalis (Mellor, 1973).

1. Rate of emergence of microfilariae from the skin of zebu cattle.

Venkataratman and Kershaw (1961) reported that the microfilariae of O. gutturosa can emerge from cow skin within a few hours. Nelson et al. (1966) found that a major factor which must be taken into account in making accurate determinations of microfilarial density is the time allowed for emergence of microfilariae from infected skin. Eichler and Nelson (1971) found that there was a slow rate of emergence of microfilariae from the skin snip due to the tough nature of cattle skin. Mellor (1973) recovered 29.95% of microfilariae of O. cervicalis from horse skin within 4hours. Anthony and Cello (1975) used 6 hours for quantitative determination of dermal microfilariae of O. cervicalis.

Southgate (1974) stated that the percentage of microfilariae recovered rises from 22.1% to 67.8% with increasing efficiency of technique for O. volvulus. Bianco et al. (1980) found that a rise in temperature by incubating skin snips at 37°C for one hour can encourage the rapid emergence of microfilariae. Duke (1962) reported that teasing the skin snip will increase the rate of emergence of microfilariae of O. volvulus. Buckley (1938) found the majority of microfilariae of O. gibsoni would emerge within 12 hours. El Bihari and Hussein (1976) found 3 hours at 37°C to be a good time for O. armillata microfilariae to emerge from

the skin of a cow.

In the present study four different media were used with different intervals of time in order to determine the best medium and minimum time needed for the emergence of microfilariae from infected skin of zebu cattle.

A) Materials and Methods

Media Used :

1. Normal saline (0.08%NaCl)
2. Normal saline + 20% sheep serum
3. Tyrode's medium
4. Tyrode's medium + 20% sheep serum

Cow skins were collected from the autopsy room of the University Veterinary College, Khartoum.

From three positive cows, two 7 year olds and one 9 year old, the whole skin of the hump region was removed. Then from each two skin snips were taken, using a snipper, and each snip was divided into four equal parts; each part from these was processed as a separate skin snip. These were shaved, freed from subcutaneous fasciae, shredded with scissors, weighed, then placed in one of the four media mentioned above, in clean tubes with a top cover and incubated at room temperature ($27^{o}C$). The samples were then removed to the same fresh medium after 0.5, 1.5, 3, 7, 9, 22 or 24 hours.

The number of microfilariae in the whole quantity of

Table 5.

EMERGENCE OF MICROFILARIAE IN MEDIUM OF NORMAL SALINE
(MEAN SKIN WEIGHT = 0.328 gm)

Time (hours)	1st Experiment		2nd Experiment		3rd Experiment		Mean % of mff emerged
	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	
0.5	375	55.89	182	44.5	107	34.1	44.82
1.5	543	80.92	314	76.8	213	67.38	75.18
3	598	89.00	365	89.24	235	74.84	84.36
7	628	93.00	386	94.40	279	88.22	91.87
9	644	95.00	394	96.33	291	92.67	94.67
22	666	99.00	399	98.30	307	97.77	98.36
24	671	100.00	402	100.00	314	100.00	100.00

Table 6

EMERGENCE OF MICROFILARIAE IN MEDIUM OF NORMAL SALINE PLUS 20% SHEEP SERUM (MEAN SKIN WEIGHT = 0.505 gm)

Time (hours)	1st Experiment		2nd Experiment		3rd Experiment		Mean % of mff emerged
	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	
0.5	665	58.44	56	24.13	23	9.79	30.79
1.5	949	83.39	160	68.96	124	52.76	68.37
3	996	87.52	197	84.91	180	76.59	83.00
7	1034	90.86	215	92.67	194	82.55	88.69
9	1057	92.88	218	93.97	208	88.51	91.48
22	1115	97.98	227	97.84	220	93.63	96.48
24	1138	100.00	232	100.00	235	100.00	100.00

Table 7

EMERGENCE OF MICROFILARIAE IN MEDIUM OF TYRODE'S SOLUTION
(MEAN SKIN WEIGHT = 0.405 gm)

Time (hours)	1st Experiment		2nd Experiment		3rd Experiment		Mean % of mff emerged
	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	
0.5	544	27.80	588	41.21	400	13.50	27.50
1.5	1110	56.75	1101	77.15	1266	42.71	58.87
3	1855	89.72	1270	88.99	2586	87.23	88.65
7	1873	95.76	1306	91.52	2748	92.71	93.33
9	1906	97.44	1333	93.41	2865	96.66	95.84
22	1950	99.74	1373	96.22	2940	99.19	98.40
24	1956	100.00	1427	100.00	2964	100.00	100.00

Table 8.

EMERGENCE OF MICROFILARIAE IN MEDIUM OF TYRODE'S SOLUTION PLUS 20% SHEEP SERUM (MEAN SKIN WEIGHT = 0.418 gm)

Time (hours)	1st Experiment		2nd Experiment		3rd Experiment		Mean % of mff emerged
	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	No. of mff emerged	% of mff emerged	
0.5	849	50.36	356	34.50	27	5.37	30.07
1.5	1397	76.92	502	48.64	124	24.65	50.07
3	1553	92.11	866	83.91	399	79.32	85.11
7	1611	95.55	913	88.46	473	94.04	92.68
9	1621	96.14	922	89.34	496	98.60	94.69
22	1676	99.41	932	90.31	498	99.00	96.24
24	1686	100.00	1032	100.00	503	100.00	100.00

medium was then counted using a single counting chamber (one ml capacity) on a microscope slide. The result for each medium is summarised in Tables 5 - 8.

B) Discussion

From the results shown in the Tables for the four different media, the proportion of microfilariae which emerged within three hours to the total which emerged after 24 hours was found to be similar in all the four media. The number which emerged constituted approximately 84% of the total number of microfilariae emerging within 24 hours. It is clear that the proportion was not low, as was reported by Eichler and Nelson (1971) and Mellor (1973) who recommended 24 hours incubation of skin snips for total emergence of microfilariae. Collins (1973) found that some microfilariae of O. cervicalis remained in the skin of positive samples after soaking for 30 hours. However, 24 hour incubation of skin snips is time consuming and there is a risk that the medium may be exposed to contamination with bacteria and yeast, thus reducing the activity and emergence of microfilariae.

The high rate of emergence of microfilariae in the experiment within a short time could be due to the relatively high ambient temperature which encourages rapid emergence of microfilariae (Bianco et al., 1980). El Bihari and Hussein (1976) found 37°C for 3 hours gave maximum emergence

of microfilariae.

Through measuring the concentration of microfilariae in different depths in the skin, it was shown that the majority of microfilariae in Sudanese cattle were found in the superficial layer during most of the year (0.5 mm deep). This is a similar finding to that of Buckley (1938) who found that microfilariae of O. gibsoni which were in the superficial layer could emerge from a cut surface of skin within a short time.

So, since the data were based only on the numbers that emerged from the snips and no attempt was made to find how many were left behind, the conclusion may not be valid, and 24 hour incubations are likely to be time consuming.

An important difference found between the four media was that the microfilariae in Tyrode's plus 20% sheep serum can survive for 16 hours and in saline with serum for 10 hours, but in the saline without serum the microfilariae become sluggish and die within 6 hours.

(b) Distribution of microfilariae of O. gutturosa in zebu cattle

Venkataratman and Kershaw (1961) in the U.K. and Charles Hibler (1965) from Arizona, reported that the microfilariae of O. gutturosa and O. cervipedis occur mainly in the skin of the ears and neck and nearby parts of the body where the adults are localized. However these findings differ from those of Eichler and Nelson (1971) who reported that the localization of microfilariae was in the umbilical region of cattle in the U.K., regardless of the site of the adult worms.

In the Sudan, the distribution of O. gutturosa microfilariae has been reported by Hussein et al. (1975), who found that the adult worms were confined to the connective tissue of the nuchal ligament. The highest densities of microfilariae were found in the skin of the ears, followed by the scrotum, tail, and the lowest number in the umbilical region. El Bihari and Hussein (1978), as a result of examining 10 complete hides positive for O. gutturosa, found that microfilariae occurred in large numbers in the midline of the hump and immediately behind it.

Due to this point regarding microfilariae distribution, the purpose of the present study was to examine further in animals infected with O. gutturosa to map the microfilarial densities over the body.

A) Materials and Methods

Random examinations were made of animals of all ages, both males and females, at Omdurman Central abattoir in the Sudan.

In addition, a skin snip survey was also carried out in Shambat farm, and Kuku farm on live animals.

In Omdurman abattoir the animals were put into age groups according to the stage of their cropping teeth (Fig. 4) using the system published by the U.S. Bureau of Animal Industry.

Twenty-five animals were examined at Omdurman central abattoir for the distribution and concentration of microfilariae in different sites of the body (Fig. 20). The skin snips from different regions of the animal's body were taken using snippers (10 mm) which gave a standard depth and surface area. When the viscera were removed from the animal, the spleen was set aside while still attached to the rumen by gastro-splenic ligament, and this was examined carefully (Fig. 2). Both halves of the cervical ligament were removed as completely as possible from each animal, as far back as a point level with the 8th or 9th thoracic vertebrae.

It was possible to examine and sample skin snips from different regions and examine the gastro-splenic ligament of the same animals, for the sake of accuracy of the work 15 animals were examined per visit.

All samples of skin were shaved free from subcutaneous

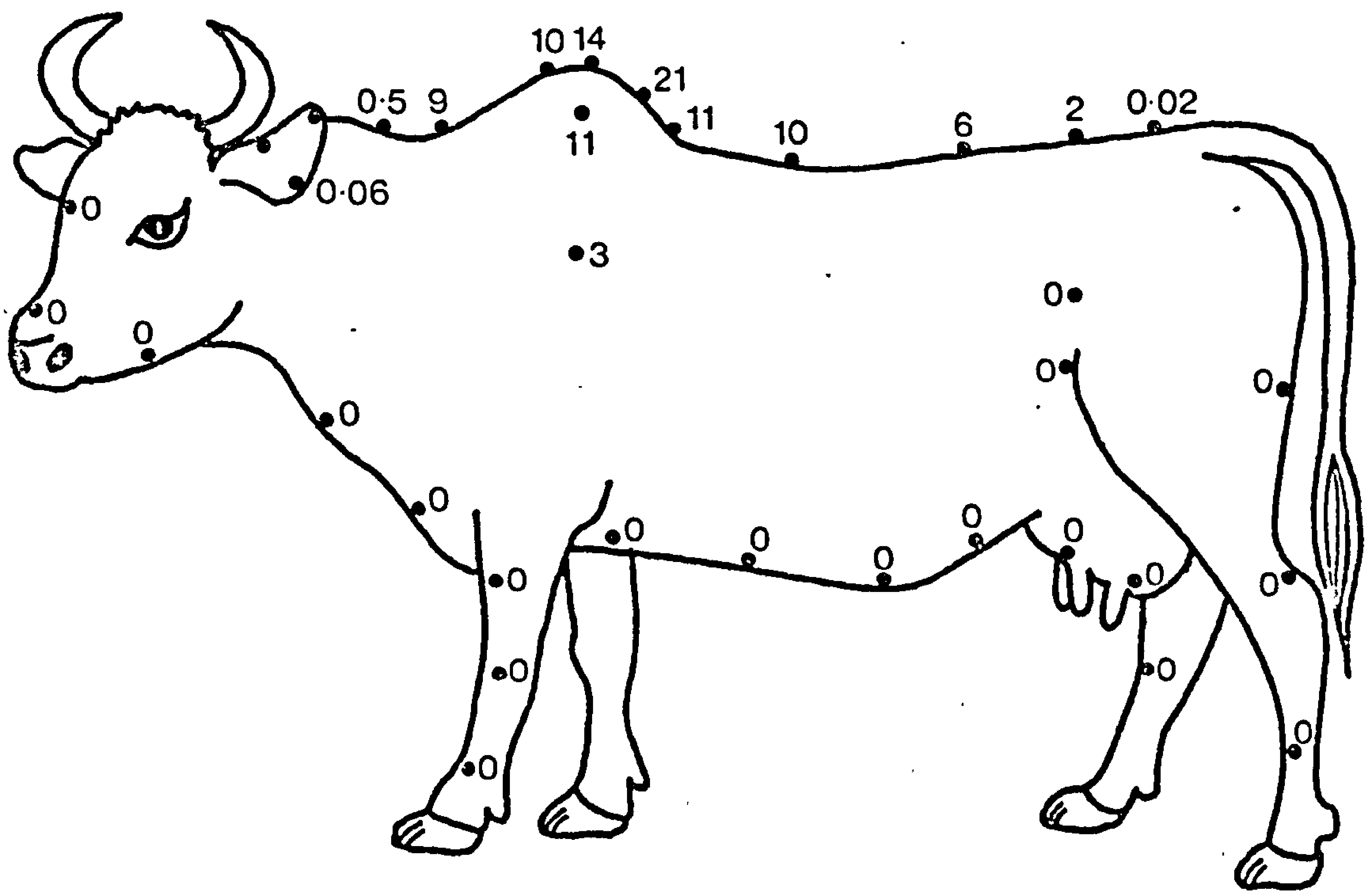


Fig. 20. Distribution of <u>Onchocerca gutturosa</u> microfilariae in the skin of Sudanese cattle. Dots represent area. (result of 25 animals, No. of mf./mg.)

tissue, weighed, chopped into small pieces and then placed into small amounts of tyrode's solution plus 20% sheep serum, to which a few drops of penicillin and streptomycine had been added.

The skin snips were incubated at room temperature (about 27°C) in screw-topped bottles overnight to allow the emergence of the majority of microfilariae (see page 22). Finally the microfilariae in the whole quantity of medium were counted.

2. Discussion

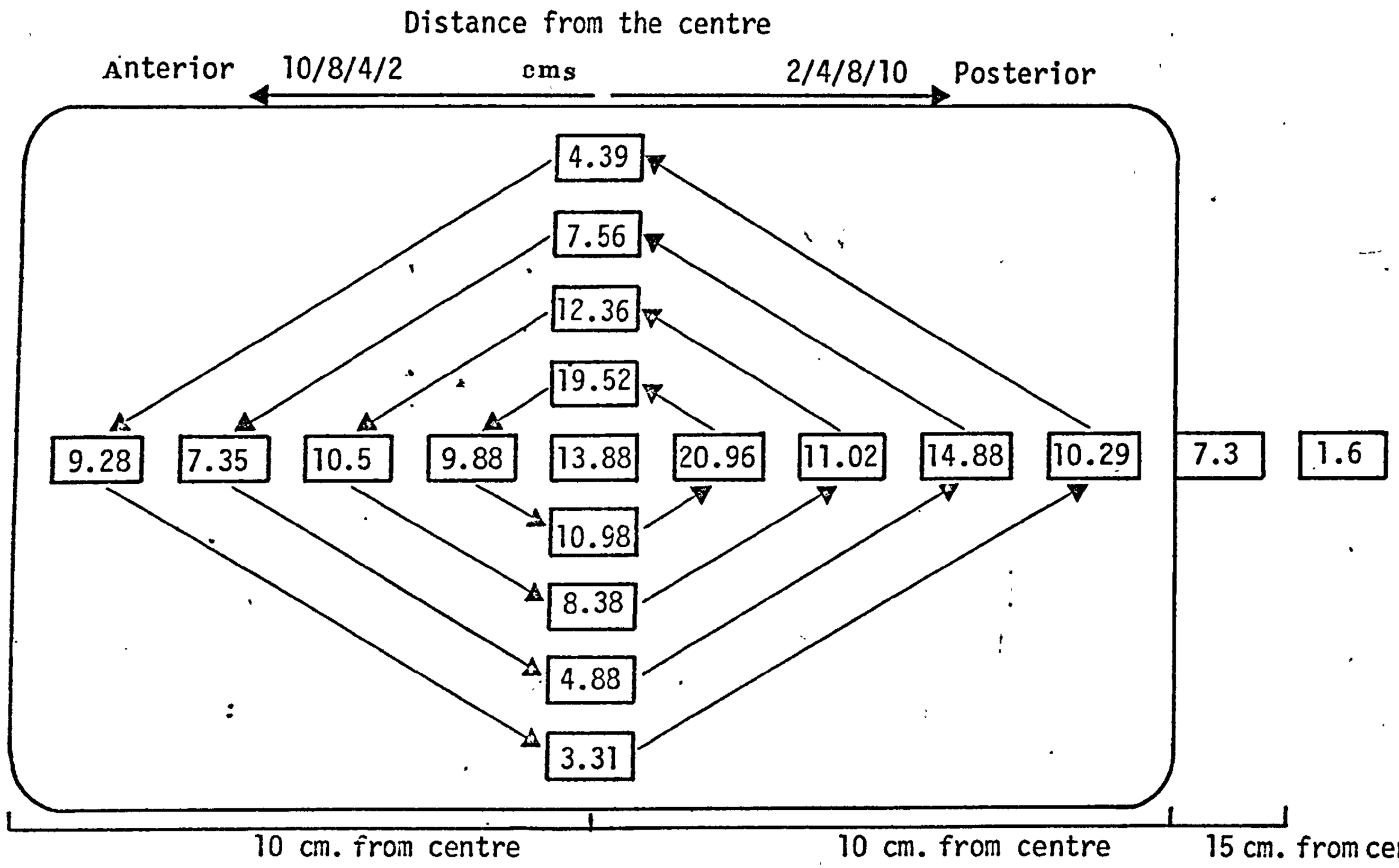
The greatest number of microfilariae counted in all animals examined of different age groups were found in the mid-line of the back at the region of the hump. Few microfilariae per sample were found on the line of the back (Fig. 20). But in places such as ears, umbilicus, chest, head and udder and other sites only occasional microfilariae were encountered, as shown in the Figure.

The result of the studies of the pattern of microfilariae distribution of O. gutturosa in Sudanese cattle reported in the present study confirm that the microfilariae were most numerous in the region of the hump as reported by El Bihari and Hussein (1978) in Sudan; Bain et al. (1978) in France; and Shastri (1978) in India.

In sampling the hump region (Figure 21), the number of microfilariae recovered progressively decreased as sampling moved downwards along the lateral

Fig.21

Distribution of microfilariae of O.gutturosa in the hump of infected cattle



Result of animals (No.of mf/mg)

sides of the hump. However, in all cases the sites of the greater microfilarial counts extended back about 2 cm from the centre of the hump than elsewhere. The fact that the microfilariae of O. gutturosa are restricted to only this area regardless of the intensity of infection should be useful in ante-mortem diagnosis.

A skin snip from the hump region should reveal whether an animal is suffering from O. gutturosa infection or not, even in the case of mixed infection with O. armillata, where microfilariae occur in the same anatomical area. O. gutturosa could easily be identified as will be discussed later.

(c) Relationship between number of adult worms and microfilariae level

The microfilariae of O. gutturosa were found exclusively concentrated in the upper part of the animal's body, particularly in the hump region in Sudanese cattle (El Bihari and Hussein, 1978), with a few in the ears; while no microfilarae were found in the ventral part of the animal.

This finding is in agreement with reports from other parts of the world, e.g. Australia (Bremner, 1955; Ottley and Moorhouse, in press; U.K. (Venkataratnam and Kershaw, 1961); France (Bain et al., 1978); India (Shastri, 1978); Kenya and Somalia (Clarkson, 1964); Tanzania (Mwaiko, 1979); Sudan (Hussein et al., 1975; El Bihari and Hussein, 1978).

The conclusions of Eichler and Nelson (1971), who found that the microfilariae of O. gutturosa in British cattle were concentrated mainly in the ventral part of the animal's body with very few or no microfilariae in samples collected from other regions, including skin from the cephalic part of the animal, are unsupported by the present study in Sudan.

In Sudanese cattle most adult worms are in the connective tissue of the cervical ligament and stifle joint. For the study of the relationship between the number of adult worms and microfilariae, samples were collected from animals with adult worms localized only in the connective tissue of the ligamentum nuchae and without worms elsewhere (gastro-splenic and stifle joint).

All worms were extracted using a digestive technique as described before, and the microfilariae were counted from skin snips taken from the hump of the same animal and counted as mf/mg.

From Table 9 and Figure 22, although the microfilariae relationship is clear, the number of microfilariae cease to increase in animals with more than 45 adult female worms. Although the number of adult worms is still high, the interpretation of this finding could be due to calcification of the worms in old animals.

From the study of the worm burden, the calcification of adult worms was not confined to old cattle only, because calcified worms were found in a 3 year old animal; but the number of calcified worms increases with increasing age.

Figure 22.

Relationship between the number of adult females and the mean number of microfilariae /mg.

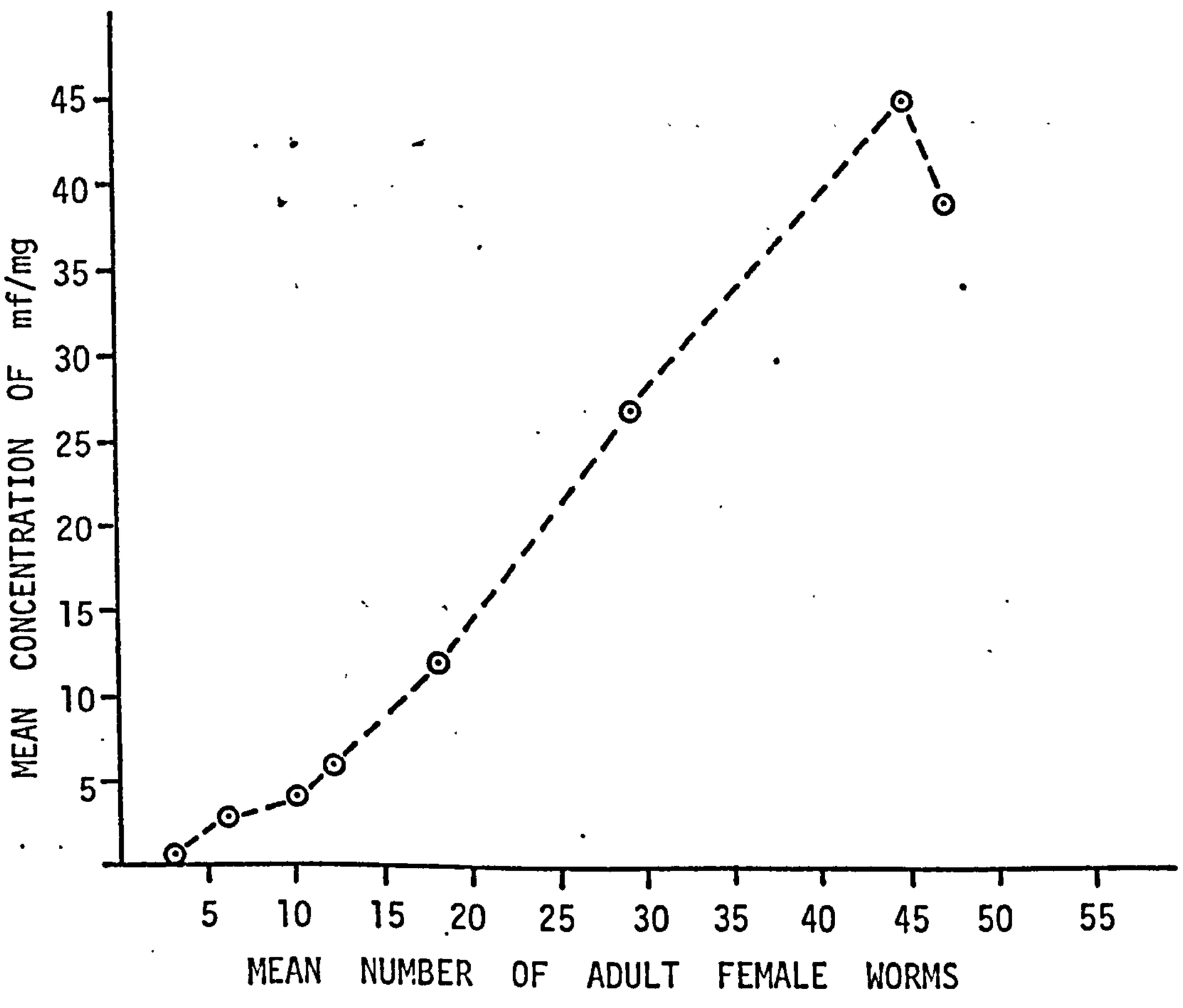


Table 9.

RELATIONSHIP BETWEEN DENSITIES OF MICROFILARIAE, AGE OF THE ANIMAL AND THE NUMBER OF ADULT WORMS

No. of animals examined	Age of animal (years)	No. of adult females		No. of adult males		No. of mf/mg	
		Mean	Range	Mean	Range	Mean	Range
4	10	47	37-54	70	58-72	39	26-43
2	8	45	35-55	60	50-70	45	30-50
2	7	29	28-30	36	35-37	27	20-35
3	6	18	5-37	19	7-32	12	1-40
5	5	12	9-20	12	7-17	6	0.2-22
3	4	10	5-15	13	7-20	4	0.3-12.9
2	3	6	3-10	8	5-13	3	-
2	2	3	3-3	5	4-7	0.2	-

Densites of microfilariae in skin rise progressively with increasing worm burden (Figure 22) but stabilises in the high adult worm infections •possibly there is som sort of im munological reaction between the host and the parasite leading to a decrease in the number of larvae in the skin, either by destroying them or reducing the reproductivity of the adult females and building immunity against new infection.

(d) Relationship between age of animal and microfilariae level

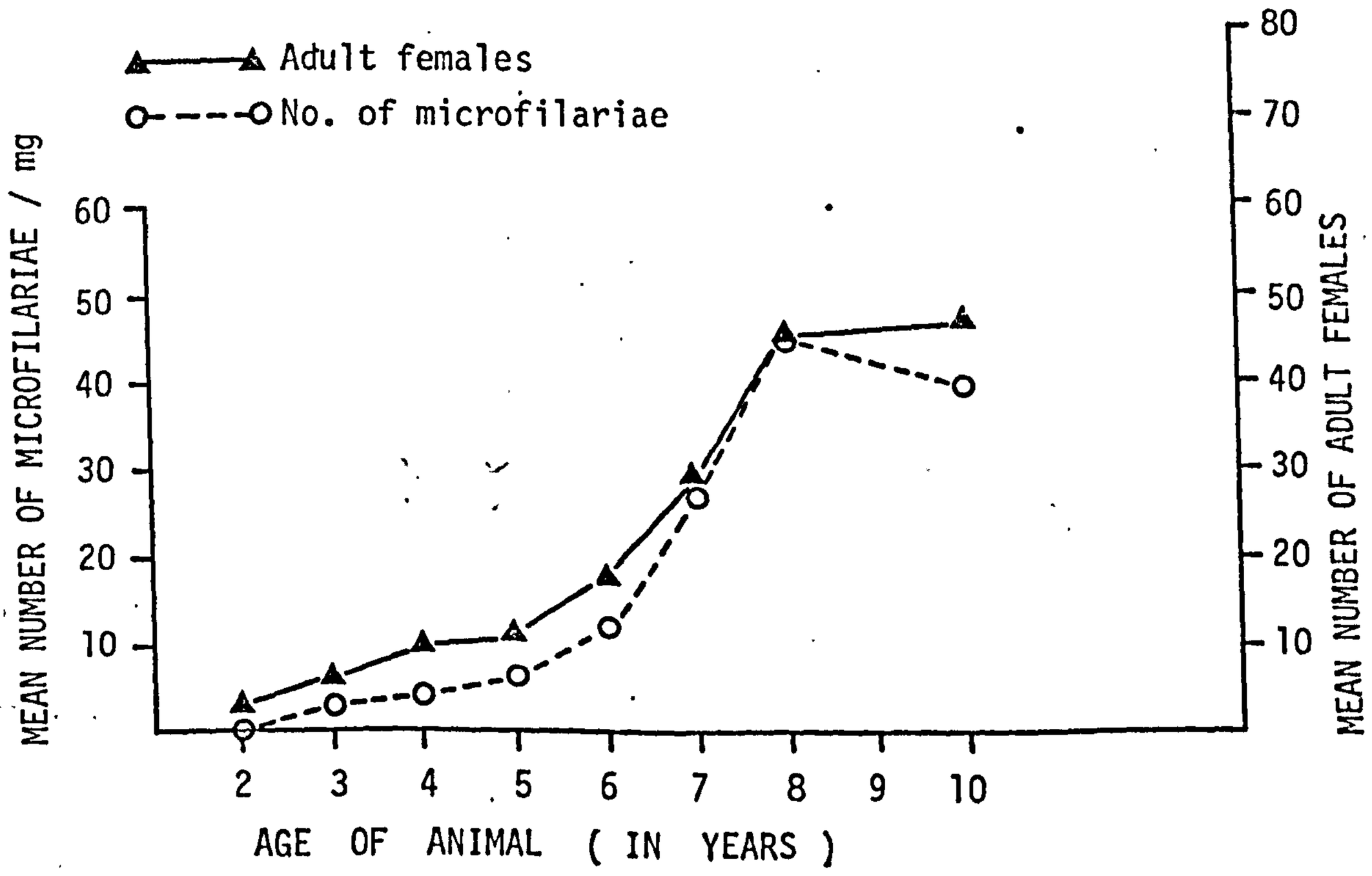
There appears to be a great variation in the numbers of microfilariae within different age groups. From studies carried out in Omdurman abattoir and Kuku and Shambat farms the ages of all animals were estimated by dental examination (Fig. 4) and in the case of Government farms the age as estimated first and then confirmed by the records.

The relationship between age and microfilarial densities is shown in Figure 23. Although the age relationship is clear, the microfilariae numbers cease to increase in the older animal, or sometimes there is a tendency for microfilarial densities in old infected animals to increase very slowly if the animal is above 8 years old; although the number of adult worms is still high.

Full interpretation of this finding must await further work, owing to the small number of observations, but it is possible that if the number of infective bites sustained by

Figure 23.

Relationship between the age of the animals and
(a) The number of microfilariae/mg
(b) The number of adult females



an individual animal living in an endemic area is relatively constant, then the microfilariae densities in the skin would be expected to build up.

Stable counts revealed in some of the older animals may have been due to their recent infection, but this is unlikely as immature adults or young worms of O. gutturosa were never seen in the old animals; also the worms in the cervical ligament of the older cows were very often calcified. The active reproductive life of worms is unknown, but some authors have suggested that the adult worms of O. volvulus can live for 11-15 years (Nelson, 1970; Duke, 1972) and it is probable that factors other than duration of infection play a part in determining the level of microfilariae in the skin, possibly there is a form of immunological balance between host and parasite (Eichler and Nelson, 1971), or it could be due to pathological changes in the skin, making the skin unattractive to the microfilariae.

(e) Histopathological studies on infected skin in cattle

In the present study, adults of O. gutturosa from Sudanese cattle were found mainly in two sites: the stifle joint and ligamentum nuchae. The microfilariae were exclusively concentrated in the hump. The most severe clinical manifestation of O. gutturosa is due to a reaction

to the presence of the microfilariaé. The adult worms are unobtrusive and usually of secondary importance (Nelson, 1970). However, numerous papers have considered in detail the pathological lesion attributed to O. gutturosa microfilariae, as was reported by Niimi and Kouno (1954), Herin et al. (1955), Datta (1939), Dikmans (1948), and Thomas (1958).

1. Materials and Methods

During the period from October 1978 to September 1979, material was collected from infected animals in the Sudan. These cattle were native and were from 9 months to 10 years old; both sexes being represented.

Skin snips were taken from the hump of infected animals; macroscopically the skin appeared normal. From each skin snip small pieces were cut and placed in a drop of Tyrode's medium on a microscopice slide and left for 30 minutes in a humidity chamber to see whether the sample was infected or not.

If infected, the rest of the skin was fixed in 10% formol saline.

From the large number of specimens thus collected, suitable samples were selected for histological examination. Serial sections were cut at 5$^{\mu}$m after processing through paraffin wax and staining with haematoxylin and eosin.

Results

Changes recorded in the skin of infected cases were usually discernible only microscopically. However, occasionally small eruptions were noted in the hump region, presumably induced by flies biting at this site.

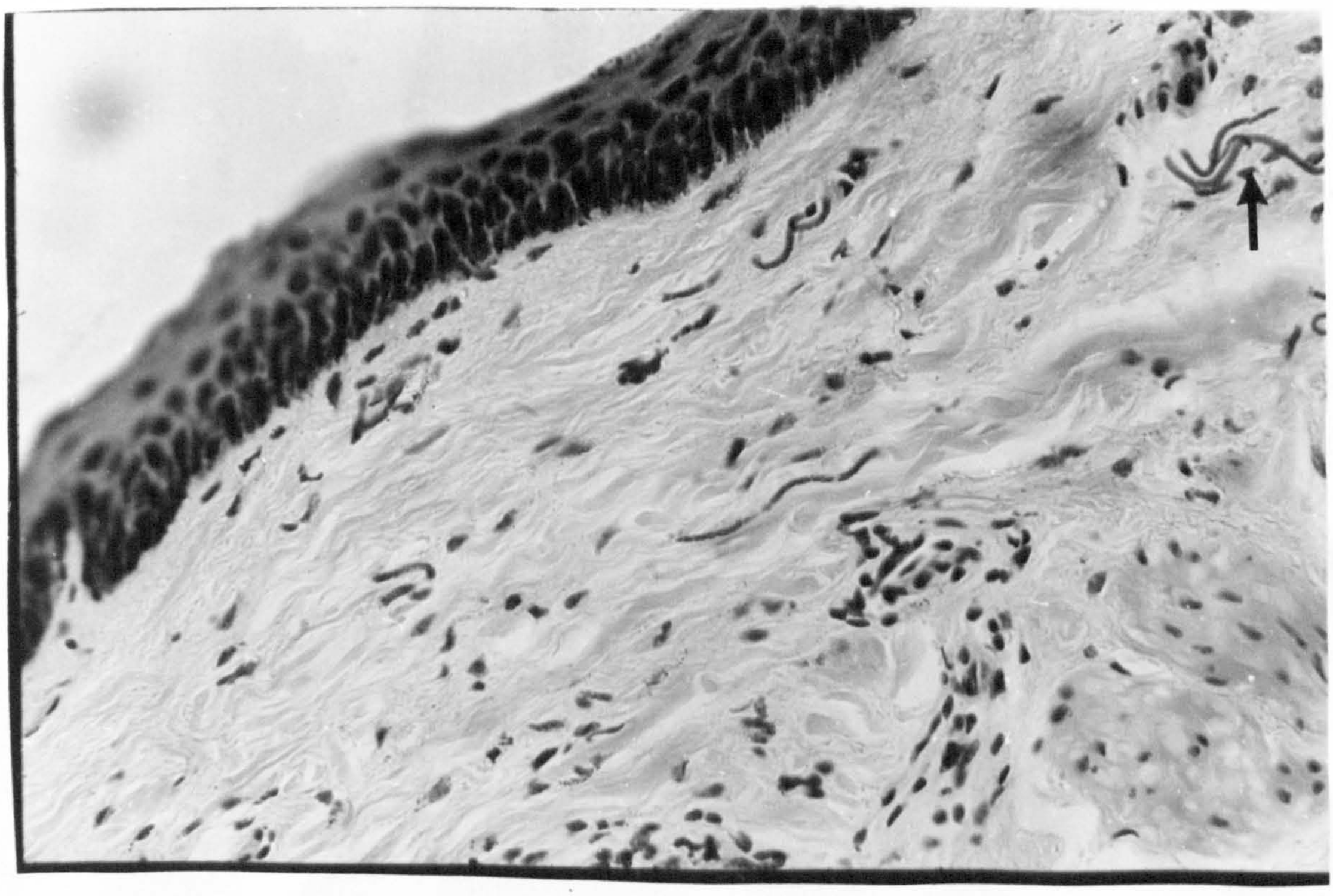
Microscopic examination of the skin of infected animals, on the other hand, showed microfilariae (Fig. 24) in different numbers, mainly in the dermis, tending to concentrate in the upper layer near the epidermis and close to hair follicles. They were also found in small amounts, however in the deeper dermal region and when intact they did not seem to have invoked a significant cellular reaction (Fig. 25). It was, however, not uncommon to find patches of cellular infiltration with fibroblasts, lymphocytes and macrophages and sometimes plasma cells scattered focally in the dermis, usually associated with blood vessels (Fig. 26). In old animals similar chronic focal infiltration was also sometimes seen in the upper layer of the dermis characterized mainly by an infiltration of small round cells.

In some samples this cellular reaction extended from the dermis upward into the epidermis, with inflammatory cells replacing the germinal cell layer. Sometimes micro-filariae were also seen just below the epidermis, very close to such an area of inflammation. Besides faintly-stained microfilariae fragments were often seen embedded in small foci of infiltration composed mainly of phagocytic cells in the superficial part of the dermis.

In some sections, large numbers of intact, as well as fragmented, microfilariae were found scattered in different parts of the dermis within the connective tissue and also in the subcutaneous region. Again, most of those lying in the deeper parts of the skin were free from local reaction, but an intense local inflammation in the superficial layer of the dermis and around small capillaries was sometimes found.

Those microfilariae seen near the dermal connective tissue, in addition to being surrounded by small cells and macrophages, the connective tissue contained microfilariae sometimes showing a more acidophilic homogeneous appearance suggesting some degree of hyaline degeneration. Eosinophils, on the other hand, were hardly seen in inflamed skin, while some of the dermal blood vessels were thick-walled and showed hyperplastic endothelial proliferation. It was to be noted that when there was an inflammatory response in the skin, it was predominantly within the superficial layer of the dermis just below the epidermis and that any microfilariae found in the vicinity of such a reaction had probably degenerated, whereas microfilariae in the deeper dermal layer and subcutaneous tissue were apparently intact and usually free from cellular response. Accordingly, it seemed that the tissue reaction induced by degeneration of microfilariae in the skin produced only a superficial type of dermatitis and this might have partly contributed to the focal thickness, sometimes observed grossly in the upper

Figure 24.



Section of the skin showing the microfilariae concentrated in the upper layer in groups

part of the dermis.

It also suggests that microfilariae located in the uppermost dermal layers were more vulnerable to the tissue reaction than those located in the deeper parts of the dermis.

3. Discussion

Al Zubaidy (1973, Ph.D. thesis) made a comprehensive study of the pathology of O. gutturosa in British cattle. Detailed pathological studies of bovine onchocerciasis due to O. gutturosa was first studied in Sudan by Hussein et al. (1975), with whom the author fully agrees as to the wider prevalence and incidence of the disease. As a matter of fact, aged animals rarely escaped. However, there appear to be no clinical manifestations associated with the presence of the adult stage of O. gutturosa in the nuchal ligament.

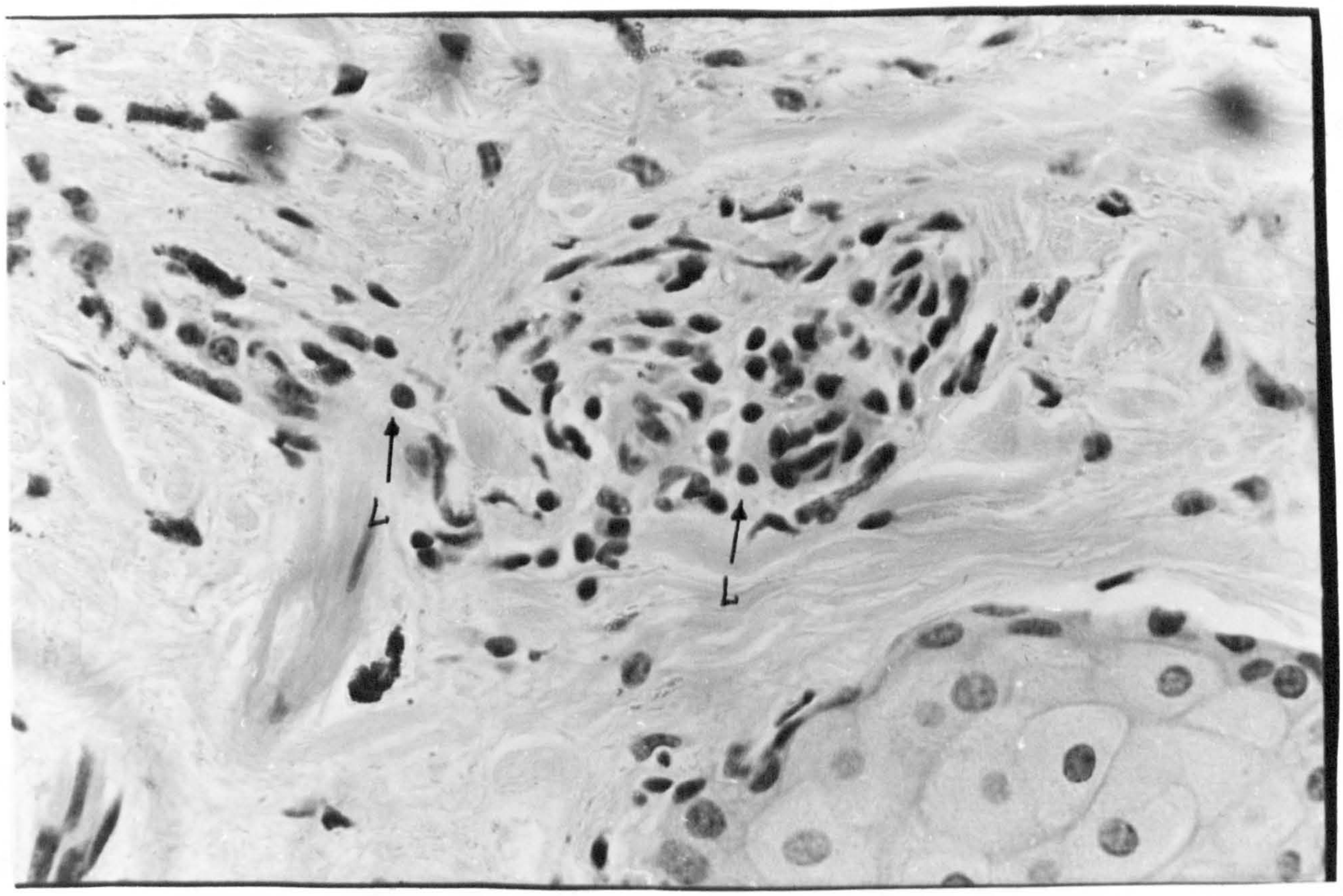
The author agrees with Mohamed (1931), Al Zubaidy (1973) and Cheema (1978) that the live worms seem to invoke no inflammatory reaction, while degenerated worms may be surrounded by round cells, macrophages, plasma cells and eosinophils. This can be described as an acute exudative and chronic granulomatous reaction; these changes may be attributed to the hypersensitivity response of the host animal to repeated infection and foreign body reaction to degenerated and dead onchocercal worms (Nelson, 1970).

On the other hand, the pathological lesions due to

Figure 25.

Section of the skin showing no reaction around microfilariae in the deeper layer

Plate 26.



Section of the skin showing cellular infiltration with fibroblasts(L)lymphocytes and macrophages, and a few plasma cells, associated with blood vessels

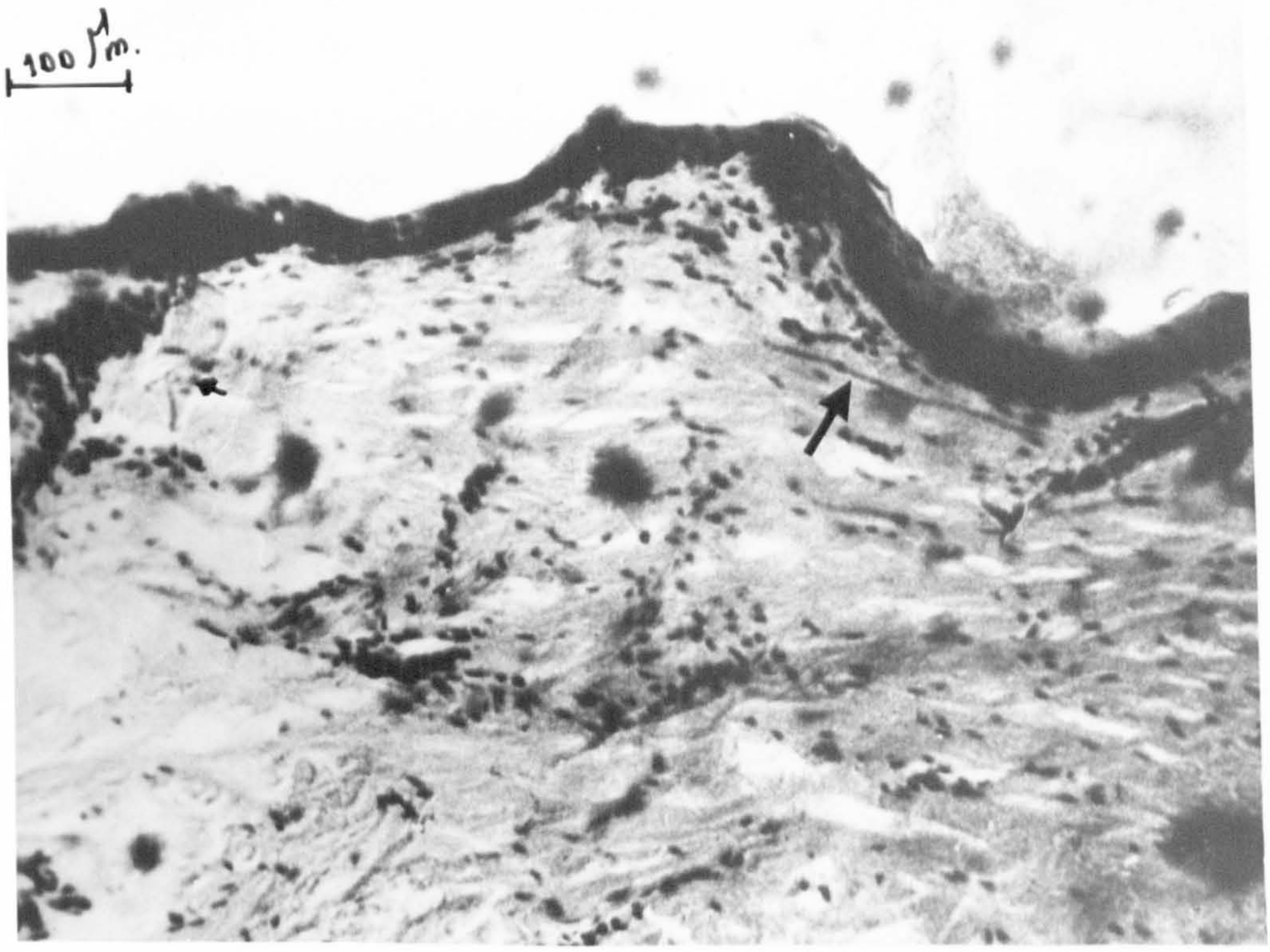
microfilariae such as severe dermatitis responsible for considerable damage to the hide, have been reported by Niimi and Kouno (1954) and Herin et al. (1955). Following the examination of the skins of 384 infected cattle during one year's observation, no serious pathological changes were noticed even in highly infected animals (20.9 mf/mg) attributable directly to the parasite, except small hairless patches at the site of biting flies (hump), and this could be a reaction to the flies (Riek, 1954).

In the skin no neutrophils could be observed, nor any other sign of active inflammation. Also, there was no obvious seasonal fluctuation in the number of microfilariae in the skin, as has been observed in British cattle by Eichler and Nelson (1971) and Al Zubaidy (1973).

The microfilariae were always in the superficial layer of the dermis even during the winter, when the mean temperature is about $12^{o}C$ (Fig. 27).

Thus there is no doubt that mature O. gutturosa and their larvae are responsible for pathological changes in the ligamentum nuchae and skin as observed in the present investigation. There is no evidence, however, that the microfilariae of O. gutturosa reach the eyes of infected cows, as has been observed with the horse species, although a high concentration of microfilariae in zebu cattle are found in the hump.

Figure 27.



Skin section showing the microfilariae in the superficial layer in the middle of winter

(f) Morphology of microfilariae of O. gutturosa

The size variation of microfilariae was studied to investigate a controversy concerning them. El Bihari and Hussein (1976, 1978) reported that microfilariae of O. gutturosa and O. armillata were both concentrated in the skin of the hump. While examining the skin from the hump region of infected animals, I found that the microfilariae were of two types. This drew my attention to the probable presence of two species of Onchocerca in the same animal in most infected cattle in Sudan. Eighty per cent of Sudanese cattle were found to be infected with O. armillata by Hussein et al. (1975) and 95% by Malek (1958) and by Mostafa et al. (1966). Blacklock (1926) reported two different sizes of microfilariae in the uterus of adult worms of O. volvulus and Eichler (1970) found similar differences for microfilariae of O. gutturosa. However, Mellor (1973) failed to find this variation in O. cervicalis microfilariae.

There is controversy concerning whether or not the cervical and stifle joint forms of O. gutturosa are synonymous. Eberhard (1977) described Onchocerca from the stifle joint in USA as a new species, O. stilesi. Webster et al (1977) in Canada and Ottley and Moorhouse (1978) in Australia consider Onchocerca species from the ligamentum nuchae and from the stifle joint are morphologically similar.

All these points necessitated a comparison of uterine and skin microfilariae of O. gutturosa and O. armillata.

1. Materials and methods

To facilitate comparison of microfilariae, it was important to find a suitable method for fixing and staining them.

Two methods were investigated:

(1) The first, which was used by Eichler (1970), is as follows:

A thick suspension of microfilariae in sheep serum is mixed with an equal volume of 2.5% formol saline. Then smears of the suspension are made on microscope slides and allowed to dry. The smears are then stained for 30 minutes in Giemsa and diluted with phosphate buffered water (pH 7.4) in the ratio 1:20.

(2) In the second method, dried smears containing microfilariae were stained with haematoxylin for 15 minutes, followed by washing and blueing in tap water (3 minutes). They were then differentiated in 0.5% acid alcohol before being counter-stained with 1% eosin.

Morphological features of stained microfilariae were clearly shown.

Both groups of stained microfilariae were compared with wet preparations of unstained microfilariae which had been killed by heating (larvae killed by this method lie straight and could be measured quickly). Eichler (1970) and Mellor (1973) indicated that the fixing and staining can cause a reduction of microfilarial length in the order of 13%. In fact, in the specimens measured in this study

this reduction was very small, which should not cause obvious differences in length. Bremner (1955) obtained similar results.

Uterine microfilariae were prepared from three females from the cervical ligament and three from the stifle joint of cattle. The worms were extracted complete and alive by using a collagenase digestion technique (Schulz-Key et al. 1977) (see page 20). In addition, incomplete females from each site were also collected.

It was necessary to obtain worms from both sites in order to determine whether the differences between uterine and skin microfilariae applied to both.

In each case the worms were pooled in Tyrode's solution plus 20% sheep serum in petri dishes and repeatedly cut with a scalpel into small lengths (0.5 cm). They were left for about 30 minutes to allow the microfilariae to emerge. The microfilariae were then separated from the broken adult tissue by sieving. Two suspensions of skin microfilariae were also extracted from the skin of infected cows. The two suspensions were:

1) from cows with adult worms of O. gutturosa in the ligamentum nuchae only;

2) from cows with adult worm in both sites, the ligamentum nuchae and stifle joint.

The skin snips were taken from the hump of the animal after all necessary precautions were taken: removing the

Table 10.

LENGTH AND WIDTH OF MICROFILARIAE OF O. GUTTUROSA

Origin and types of microfilariae	No. of mff measured	Length in microns		Breadth in microns		Extreme ranges, lengths and widths calculated by Eichler (1970) for British cattle	
		Mean ± SD	Range	Mean ± SD	Range	Length (µm) Range	Width (µm)
Uterus of adult female							
Cervical							
small	16	168 ± 5.34	160-173	3.21 ± 0.1	3.12-3.4	180-275	5.75-6.25
large	20	226 ± 16.56	218-231	6.25 ± 0.02	6.2 -6.3	285-325	5.75-7.00
Stifle Joint							
small	26	175 ± 15.8	162-190	3.22 ± 5.88	4.9 -6.4		
large	20	229 ± 11.21	218-248	5.88 ± 0.7	3.13-3.9		
Skin	40	227.15 ± 26.09	186-264	3.4 ± 1.09	3.13-3.9	175-240	4 - 8

dust and mud and the hair shaved. The skin was then cut into small pieces to facilitate the emergence of microfilariae, placed in a medium of Tyrode's solution plus 20% sheep serum, and left for 8 hours. Then the suspension was centrifuged and thick and thin smears were made on microscope slides. All measurements were made using a micrometer eye piece under X400 magnification.

2. Results

Table 10 shows a comparison of the length and width of O. gutturosa microfilariae from the skin and uterine microfilariae from both stifle and cervical ligaments.

The author is in agreement with Eichler in dividing the microfilariae of O. gutturosa into three classes: 'large' and 'small' uterine microfilariae and skin microfilariae. This division of uterine microfilariae was not evident in O. cervicalis (Mellor, 1973). In order to elucidate this division in uterine microfilariae and to ensure that Onchocerca recovered from the stifle joint and those recovered from the cervical ligament were the same species, the length and width of 'small' forms of cervical and stifle microfilariae were compared with each other; in both cases there was no significant difference.

Using the same technique for significance, the

length and width of 'large' and 'small' forms of cervical and stifle joint microfilariae were compared and were found to be significantly different in both cases. Comparison of the length and widths of 'small' uterine forms were made with those of skin microfilariae and these were also found to be significantly different.

3. Discussion

A) Uterine microfilariae:

The size range of both uterine forms of microfilariae from cervical and stifle joint worms does not differ greatly from those recovered by Eichler& Nelson (1971) from British cattle, especially the 'small' forms which were 180-275 μm x 3.75-6.25 μm and 285-335 μm x 5.15-700 μm 'large' forms. This is clearly slightly longer and broader than 'large' forms of Sudanese cattle microfilariae.

The 'large' forms of uterine microfilariae closely resemble embryos in the last stages of development before hatching from the egg. This suggests that the large forms of microfilariae are immature. Small ↑ Microfilariae were recovered from the anterior part (3 cm) and the vaginal end of the uterus of adult females, indicating that these types of microfilariae are presumably mature, and the absence of large forms in these sites of the uterus of the adult female ensures that the large forms are immature forms.

There is no clear intermediate form of microfilariae, as was observed by Eichler (1970), suggesting that the change from the 'small' form to the 'large' form must be

relatively rapid.

B) Skin microfilariae:

Only one form of microfilariae was found in the skin and this is longer than the small forms obtained from the uterus of adult worms. El Bihari and Hussein (1978) concluded that skin microfilariae of O. gutturosa were longer than uterine forms, and the present study confirms the finding of these authors.

There are several published accounts of species of Onchocerca in which comparisons have been made between uterine microfilariae and those available to the vector. Eichler (1970) indicated that the skin formsare shorter than the small forms obtained from the uterus. Bremner (1955), from his measurement of uterine and skin microfilariae, concluded that the larvae do not alter in their body length between the time of emergence from the female and when they appear in the skin.

A similar conclusion was reached by Gibson (1952), who studied microfilariae of O. gutturosa, O. reticulata and O. volvulus, and Chodnik (1957), who studied the differences in the microfilariae of O. armillata.

The skin microfilariae have a general appearance similar to the 'small' forms of uterine microfilariae, the only clear difference being in the length, as the skin microfilariae are longer.

The occurrrence of this morphological difference between skin and uterine microfilariae must be taken into

account when trying to relate microfilariae to their parent worms. If microfilariae of an unknown type are found in the blood or skin of an animal, differences between them and uterine microfilariae subsequently recovered from adult worms, does not necessarily mean that the animal was harbouring more than one species of parasite. It is therefore important to have a clear understanding of the morphology of both skin and uterine microfilariae.

To ensure that the maturity change occurs in the microfilariae during their migration from the uterus of the adult females to the skin before being infective to the vector, was confirmed by the study of the comparison of uterine and skin microfilariae with respect to their infectivity to their natural intermediate host.

Adult females of S. ornatum pupated in the laboratory were infected with two types of microfilariae of O. gutturosa of the British strain by the intra-thoracic injection technique. The first group were derived from the uterus of adult female worms and the second group was from the skin of infected cattle to serve as a positive control. By intra-thoracic injection of 20-40 larvae per fly suspended in a minimum quantity of Tyrode's solution and cow serum, the two groups of infected flies were maintained in an insectary at 23°C and 85% relative humidity on 10% sugar solution. All surviving flies were examined 9 days after infection. Almost all flies infected with skin microfilariae contained third stage larvae in the head and in the

abdomen (with 3-40 larvae per fly). However, those infected with uterine microfilariae contained no developing larvae, although active microfilariae were recovered from the head, thorax and abdomen.

It is concluded that microfilariae in the uterus of the female worms have not yet acquired the capacity to develop further in their intermediate host. Apparently this property has been gained by the time that the parasites have migrated to the skin 4-8 weeks later, as estimated by Duke (1968) for O. volvulus.

4. General morphology of skin and uterine microfilariae

Studies were made initially on microfilariae freshly emerged from females, dissected from cattle within one hour of slaughter and placed in sheep serum for 30 minutes at room temperature. Isolated microfilariae remained alive for a considerable time when kept in medium containing serum (16 hours).

A) Materials and methods

All samples for morphological studies were prepared as described above (page 99).

In fact accurate and detailed morphological study of microfilariae will be very difficult unless good stained preparations are obtained. Staining with Giemsa's method gives excellent demonstration of internal structures of microfilariae and also shows fine cuticular striation.

The 'small' form of uterine microfilariae from the cervical and stifle joint worms showed no definite and constant differences, so they can be considered as one type. This form of microfilaria representsthe smallest size form of the three, having a blunt rounded anterior end and finely tapering tail. The measurement showed the mean body length to be 168 μm (minimum 160 μm, maximum 173 μm) with a mean thickness of 3.21 μm.

5. Comparative morphological studies on microfilariae of O. gutturosa, O. armillata and O. raillieti

The genus Onchocerca is represented in Sudanese cattle by O. gutturosa. (Neuman, 1910) found in the cervical ligament and connective tissue of the stifle joint, and O. armillata found by Railliet and Henry (1909) in the aortic intima.

Observations commenced in the Sudan some years later when Hussein et al. (1975) demonstrated that a large proportion of cattle infected with O. gutturosa were also infected with O. armillata, and the prevalence appeared high (86%).

The microfilariae of these two species occur in the skin and are concentrated at the same place (hump), as reported by El Bihari and Hussein (1978) and confirmed in the present study. Ninety per cent of Sudanese donkeys were found to be infected with O. raillieti (Saad El Din, personal communication).

The presence of three species of Onchocerca in the Sudan means that a method to identify the microfilariae of each is essential for any meaningful studies on their distribution in the skin and on their transmission. It is possible that the same vector may be involved in the transmission of all three species and therefore it is important to be able to differentiate the stages of the three species in the vector. The problem is that it is not possible to differentiate between the developing larvae, so the study must be concentrated on the microfilariae.

In 1978 an attempt was made to study the life cycle of O. gutturosa in Culicoides kingi. It was necessary to investigate wild caught flies for the infection experiments.

The vector of O. gutturosa feeds freely, particularly on donkeys and cattle.

Animals harbour microfilariae in superficial layers of their skin (Fig. 26), presenting the possibility that the naturally infected flies of the area could have acquired their infection from any one of these three species of Onchocerca. It was therefore deemed advisable to make a comparative study in order to allow proper interpretation of further study on the development of O. gutturosa in wild C. kingi flies.

A) Materials and methods

Skin snips were taken from the hump region for O. gutturosa and O. armillata and skin snips from the ventral parts

of infected donkeys for O. raillieti. Samples for morphological studies were prepared by methods similar to those described for the comparison between skin and uterine microfilariae.

The relative position of the cephalic space, the position of the nerve ring, length and width, besides extent of caudal and cephalic nuclei arrangement were recorded. The number of nuclei between the cephalic part and the nerve ring were used as a method for differentiation.

Measurements of microfilariae were made with the assistance of an eye piece micrometer and the drawing was made with the aid of a Camera Lucida (Figure 28).

Specific identification of microfilariae found in the skin of the host was established by comparing them with microfilariae taken from the uteri of gravid Onchocerca females obtained from the cervical ligament (O. gutturosa), or aorta (O. armillata) of the cow and the cervical ligament of the donkey (O. raillieti).

B) Comparison between O. gutturosa and O. armillata

The number of microfilariae of O. gutturosa and O. armillata in a fresh preparation of the skin of the hump was studied. This confirmed that the microfilariae of O. armillata, like other species in the genus, are skin dwellers. They are most numerous in the skin of the hump, and it seemed that Culicoides is likely to be the vector of Onchocerca armillata as well as of O. gutturosa.

The number of microfilariae recovered from 15 animals were examined in mixed infections of O. gutturosa and O. armillata and the results are shown in Table 11. As can be seen, in all cases microfilariae of O. gutturosa were often present in large numbers (96%), while those of O. armillata were few in number (4%). Similarly to observations made by Shastri (1978), it was found that the microfilariae of both species could easily be identified by their size, width and the appearance of their extremitites (Figure 28), O. armillata were longer and broader and had characteristic cephalic dilatation (Table 11).

Table 11.

THE NUMBER OF MICROFILARIAE PER SNIP OF BOTH SPECIES IN THE SKIN OF THE HUMP

No. of animals	Number of microfilariae per mg					
	O. gutturosa			O. armillata		
	Mean ± SD	Range	%	Mean ± SD	Range	%
15	18.5±12.27	2.2-43.5	96	0.66±0.18	0.2-1.46	4

6. Comparison of the number of nuclei between the cephalic space and the nerve ring for O. gutturosa, O. armillata and O. raillieti.

Schacher et al. (1967) used this technique as a test for intra-specific grouping and evaluation in Wuchereria bancrofti,(and erected a new species W. lewisi); Eichler (1970) used thesame technique for comparison between O. volvulus, O. gutturosa and O. cervicalis and the result showed highly significant differences in all cases.

The result suggested that it would be valuable to see if the number of nuclei between the nerve ring and the cephalic space of microfilariae constituted distinguishing features between O. gutturosa, O. armillata and O. raillieti. The nuclei of O. armillata and O. gutturosa are generally less condensed and somewhat widely separated, but the nuclei of O. raillieti were often difficult to separate, particularly in the region between the cephalic space and the nerve ring because they were close to each other and coalesced. In spite of this we managed to count the number with some difficulty.

It is not difficult to make a reasonably accurate count of the number of nuclei anterior to the nerve ring when a high power is used (oil immersion). Counts of anterior nuclei were made after making the mean of three counts of the same microfilariae for the larvae of O. gutturosa, O. armillata and O. raillieti. The results are shown in Table 12.

Table 12.

NUMBER OF NUCLEI BETWEEN THE CEPHALIC SPACE AND NERVE RING OF MICROFILARIAE OF O. GUTTUROSA, O. ARMILLATA AND O. RAILLIETI

Type of microfilariae	No. of mff examined	No. of nuclei Mean ± SD	Range
O. gutturosa	20	62.17 ± 6.32	54 - 74
O. armillata	15	56.13 ± 3.5	49 - 62
O. raillieti	20	37.625 ± 2.12	33 - 39

It is concluded from this study that the number of nuclei between the nerve ring and the cephalic space of microfilariae can form a basis for distinguishing between the two species, O. gutturosa and O. raillieti as they show no overlap between the range.

7. The arrangement of cephalic and caudal nuclei

Gibson (1955), Bremner (1955) and Eichler (1970) used the variation in form, size and arrangement of the cephalic and caudal nuclei as criteria for separating the different species they studied.

In the present study the same technique was used in studying the differences in the three species. All the figures were drawn from stained microfilariae with Giemsa's

stain.

O. armillata :

The cephalic end of the microfilariae was distinctly wider than the rest of the body (Fig. 28) which allowed it to be separated from the other two species. The most anterior nuclei are somewhat variable, consisting mainly of single anterior nuclei, followed by a single row in the middle of the larvae, and then there is a big space between the first nucleus and the anterior part (about 10 μm).

O. gutturosa :

The cephalic part has two large anterior tandem nuclei, followed by a single large nucleus in the mid-line of the larvae. But very few larvae had a single anterior nucleus, and the space between the first nucleus and the anterior end was about 5 μm (Fig. 28).

O. raillieti :

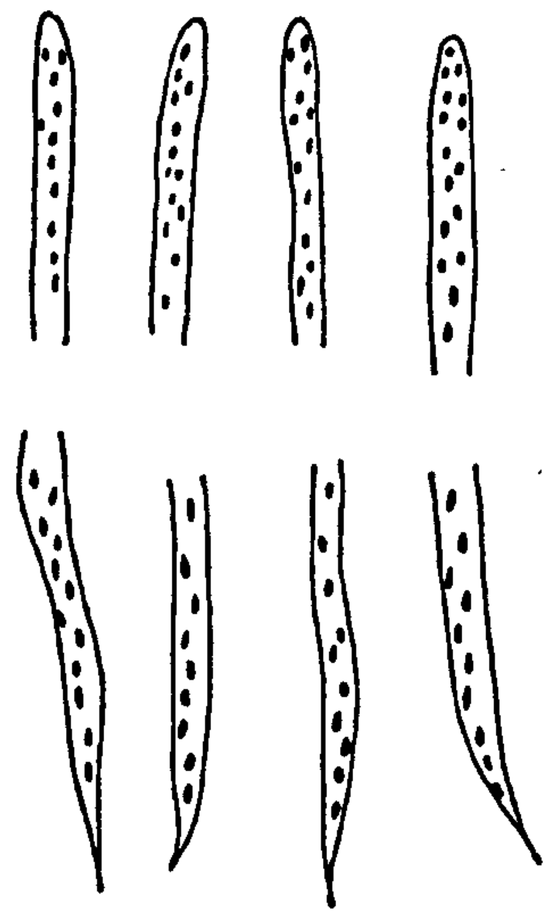
These usually have a large solitary nucleus followed by a tandem pair and the cephalic space is very small (about 2 μm) (Fig. 28).

All the terminal nuclei of O. armillata are equal in size but the last one is constricted in the middle and considerably longer than the preceding two ; the tip of the tail was also blunt.

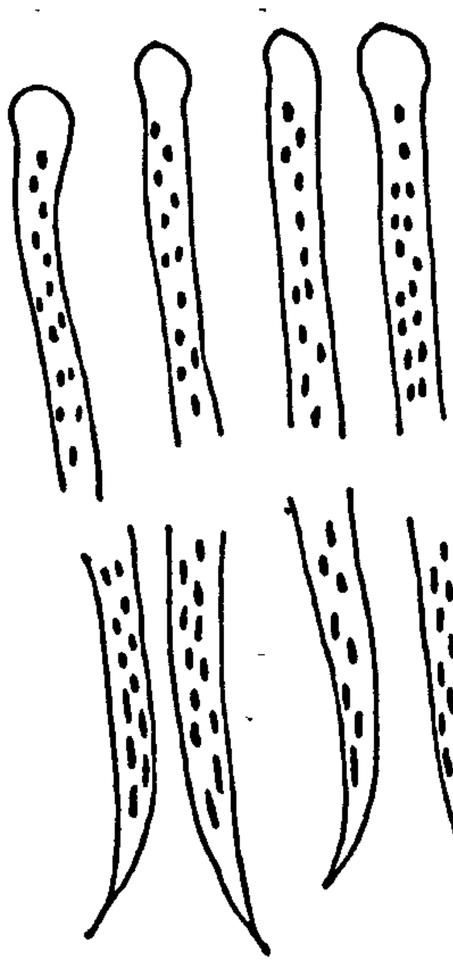
The caudal nuclei of O. gutturosa conformed to the arrangement and form described by Gibson (1952) and Bremner

Figure 28.

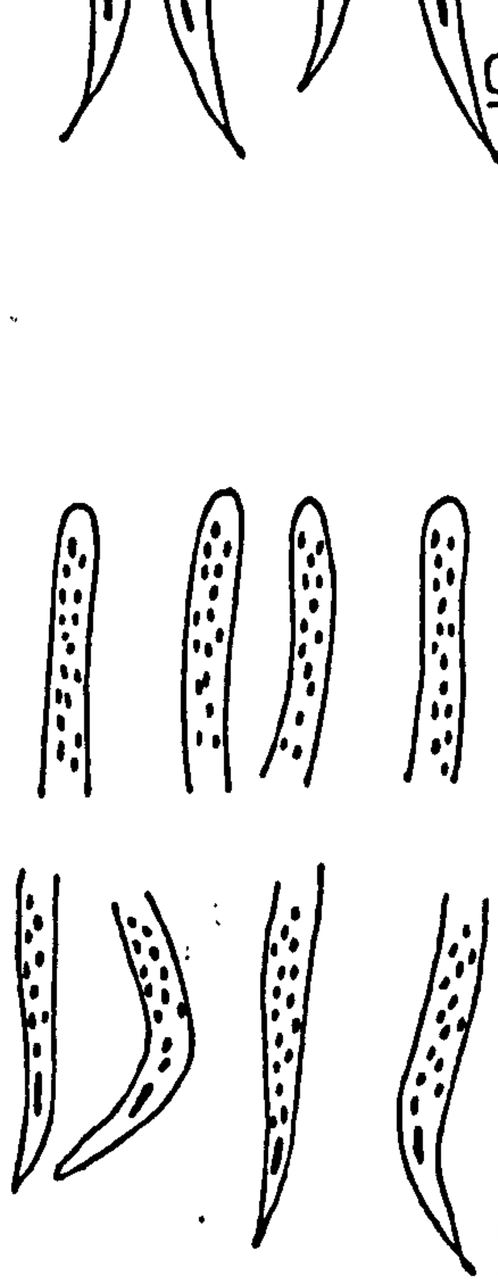
ARRANGEMENT OF CEPHALIC AND CAUDAL NUCLEI



O.raillieti



O.armillata



O. gutturosa

(1955) in having a long terminal bar consisting of four tiny discrete nuclei (Figure 28) and this differing from the other two species. The last nuclei are large and somewhat triangular in shape, preceded by two large nuclei in the middle and smaller in size than the last one.

In O. raillieti, the terminal tail nuclei are large and irregular in shape and often preceded by three relatively smaller nuclei. All terminal nuclei gave the impression of being more darkly stained than the others.

8. Measurement

Detailed morphological studies of microfilariae are possible with stained preparations.

All measured microfilariae were fixed and stained with Giemsa's stain; the preparation and method as already outlined.

The dimensions of the important locations of the three species of microfilariae obtained from 40 mff of O. gutturosa, 30 mff of O. armillata and 20 mff of O. raillieti are given in Table 13.

From their morphological appearance, microfilariae of O. armillata were readily distinguishable from the other two species by their greater length, width, large cephalic space, blunt rounded anterior end and tapering finely pointed tail.

The microfilariae of O. gutturosa and O. raillieti were also easily distinguishable from each other. O. gutturosa microfilariae were longer, but thinner and had a

Table 13.

MEASUREMENTS OF SIGNIFICANT STRUCTURES IN MICROFILARIAE OF O. GUTTUROSA, O. ARMILLATA AND O. RAILLIETI

Structure	Measurements in micron of the three species		
	O. gutturosa	O. armillata	O. raillieti
	Mean ± SD	Mean ± SD	Mean ± SD
Body length	227 ± 26.8	340 ± 29.5	208 ± 10.8
Width	3.4 ± 1.09	5.13 ± 0.8	4.3 ± 0.5
Anterior to the nerve ring	55.3 ± 2.9	87.8 ± 11.17	48.9 ± 4.5
Tail	5.6 ± 1.2	6.5 ± 0.95	7.6 ± 0.75
Cephalic space	4.0 ± 1.0	7.0 ± 1.2	2.4 ± 0.5
No. of microfilariae examined	40	30	20

bigger cephalic space and greater number of nuclei anterior to the nerve ring. The nerve ring is situated in a more posterior position and the size, form and arrangement of anterior and posterior nuclei are quite distinctive from the O. raillieti microfilariae. O. raillieti are shorter and broader than O. gutturosa and the most prominent feature was the nerve ring which was sharply clear and placed near to the cephalic space and always at an oblique angle. The cephalic space was always very narrow. The G. cells and anal pore, which are clearly visible in microfilariae of O. gutturosa and O. armillata, could not be detected in some microfilariae of O. raillieti.

9. Remarks :

The present study was based on a large collection of microfilariae recovered from two hosts, cattle and donkeys, and revealed many variations.

The measurements of O. armillata reported in the present study agreed with the range reported by Buckley (1938), Patnaik (1962), El Bihari and Hussein (1978) and Shastri (1978). They indicate that O. armillata microfilariae are the largest in the group including O. sweetae, O. ochengi, O. gutturosa, O. gibsoni, O. volvulus and O. cervicalis (Spratt et al., 1978; Bwangamoi 1969; Gibson, 1952; Bremner, 1955; Mellor, 1974).

Gibson (1952) and Bremner (1955) concluded that the microfilariae of one different species which they studied

could be readily distinguished by the form, size and arrangement of cephalic and caudal nuclei. This conclusion is quite applicable to the microfilariae of O. armillata, O. gutturosa and O. raillieti.

CHAPTER 3

ONCHOCERCA GUTTUROSA NEUMANN, 1910

STUDIES OF THE LIFE CYCLE IN SUDAN

Onchocerca gutturosa was first reported from Sudan by Mohamed (1931); and subsequently by Hussein et al. (1975) and El Bihari and Hussein (1978).

The literature shows, however, that O. gutturosa has a much wider distribution in Africa, especially in those countries neighbouring the Sudan (Fig.28) such as Egypt (Mohamed, 1931); Uganda (Bwangomoi, 1970); Ethiopia, Chad and Central Africa (El Bihari and Hussein, 1978); Kenya and Somalia (Clarkson, 1964); and Tanzania (Mwaiko , 1979).

In spite of the wide distribution and high prevalence of this parasite, the vector is still unknown in Africa, except for some speculative studies reported by Wegesa (1966) and Mwaiko (1979).

The mode of transmission of various species of bovine onchocerciasis has been a subject for speculation and experiment for many years. A detailed historical review of previous work is unnecessary here as it has been reviewed by Mohamed (1931) and nothing has been added since.

The life cycle of O. gutturosa in Simulium ornatum and O. cervicalis in C. nubeculosus was worked out by Steward (1933-1937) in England. Recent work on S. ornatum

and C. nubeculosus at the London School of Hygiene and Tropical Medicine's field station at Winches Farm, St. Albans, have confirmed this and shown that these species are the local vectors of O. gutturosa (Eichler, 1973) and O. cervicalis (Mellor, 1974).

Buckley (1938) first described the development of O. gibsoni in Culicoides pungens in Malaya. Spratt et al. (1978) concluded from their observations, that Culicoides "sp. M" is a natural intermediate host of Onchocerca sweetae in the Northern Territory of Australia.

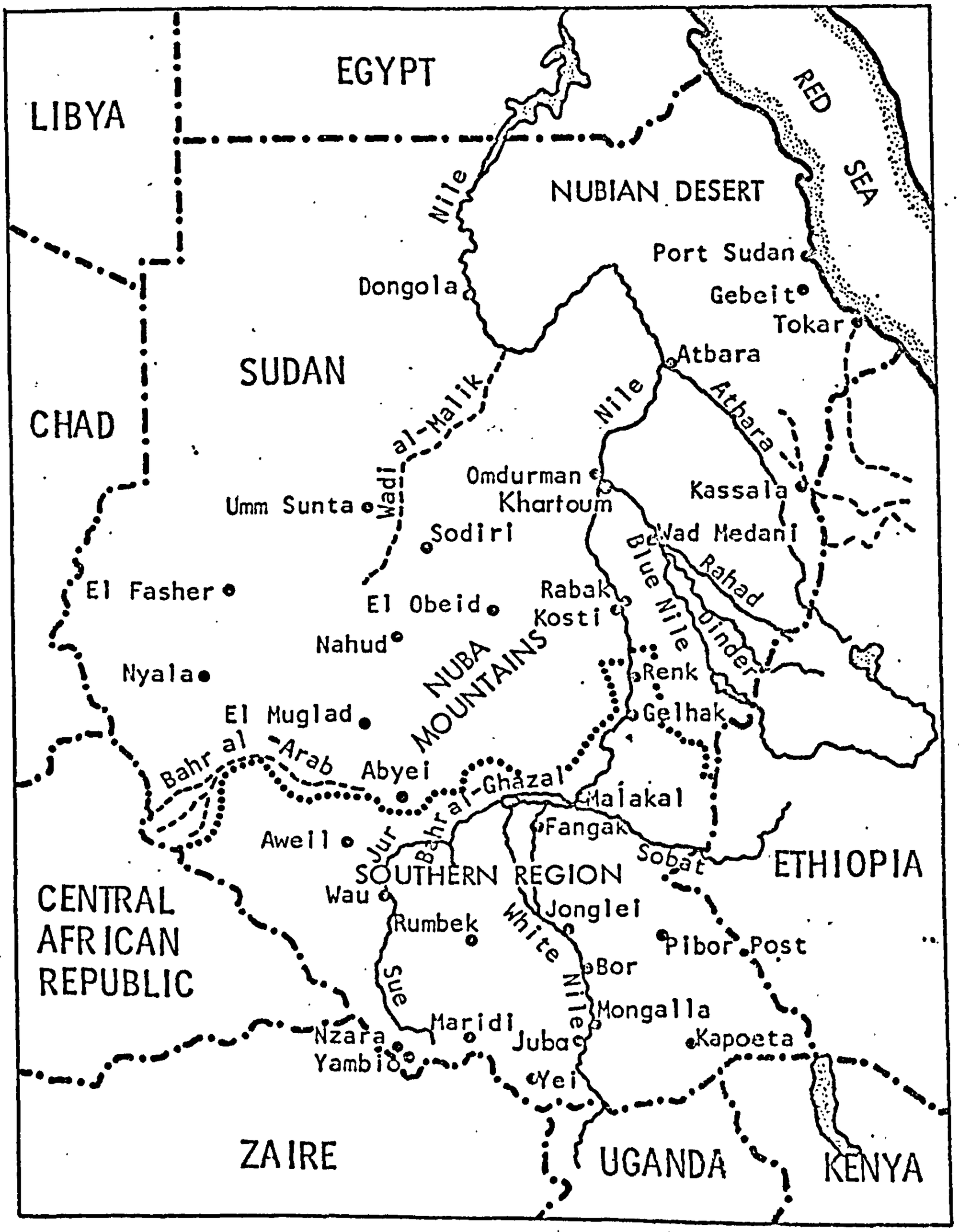
Bain (1979) in France suggested that O. gutturosa is more likely to be transmitted by Culicoides than Simulium as reported in England, as the microfilariae occurred in the back skin of the animal.

Mwaiko (1979) in studies of O. gutturosa in Tanzania, suggested S. vorax and S. nyasalandicum as possible vectors for bovine and human onchocerciasis. Wegesa (1966) observed S. vorax feed in large numbers on cattle, which it bites in the ears. Apart from the suggestions of Mwaiko (1979) and Wegesa (1966), there have been no further studies on the life cycle of animal onchocerciasis in Africa.

Onchocerca gutturosa was reported in the Sudan a long time ago (Mohamed, 1931). However, the first detailed studies were carried out by Hussein et al. (1975) and El Bihari and Hussein (1978), but there have been no further reports on the vector.

Figure 28.

The Democratic Republic of the Sudan



The current study presents field and laboratory investigations in the Shambat area (Khartoum North), to determine the method of the spread of the disease.

A substantial number of cattle farms have been established recently around Khartoum to meet the high need for both meat and milk. The Shambat farm of the University of Khartoum's Faculty of Agriculture and Veterinary Science, has a relatively large population of resident cattle, generally between 200 - 400. They share the station with at least 50 - 100 sheep and a few horses and donkeys. Unfortunately the farm is located in an area with high densities of Simulium griseicolle (El Bashir et al. 1976) and Culicoides kingi (El Bihari, personal communication). Both species are the dominant species in the area. Fifteen species of Simulium were reported in the Sudan (Lewis, 1948) and at least 12 different species of Culicoides (Kirk, 1957); but Culicoides kingi was not included in the list because Khartoum was excluded from the survey.

Culicoides usually appears in large numbers during the rainy season (July, August and September). The condition for C. kingi was favourable for breeding and the extension of animal onchocerciasis when the geographical and climatic conditions are good. Waste products often become collected in refuse piles in the neighbourhood of the animals and remain for varying periods of time. This attracts many flies, the larvae of which may live in these heaps (Edward et al., 1939; Downes, 1950).

However, the species of Culicoides which occur in this region and which are concerned in the transmission of onchocerciasis are abundant (El Bihari, personal communication). Hence it was evident that Shambat and the adjacent areas not only furnish conditions which are excellent for the breeding of C. kingi, but that they also constitute localities in which most favourable opportunities exist for the flies to come into contact with a large number of animals.

Several newly-born animals had recently suffered from infection (Fig.29). This was supported by finding naturally infected flies in the same area and also by finding the rate of infection among calves up to one year old was about 9%, in the farm in which our work was especially performed.

Hence in the present study an attempt was made to investigate the flight and biting pattern of the vector, and to determine the effects of climatic factors on such activities; however, it is not proposed to deal with the purely entomological aspects concerning bionomics and taxonomy of the vector. These aspects have been extensively reviewed by Edwards et al. (1939) and by Khamala and Kettle (1971).

A. The vector of Onchocerca gutturosa in the Sudan

Onchocerca gutturosa is widespread in Sudanese cattle, but the vector is yet to be determined (El Bihari and Hussein, 1978).

Figure 29.

Newly-born calves at Shambat farm. Up to one year old, 9% were found to be infected with O. gutturosa

A vector of O. gutturosa in Britain was reported to be S. ornatum (Steward, 1937; Eichler, 1973b). However, Bain (, 1979) has suggested that these workers were actually working with O. lienalis, while O. gutturosa is more likely to develop in a Culicoides species. This is based on the observation that in some cases the microfilariae of O. lienalis congregate in the skin of the umbilicus (the feeding site of S. ornatum), while those of O. gutturosa concentrate in the shoulder and head region. The microfilariae in Sudanese cattle are similarly located (El Bihari and Hussein, 1978) and it is suggested that a Culicoides species rather than a Simulium species is the vector of bovine onchocerciasis in the Sudan.

The predominant Simulium species in the Shambat area of Khartoum North in the Sudan, is S. griseicolle (Lewis, 1948; El Bashir et al., 1976), which is known to feed on birds and is commonly observed feeding everywhere around the umbilicus and perineum of cattle, and can also be a troublesome bird biter (Lewis, 1948). Crosskey and Crosskey (1958) have seen infective larvae in S. griseicolle, but it is doubtful if these were of O. volvulus because no evidence was found that the flies bite man. In contrast, Culicoides kingi prefers the hump region of cattle as a biting site. Although Hussein et al. (1975) reported O. gutturosa microfilariae from the umbilicus, this was not confirmed in a more detailed study (El Bihari and Hussein, 1978).

The ability of both insects to transmit O. gutturosa is being investigated and the results are presented here.

(á) Materials and Methods:

This work was carried out at the University of Khartoum farm at Shambat. The infection rate among milking cows was estimated to be 75% (El Bihari and Hussein, 1978). Most of these animals were born and reared on the farm, with no history of ever leaving Khartoum. It was apparent that most cattle are infected with O. gutturosa locally and the high percentage of infection indicated that the parasite was being freely transmitted.

Adult flies were periodically collected during the course of this investigation. The Culicoides were kindly identified by Dr. J. Boorman of the Animal Virus Research Institute, Pirbright, Surrey, England. The investigation of which an account is given here was commenced in 1978 and continued through 1979.

In order to ensure that cattle from which insects were collected were infected with O. gutturosa, skin snips were examined for microfilariae. The choice of certain animals for use in experimental work with a vector depended upon the degree of infection with microfilariae in the skin, for it was thought that the insect which fed upon cattle whose skin was heavily charged with microfilariae would naturally be more likely to pick up some of those during feeding. Accordingly an attempt was made to measure the

degree of skin infection by counting the number of microfilariae which emerged from the skin snip cut from the living animal. All the animals on Shambat farm were examined during the survey.

Examination of the animals in spite of variations in the microfilarial distribution in the skin, revealed that certain animals were more heavily infected with microfilariae than others, and two of these were employed for the routine collection of feeding insects and in the experimental studies. The two cows were seven and nine years old, and the concentration of microfilariae was 27 and 30 mf/mg respectively.

The microfilariae from the skin were identified as those of O. gutturosa. It can be safely said, therefore, that the predominant microfilariae infection in cattle in Shambat farm is derived from adult O. gutturosa.

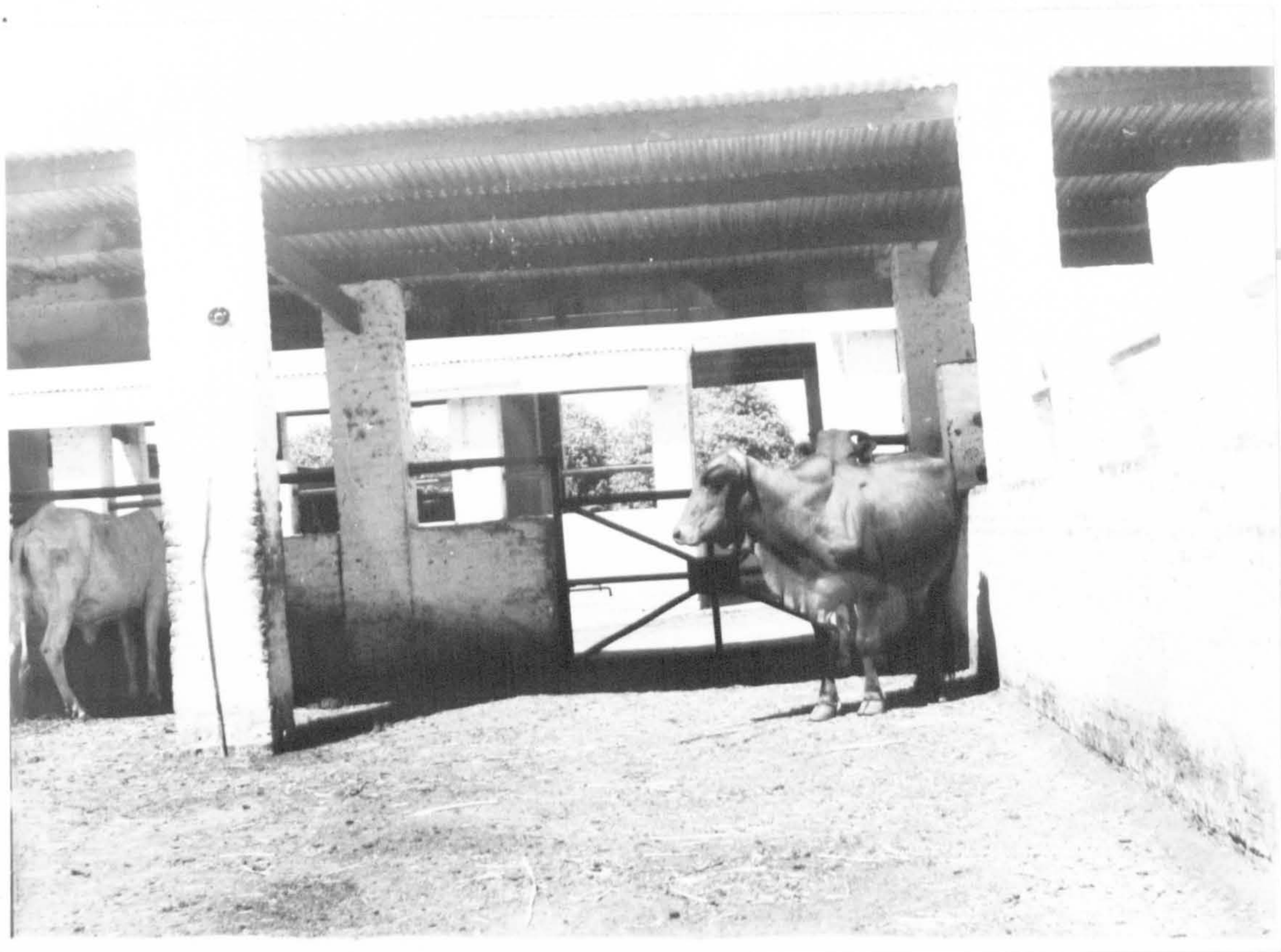
I. Examination of arthropods from natural infections

Method used for collection :

1) From bait animals

Collections were made directly from the animals from about 17.30 - 18.30, usually half an hour before sunset. The number of flies coming to feed on the animals during this time was surprisingly high. The total catching period might appear short, but it was during this time that the flies were most abundant on the animals. Later all the flies moved towards the light in the shed roof.

Figure 30.



Bait animals used for the collection of wild arthropods within the pen

Practically all the collections were made inside the shed (Fig. 30) by two people who caught all the flies feeding on the hump of the animals.

As they landed on the hump region movement of the cows was prevented by feeding them. Several catches were made, flies of each catch being stored separately in small waxed paper cases (4 x 3 in) which were closed with sandfly nets (nylon), and to keep the flies off the bottom of the cage a long piece of filter paper was placed inside the cage. The cages were kept at room temperature (27°C) for subsequent identification of the flies in the laboratory the following morning.

2) Collection from the light

The light was a neon electric light one metre long situated 120 cm above the back of the animal inside the shed roof (Fig. 31).

The best time for collection was found to be between 18.30 and 19.30, half an hour after sunset, when the number of flies was very high. Because at that time the flies were attracted to the nearest source of light, it was easy to see the flies move toward the light in groups.

The same people who collected the flies from the animals also collected flies from the light by using a sucking tube method. There was great variation in the number of flies caught on different nights. On a favourable day an hour's collection would yield 1000 flies, but

Figure 31.

The light above the back of the animal inside the pen from which adult flies were collected

on windy days the number was greatly reduced. Most of the flies collected from the light were blood unfed and were used in laboratory infections.

All the flies were kept overnight at room temperature ($27^{o}C$) and on each cage was a label with the date of collection, source of the flies (either from bait animal or the light), time of collection and the name of the collector.

The next morning the flies in each cage were immobilized either with ether vapour, CO_2 or cold; then under dissecting microscopes they were sorted into separate species and the number of each was entered in the main record. Then each species was separated into blood-fed and non-blood-fed and each 50 flies were kept in separate cages.

I was very fortunate in having the assistance of the group who worked with Dr. El Bihari for a long time in the collection and identification of Culicoides.

From the record of blood-sucking flies obtained, only two species of flies were found to be abundant in most catches, namely C. kingi and S. griseicolle. It is of considerable practical value to know if the two species are likely to be important vectors of animal onchocerciasis in the Sudan.

Three different sizes of Culicoides were caught. At first they were thought to be different species, but Dr. Boorman (Pirbright) confirmed that they were all C. kingi.

The results, recorded in Tables 14 - 16 show that only

Table 14.

ARTHROPODS COLLECTED FROM THE BAIT ANIMALS INFECTED WITH O. GUTTUROSA AND EXAMINED FOR MICROFILARIAE AND DEVELOPING FILARIAL LARVAE

Arthropod species	No.of flies collected	No. of flies with Bl.meal	No. of flies infected	Degree of larval development
C. kingi	391	124	8 (6%)	2 flies with mf 4 flies with L2 2 flies with L3
S. griseicolle	21	-	-	

Table 15.

ARTHROPODS COLLECTED FROM THE LIGHT IN THE ROOF OF THE ANIMAL HOUSE

Arthropod species	No. of flies collected	No. of flies examined		No. of flies infected	Degree of larval development
		Blood-fed	Blood-unfed		
C. kingi	1,132	60	510	15 (25%)	7 flies with mf 1 fly with L1
S. griseicolle	977	-	200	2	5 flies with L2 2 flies with L3 2 S. griseicolle with mf

Table 16.

DEVELOPMENTAL STAGES OF FILARIAL LARVAE IN WILD COLLECTED C. KINGI AND S. GRISEICOLLE

Arthropod species	No. of flies infected	Degree of larval development			
		mf	L1	L2	L3
C. kingi	23	9	1	9	4
S. griseicolle	2	2	-	-	-

two species were involved, but C. kingi was the more abundant feeder on the hump, which is quite clear from the high number if compared with the few S. griseicolle caught from the hump region.

i. Anaesthetising and sorting :

Flies collected from the animals and from the light were usually kept in the laboratory until the next morning with very little mortality.

Most flies were anaesthetised by ether vapour for short periods without actually killing them, and a few of the flies were anaesthetized either by CO_2 or cold. The reason for choosing ether as the anaethetizing agent can be seen from the result of a small experiment shown in Table 17 and Figure 32; both ether and CO_2 were better than cold, but due to the shortage of CO_2 we used ether.

In order to separate blood-fed from blood-unfed flies, and to isolate different species, the flies were immobilized and transferred rapidly to a large petri dish for sorting. The sorting was carried out under a dissecting microscope, each specimen being removed by lifting it off by penbrush. It was then transferred to another clean cage labelled with the same information used in the first cage.

The flies usually recovered 2-3 minutes after being anaesthetized, so the sorting had to be carried out rapidly. If it was not completed within this time, a piece of cotton-wool soaked in ether was placed over the petri dish for a

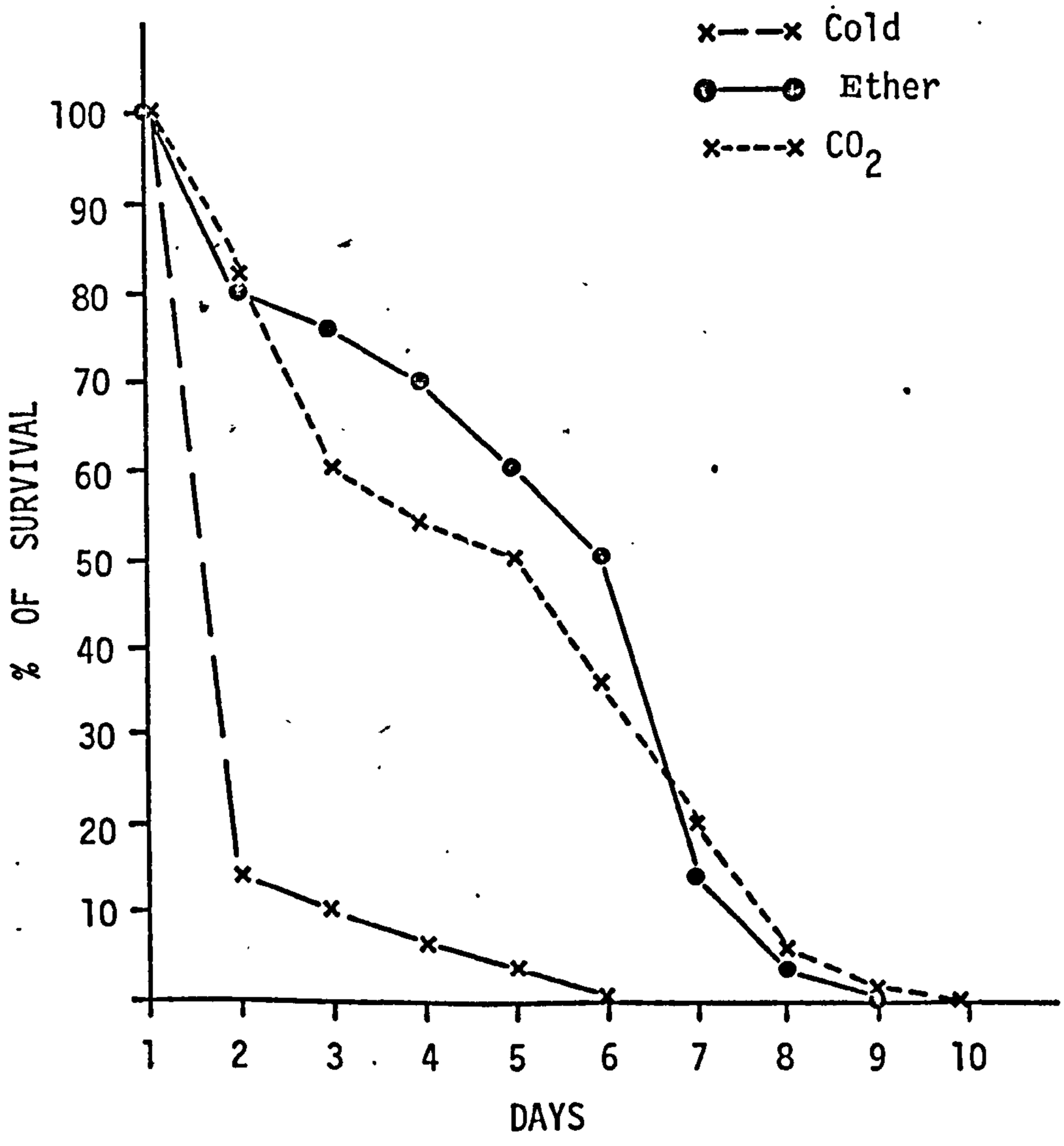
Table 17.

EFFECT OF ANAESTHETIC AGENT ON SURVIVAL OF CULICOIDES KINGI

Days after anaesthetic	Cold		Ether		CO_2	
	No. of flies	%	No. of flies	%	No. of flies	%
1	50	100	50	100	50	100
2	7	14	40	80	42	82
3	5	10	38	76	30	60
4	3	6	35	70	27	54
5	2	4	30	60	27	54
6	-	-	25	50	25	50
7	-	-	7	14	18	36
8	-	-	2	4	10	20
9	-	-	-	-	3	6
10	-	-	-	-	-	-

Figure 32.

Effect of anaesethetic agent on survival of Culicoides kingi



while, then the sorting started again. The flies were remarkably resistant to repeated small doses of ether and recovered full activity without any apparent ill-effects.

ii. Method of keeping the flies alive :

After sorting out all blood-fed flies, it was possible to maintain them within the temperature range of 22 - 27°C without any form of artificial heat in the laboratory. The flies needed to be kept alive to give a chance for microfilariae to develop if they had been picked up by the fly during a blood-meal, and dead or moribund flies were dissected immediately.

All flies were kept and fed with 10% glucose solution soaked into a cotton-wool pad and placed on the top of the cage and changed daily. By this method very few of the flies survived for 9 days. This was probably due to the lack of necessary conditions of humidity.

From the experiment of humidity, slightly better results were obtained when flies had been kept in an optimum humidity (85%), as shown in Figure 34.

iii. Dissection :

All unfed flies were dissected immediately for possible natural infections. The blood-fed flies were dissected at 24 hour intervals, starting 12 hours after collection for 9 days (because no fly survived more than that), using the technique used by Muller and Denham (1974).

At first the flies were dissected singly in a drop of

of Tyrode's solution, after dividing into head, thorax and abdomen, under a dissecting microscope. The most satisfactory instrument for dissecting the flies was found to be rustless steel watch forceps. Also a pair of needles made from small sharpened entomological pins in wooden handles were used for teasing out each portion in a separate drop of Tyrode's solution on the same slide; then the slide was left for a few minutes in a humidity chamber. It was then examined for O. gutturosa parasites. Their number, distribution and stage of development were recorded. Microfilariae and any other stage of development were transferred to fresh, very small, drops of Tyrode's solution. They were picked up with a fine cat's whisker, placed on a clean cover slip and a drop of 70% glycerine alcohol was added. The alcohol evaporated rapidly and the larvae were left in a minute drop of glycerine. The cover slip was then inverted over a cavity slide and the slides were kept for further examination later.

When it was realized that large numbers needed to be dissected, the process was speeded up by crushing the whole fly in a drop of Tyrode's solution. Developing stages were picked up and kept as above. Microfilariae picked up with blood meal were fixed on the slide after removal of the insect debris, dried and treated as an ordinary blood film.

The blood in the gut of the fly was usually in a semi-solid mass when the fly was dissected and it had to be mixed thoroughly with water in order to free any larvae

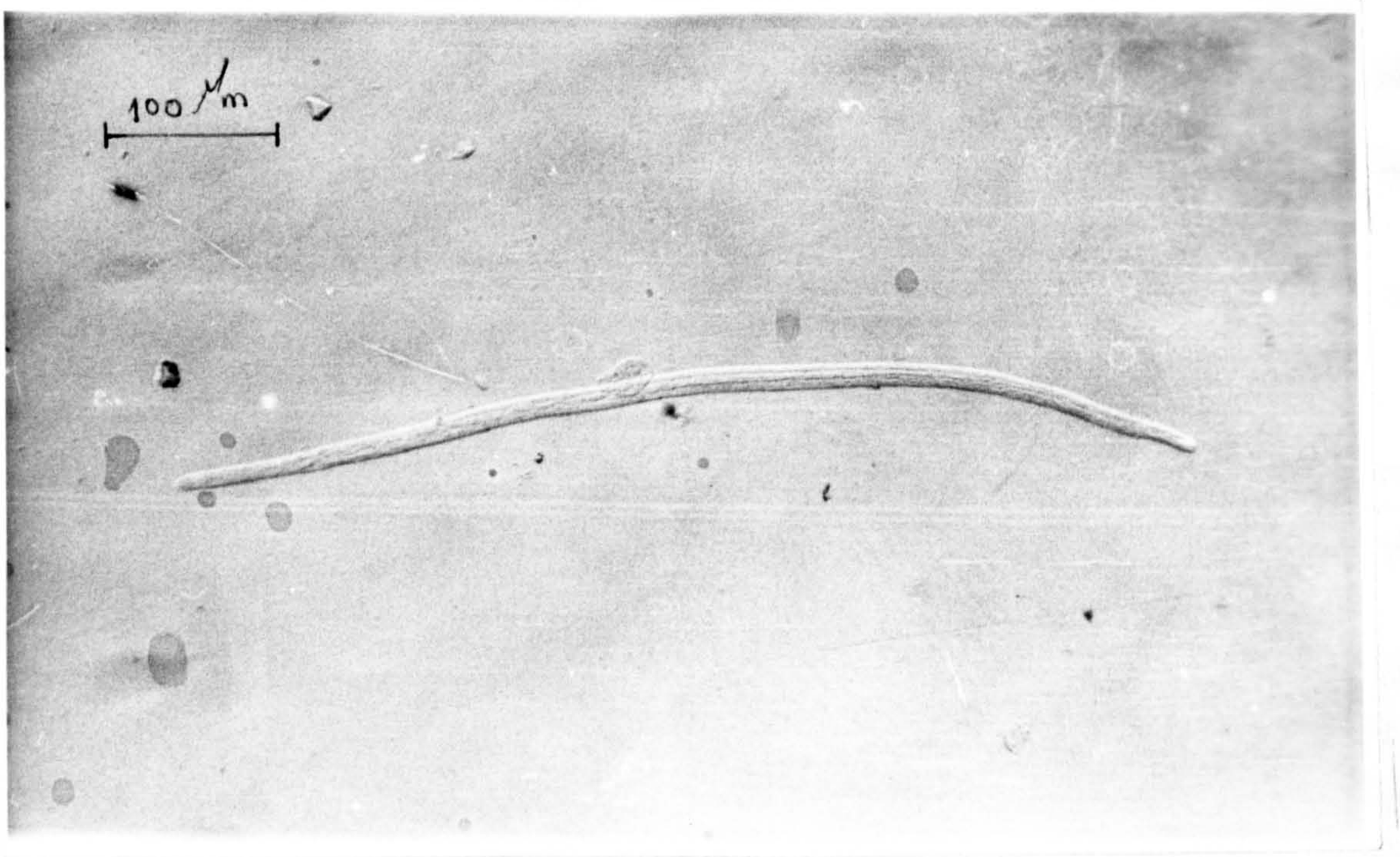
which might be present.

iv. Observations

During the first few months of investigation, two species of arthropods were found feeding on the backs of the animals: Culicoides kingi and Simulium griseicolle. The results of the collection of flies from the bait animals is shown in Table 13. The numbers of C. kingi were much higher. All S. griseicolle collected from the bait animals were unfed flies (Table 13). One hundred and twenty-four fed C. kingi were found, while 8 (6%) of them had various larval stages. Microfilariae were recovered from the abdomen and thorax of C. kingi dissected with a fresh blood meal. Developing first (sausage) stages were recovered from the thoracic muscle of the flies dissected up to three days after the meal. The pre-infected larvae were recovered from the thorax after four to five days, and infective larvae were recovered from the head and proboscis of the flies dissected after 7 - 9 days; each fly contained only one stage. All third stage larvae appeared normal (Figure 33) and when fixed in 70% glycerine alcohol and measured with an eye piece micrometer they were found similar in size to larvae recovered from S. ornatum, the British natural vector (Steward, 1937; Eichler, 1973; Bianco et al., 1980). The dimensions are given in Table 19.

The percentage of infection is low; Steward (1933) reported only 5% of Culicoides nubeculosus developed infective

Figure 33.



Infective stage larva recovered from the proboscis of wild Culicoides kingi after seven days

stage larvae fed directly on infected horses, but in this study 6% of blood-fed midges caught from two infected cows with microfilarial densities of 27 and 30 mff/mg of skin had infective larvae.

Culicoides kingi and S. griseicolle were collected from the light (Table 14). From 60 blood-fed C. kingi 15 (25%) flies were infected, while no blood-fed S. griseicolle were found (although two Simulium with microfilariae were found).

Although it is smaller in size, Culicoides kingi showed the highest intake of microfilariae from the hump of the animals. In dissecting blood-fed flies which were found to contain larvae and also showed the greatest ability to develop microfilariae intake until infective stage and were presumably available for transmission without showing malformed, stunted or retarded development, Culicoides midges do ingest skin-dwelling microfilariae and are known as vectors of several Onchocerca species, (Muller, 1979) and Bain (1979) suggested that it is a vector of O. gutturosa.

Simulium griseicolle showed poor infection rates. This offers an explanation for the failure to find flies naturally infected with infective stages, although it is abundant in the area (Lewis, 1948; El Bashir et al., 1976). It tends to bite low on the animal's body. In addition most flies examined were negative for larvae or showed evidence of delayed development by finding the microfilariae

without any sign of development. Simulium species are known to occur only in the riverside area (Lewis, 1948) while a high prevalence of the parasite is known to occur in animals away from the river.

All these factors suggest that S. griseicolle is a poor natural vector for O. gutturosa in the Sudan.

II. Experimental infection of insects

This work was carried out within the premises of the farm of the University of Khartoum, Sudan.

Two species, C. kingi and S. griseicolle, were incriminated as possible vectors of O. gutturosa. Both were available locally in large numbers feeding on the hump region of cows - the region where high numbers of microfilariae were found (El Bihari and Hussein, 1978).

From dissections of wild flies caught feeding on the hump, promising results were obtained with C. kingi by finding infective stage larvae in the head of the fly. The aim of the present experiment was to obtain more information on the behaviour and development of microfilariae of O. gutturosa in C. kingi, and whether to exclude S. griseicolle as an O. gutturosa vector in the Sudan.

Simulium flies are notoriously difficult to feed under laboratory conditions (McMahon, 1968) and this is one of the important obstacles in the way of laboratory infection. The method of feeding flies on cattle is laborious

and time-consuming and many flies have to be fed on infected cattle to obtain adequate numbers of infected larvae.

To overcome these difficulties, an inoculation technique was used, similar to those used by Nelson (1962), Townson (1975), Mellor (1976), Zielke (1977), Ried (1979), and Bianco et al. (1980).

An advantage of inoculation is that the damaging or potentially lethal gut barrier and the formation of peritropic membrane (Lewis, 1953; Mellor, 1971; Omar and Garms, 1975) are avoided.

The highest losses of microfilariae which occurred during migration from blood meal to the thoracic muscle (Duke and Lewis, 1964; Eichler, 1970) are eliminated, leading to an efficient use of available microfilariae.

1) Materials and methods

Females of both species, C. kingi and S. griseicolle, were obtained from the University farm in Shambat, using light trap collection as described on page 129. Also, trials were performed for collecting larval stages from the vicinity of the farm, but none were obtained; so all flies used were collected from the light.

In order to improve the survival of flies, different relative humidities, as described on page 176, were used. It was found that flies can survive for up to 12 days if kept at 85% relative humidity (Figure 34).

Microfilariae of O. gutturosa were obtained from the

hump skin of a freshly-killed infected animal using aseptic techniques, as described by Bianco et al. (1980). These were concentrated by centrifugation and resuspended in sterile Tyrode's solution and 20% serum, and antibiotic for microfilariae survival and to reduce the mortality of flies (Townson, 1974; Ried, 1979).

Micro-syringes were made by drawing out capillary tubing over a flame until a fine glass needle was obtained (60 - 80$^{\mu m}$). The shorter the needle the easier it was to handle.

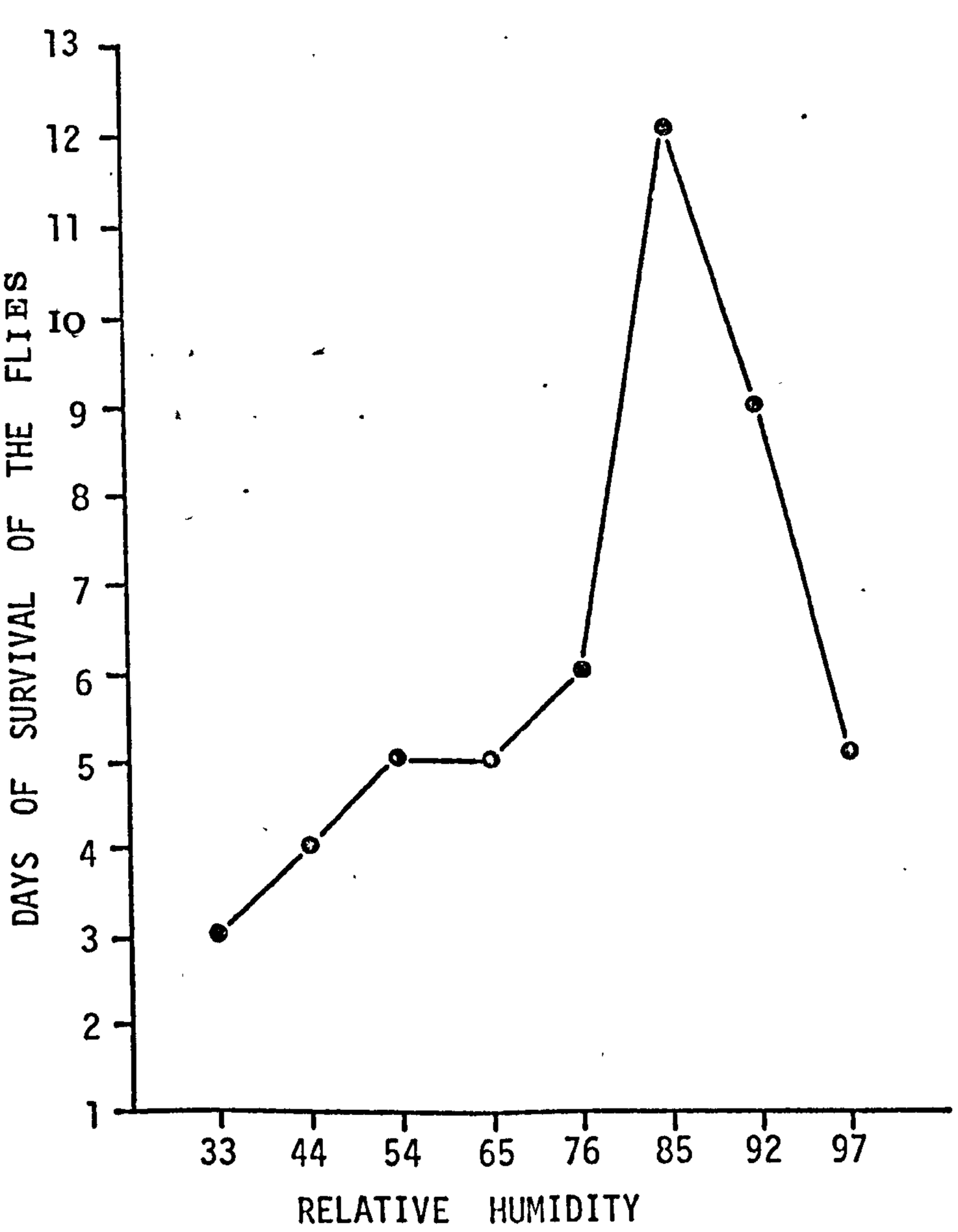
Males and blood-fed females from laboratory infection trial were excluded, using abdominal grades of Dyce (1969) but pigment was difficult to detect in many flies, especially those with an empty abdomen.

The flies were anaesthetized by ether vapour for a short time, and were than placed in lateral positions on a dissecting microscope; the infections were performed with a sterilized microsyringe. Where necessary, fine forceps were used to orient the fly for better viewing, to facilitate the use of the injecting needle, or to exclude unwanted debris or flies.

Medium containing microfilariae was drawn up into the micro-pipette and was inserted into the membraneous area below the parategite of the anaesthetized fly. Between five and ten microfilariae were injected into the thorax of each fly. The infected flies of each species were kept separate, 30 flies for C. kingi and 15 flies for S. griseicolle in

Figure 34.

Effect of humidity on suryival
of Culicoides kingi



a waxed card pill box (4 in x 3 in) closed by nylon meshes held with an elastic band. The boxes were maintained at room temperature (25 - 27°C) in a tightly closed container at relative humidity of 85% (Figure 35) and the flies were fed on 10% glucose/water solution soaked in cotton wool at the top of the cage (changed daily).

Three flies from each species were dissected immediately after infection. Active microfilariae were found in the abdomen and some freely distributed throughout the thorax. Daily checks were made of insect mortality, and dead or moribund flies were dissected immediately using Muller and Denham's (1974) method. Each fly was divided into head, thorax and abdomen in Tyrode's solution and any larvae were picked up and fixed in 70% glycerine alcohol.

2) Results

A total of 80 females of C. kingi and 50 females of S. griseicolle were inoculated. Flies were dissected at 24 hour intervals, giving priority to moribund and dead flies. It was observed that the mortality during the first 24 hours was high, which could be due to the trauma caused by inoculation.

After 48 hours, 48 (60%) C. kingi and 12.(24%) S. griseicolle were alive.

i. Morphological change during development:

All the morphological changes in the larvae occurred only in C. kingi.

Figure 35.

Infected flies were kept under humidity control to increase the survival time of the flies and thus give maximum chance for microfilariae to develop to infective stage.

Three stages in the development of O. gutturosa were seen in C. kingi (Table 17 and Figure 37). A moult occurred between each stage, and finally the infective, third stage, larvae were found.

After 48 hours three C. kingi and two S. griseicolle were dissected. Flies which had died during the previous night were also dissected, and it was noticed in each C. kingi that microfilariae showed development, especially in flies dissected after two days. They were shorter and thicker than the skin microfilariae, first stage (sausage stage Figure 37), in the thoracic muscles of the fly.

However, only dead microfilariae and occasional early first stage larvae were found in S. griseicolle.

After another two days, 12 (15%) C. kingi and 3 (6%) S. griseicolle were alive. From these three C. kingi and one S. griseicolle, besides flies which had died in the previous day, were also dissected.

The larvae begin to grow in length (Figure 36) and to elongate rapidly in the thoracic muscles. Second stage larvae (Figure 37) were found in C. kingi dissected 4 days after the infection. No developing larvae were found in S. griseicolle.

Seven days after infection, only 2 (2.5%) C. kingi and one (2%) S. griseicolle were still alive and were in a moribund stage; thus all flies were killed.

Worms moulting into third stage larvae (Figure 37)

were found on the seventh day after infection in the head of C. kingi; one larva in each fly. In contrast, no developing larvae or living microfilariae were recovered from S. griseicolle.

The infective stage larvae are more active than the earlier stages and migrate out from the thoracic muscles into the head and proboscis.

The third stage larvae obtained from natural infections and from artificial infections were compared with third stage larvae of O. gutturosa obtained by Eichler (1970) and those of O. cervicalis (Mellor, 1971).

3) Discussion and Conclusions

Bain (1979) has suggested that Culicoides is a remarkably efficient vector for O. gutturosa. Bianco et al. (1980) obtained third stage larvae from C. nubeculosus infected with skin microfilariae from a British cow.

The evidence presented here for O. gutturosa shows that microfilariae ingested by C. kingi successfully migrate into the thoracic muscles of the insect and developed to infective stage in C. kingi. However, immature and early stages can be recovered from the abdomen. This may help to explain the low recovery of the third stage larvae from infected flies. Eichler (1970) found that 75% of the microfilariae of O. gutturosa ingested by S. ornatum failed to leave the mid gut. Duke and Lewis (1964) found that 56% of microfilariae of O. volvulus also failed to leave the mid gut. Mellor (1971) found that 45%

Table 18.

SIZE CHANGES OF O. GUTTUROSA MICROFILARIAE IN CULICOIDES KINGI

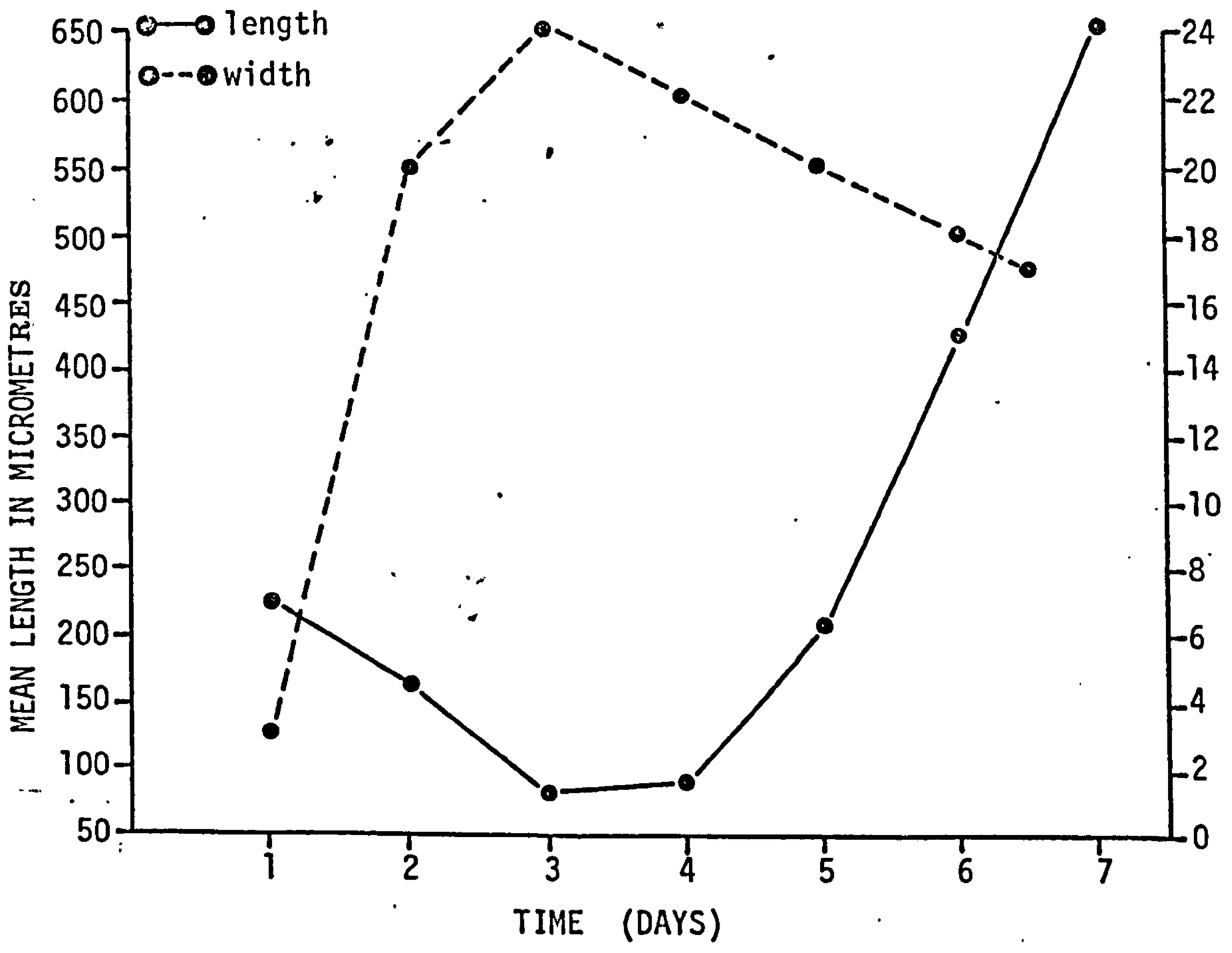
(All measurements were made with an eyepiece micrometer)

Days after infection	No. of larvae	Length		Width		Site
		Mean	Range	Mean	Range	
1	16	225	186-234	3.2	3.9-3.125	Abdomen and Thorax
2	3	180	175-190	20	14-21	Thorax
3	5	88.9	75- 90	24	25-32	Thorax
4	2	95	90-100	22	20-24	Thorax
5	6	207	200-210	20	19-22	Thorax
6	2	430.5	430-431	18	17-19	Head
7	6	653	650-685	17.14	17.1-17.3	Head

Figure 36.

Size changes of O.gutturosa in Culicoides kingi

Development and relative changes in dimensions of larval stage



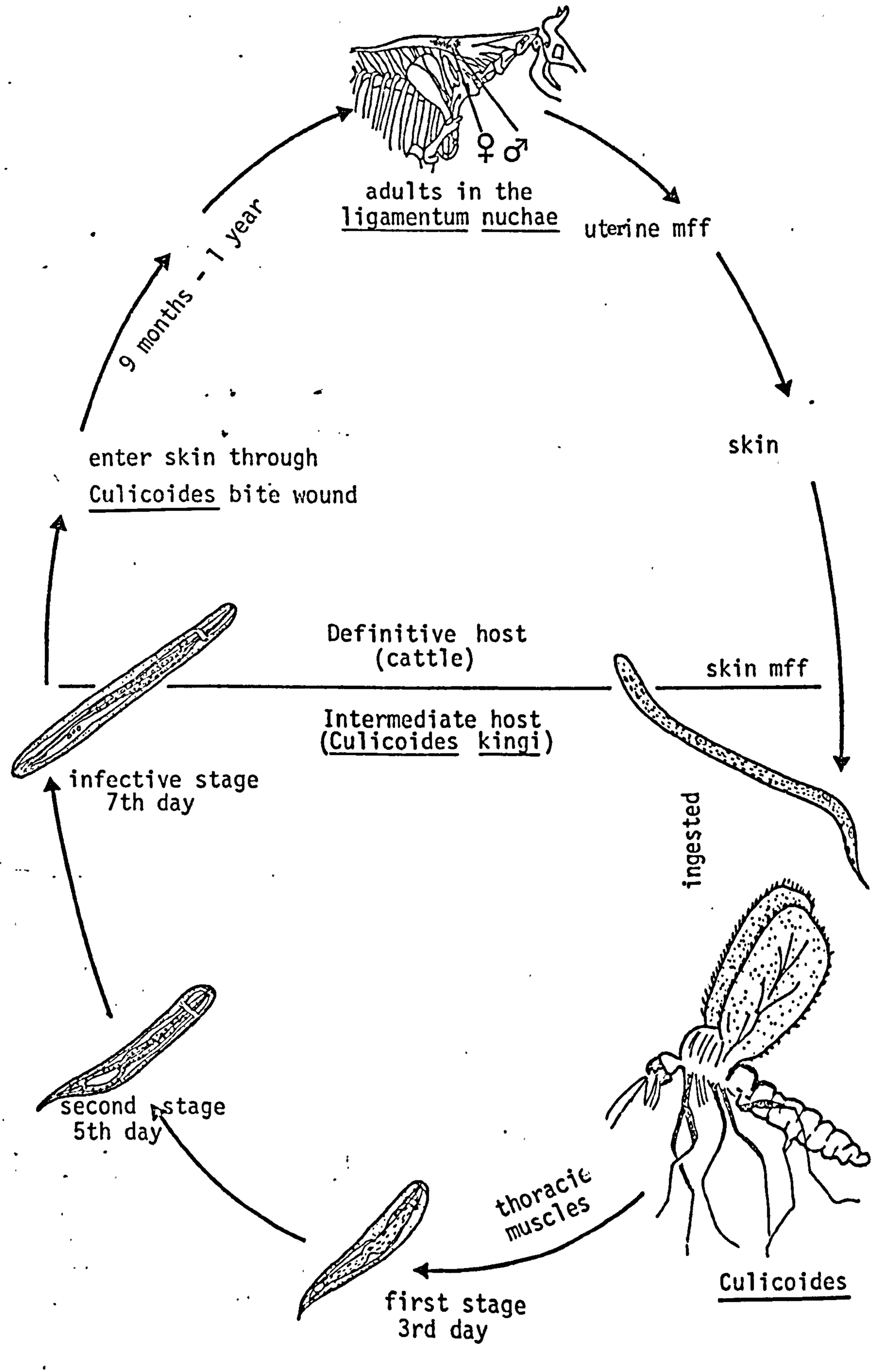
of O. cervicalis ingested by C. nubeculosus and 65% ingested by C. variipennis failed to develop for no obvious reason.

In the present study no attempt was made to study the route of migration because attention was concentrated on discovering the vector first and the migration of various species of microfilariae through the tissue of their vector has been studed by many investigators, including: Wuchereria bancrofti in Culex fatigans (O'Connor and Beatty, 1936); O. gibsoni in C. pungens (Buckley, 1938); O. volvulus in S. damnosum (Lewis, 1953); Brugia patei in Mansonia uniformis (Lawrence and Pester, 1961); O. gutturosa in S. ornatum (Eichler, 1970); O. cervicalis in C. nubeculosus and C. variipennis (Mellor, 1971).

All the larvae which developed to the infective stage become concentrated in the head and proboscis of C. kingi. This confirms the suitability of C. kingi as an intermediate host for O. gutturosa in the Sudan (Figure 37).

i. Simulium griseicolle was excluded as a possible vector for O. gutturosa in the Sudan for the following reasons: surveys carried out in Omdurman central abattoir (Hussein et al., 1975; El Bihari and Hussein, 1978) and in the present study indicate that 85% of adult Sudanese cattle are infected with O. gutturosa. It was apparent that O. gutturosa must be an endemic infection in local cattle and the high percentage of infection indicates that the parasite was being freely transmitted. Hence most of these cattle coming from the north western part of the Sudan

Fig. 37
Life cycle of Onchocerca gutturosa in Sudanese cattle



are from an area which is not the type of country in which Simulium can breed (Lewis, 1953); besides, S. griseicolle prefers biting on the lower part of cattle and usually bites indiscriminately and appears to attack birds rather than cattle (Lewis, 1948; El Bashir et al., 1976).

From the result of the wild collected flies S. griseicolle rarely picks up the infection and few microfilariae developed to the infective stage. This was confirmed by laboratory infections; no larval development being found in most infected flies as late as seven days. All the experimental and circumstantial evidence in this study is strongly in favour of Culicoides as a vector and indicate that S. griseicolle in the Sudan is not a suitable intermediate host for O. gutturosa.

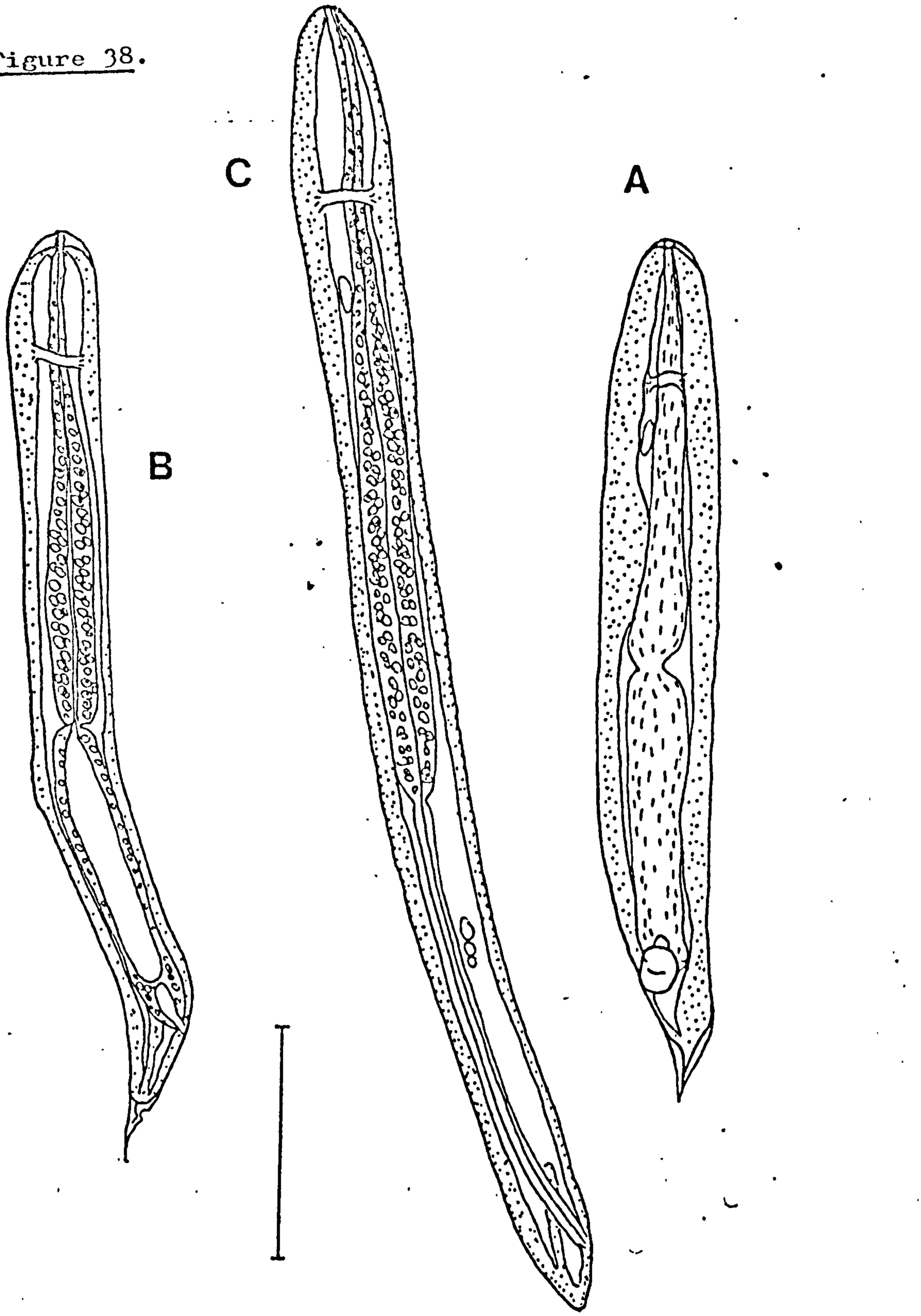
ii. Developmental changes in C. kingi

The larval development of O. gutturosa in C. kingi :
It was noticed that the change from the first to the second stage larvae and from the second to the third stage occured quickly and it was found that few flies contained more than one larva, with no double infection, so that all flies were free of infection before being infected with microfilariae.

Measurements were made of all larvae discovered during dissection and the results are tabulated in Table 18 and Figure 38.

Figure 38A, B and C illustrate the development and relative changes in the dimensions of the gut during larval

Figure 38.



A - Late first stage larvae - 3rd day (scale - 25 μ_m)

B - Late second stage larvae - 5th day (scale = 50 μ_m)

C - Third stage larvae - 7th day (scale = 215 μ_m)

development. The formation of the digestive tract is completed by the late first stage larvae. The oesophagus then gradually lengthens, relative to the intestine, the junction of the intestine and part of the oesophagus becomes much more indistinct. After the second moult, the anal plug and intestinal contents are expelled and the intestinal lumen which until this time had been relatively wide, becomes much narrower.

Bain (1969) divided the third stage larvae of O. volvulus into two groups mainly on the basis of the presence of absence of anal plug and intestine contents. She thinks that the young third stage larvae of O. volvulus are not infective.

Comparison of infective stages of O. gutturosa from the Sudan and Britain with O. cervicalis :

Various species other than O. gutturosa have been recorded from animals in the Sudan - Mostafa et al. (1966) reported O. armillata, Lewis (1953) reported O. gibsoni, and Onchocerca sp. in an antelope; Saad El Din, in a personal communication, reported O. raillieti in a donkey and the present author has found Onchocerca species in the ligamentum nuchae of a camel. In no cases are their vectors known. They are mentioned here because some may be transmitted by Culicoides kingi (El Bihari and Hussein, 1978) and so they must be differentiated when examining C. kingi for O. gutturosa infection.

Table 19.

MEASUREMENTS OF 8 INFECTIVE LARVAE OF O. GUTTUROSA MOUNTED IN 7% GLYCERINE ALCOHOL

(All measurements are in micron)

	1	2	3	4	5	6	7	8	Mean
Length	657	653	685	684	685	678	660	665	673
Width	17.1	17.14	17.16	17.2	17.15	17.12	17.1	17.14	17.1
Oesophagus	446	445.7	446	445	445	445	446	445	445.5
Intestine	207	216	210	216	212	216	207	209	211.6

Comparisons were made also to confirm that the worms does develop in the local Culicoides and support Bain's (1979) suggestions, and for the purpose of finding characteristics which would easily distinguish O. gutturosa third stage larvae from those of other Onchocerca reported in the Sudan.

Infective larvae of O. gutturosa were obtained from the head of natural and laboratory infected C. kingi. The head was removed and teased in Tyrode's solution plus 20% sheep serum, using Muller and Denham's (1974) technique, seven days after the flies had been infected with microfilariae of Onchocerca gutturosa. Only third stage larvae from the head were used because they were assumed to have reached their maximum length (Nelson, 1965). All measurements were made with the aid of an eye piece micrometer and the results are given in Table 19. When the comparison was made with O. cervicalis (Steward, 1933) only a few specimens of third stage larvae were available for comparison.

Observations

Steward (1937) gave the length of infective larvae of O. gutturosa as 675 µm which is the same size as those recovered from C. kingi in the present study, but Eichler (1970) reported a maximum of 572µm, a difference of more than 100µm.

It is quite clear that there is a difference between the third stage larvae of O. gutturosa and those of

Table 20.

COMPARISONS OF MEASUREMENTS OF INFECTIVE LARVAE OF O. GUTTUROSA NATURAL AND LABORATORY INFECTION IN SUDANESE CATTLE, O. GUTTUROSA IN U.K. AND O.CERVICALIS

	O. gutturosa Sudanese strain		O. gutturosa	O. cervicalis
	From natural infected flies (2 larvae)	From laboratory infected flies	British strain Eichler, 1970	(Mellor 1971)
Total length	662.5(660-665)	653 (650-685)	484(406-633)	692(602.5-722.5)
Width	17.1(17.1-17.1)	17.14(17.1-17.3)	17.8(16.3-20.4)	19.9(18.0-24.0)
Anterior to nerve ring				65.5(57-72)
Oesophagus	446	445.7(439-454)	321.4(282-347)	
Intestine	216	207 (200-210)	170.6(162.2-190)	208.9(163-230)
Oesophagus-intestinal ratio	2.1	2.1 (2.0-2.3)	2.1 (1.8-2.7)	2.97 (2.5-3.5)
Anal ratio	———	———	2 (1.8-2.5)	2.12 (1.4-2.9)
Tail length	———	———	32 (28-34.2)	39.7 (36-43)

O. cervicalis. In the case of O. cervicalis, the maximum length appeared much longer, than the maximum length of O. gutturosa; it was also much broader than O. gutturosa.

It is concluded that C. kingi is an efficient vector for O. gutturosa in the Sudan, and there are morphological characteristics which can readily be used to differentiate infective larvae of O. gutturosa from O. cervicalis.

iii. Development of the parasite in C. kingi

Steward (1937) and Eichler (1970) described the development of O. gutturosa in S. ornatum from microfilariae to infective stage as being 22 and 13 days respectively. In the present study, the third stage larvae were found in the proboscis of C. kingi 7 days after infection. This shorter period of development is probably due to the fact that infected flies were maintained in the laboratory at a temperature of 25 - 27°C, with 25 flies in a small box and were killed and dissected at 24 hour intervals from one to seven days.

The change from the first stage to second stage larvae and from the second to third stage occured remarkably quickly and Eichler (1970) found that infective larvae of O. gutturosa developed in S. ornatum 13 days after infection when flies were maintained at 23°C. Mellor (1971) recovered infective larvae of O. cervicalis and C. variipennis after 14 and 15 days at the same temperature. Wegesa (1966) stressed the importance of considering the

temperature when recording the duration of development of filariae in the vector.

Gnedina (1950), when studying the development of O. gutturosa in S. ornatum, maintained her flies at a temperature too low to complete the parasite larval changes and, because of this, she mistook the advanced first stage larvae at 35 days for the infective stage.

iv. Infection of Aedes aegypti with O. gutturosa

There is general agreement among workers that research on onchocerciasis has been severely handicapped by the inability to colonize a Simulium vector of both human and animal onchocerciasis (Blacklock, 1926; Steward, 1937). Recently, promising results have been obtained with non-vector insect hosts by either membrane feeding or intra-thoracic injection. Infective stage larvae have been reported for O. volvulus in Aedes aegypti (Zielke et al., 1977; Zielke, 1977) and recently for O. gutturosa in Aedes aegypti and two other species of mosquitoes (Bianco et al. 1979).

The present study was designed to determine whether large numbers of infective larvae of O. gutturosa can be produced in Aedes aegypti by manipulation of the infection procedures.

Materials and methods

The species used in this study were female Aedes

aegypti of the selected Liverpool strain (Macdonald, 1962). Young mosquitoes (6 - 8 days) were obtained from the colony maintained in the Department of Medical Helminthology. Microfilariae of O. gutturosa were obtained from the umbilical skin of British cattle freshly slaughtered in Reading abattoir. A sterile technique (Bianco et al., 1980) was used to extract the microfilariae.

Infected skins were washed, shaved, pinned to a cork board and sterilized with absolute alcohol in a laminar flow cabinet. Observing the usual aseptic procedures, large slivers of skin, which were not of full thickness, were taken from all over the surface in disposable universal containers filled with Tyrode's solution and serum, with penicillin and streptomycin (200 units per ml) and incubated overnight at 25 - 27°C.

Microfilariae were recovered from the tubes by pasteur pipette, resuspended in fresh medium by gentle shaking and passed through a filter of sponge (spontex) held in the barrel of a syringe to remove any particules of host material and bacterial contamination. There microfilariae were concentrated by centrifugation at 2,500 r.p.m. for 5 minutes, and either resuspended in blood for membrane feeding, or in one of a variety of media for intra-thoracic infection.

1. Membrane feeding experiments :

Using the standard technique of membrane feeding (Rutledge et al., 1964) the units were so designed that they

could be connected in series to enable several batches of mosquitoes, contained in waxed paper boxes, to feed at the same time.

Three trials were conducted in which females of A. aegypti were fed through chicken skin membranes, which Mc Mahon (1967; 1968) had used successfully for artificial feeding of Simulium, on suspension of microfilariae in heparinised cow blood at 37°C.

Four suspensions were used, 2500, 5000, 10,000 or 15,000 microfilariae per ml of blood (Table 21). The majority of insects fed within 30 minutes, during which time microfilariae remained active in the blood. After feeding to repletion on the suspension of microfilariae, the flies were anaesthetized and all engorged mosquitoes were kept in the same wax paper box (35 flies per box). All fed flies were kept at 23°C and 80% relative humidity and fed with 10% sugar meal soaked into cotton wool pads placed on the top of the cages. These pads were changed daily to prevent fungus growth.

Dissection of 10 mosquitoes from each group up to one hour after feeding, revealed that all had taken up microfilariae of which 17% had reached the thorax. The remainder were examined daily and dead and moribund specimens were dissected in medium 199 solution under a binocular microscope for developing larvae. The remaining mosquitoes were examined 12 days after infection, when microfilariae should have developed to third stage larvae.

The number of microfilariae ingested by each group of mosquitoes increased with increasing concentration of the blood parasite suspension, as did the mortality in the insect subsequent to the infective blood meal (Table 21). However, the yield of third stage larvae 12 days after infection was no greater in groups of mosquitoes fed on the highest concentration of microfilariae.

From Table 22 it can be seen that this appears to be due to a significant reduction in the number of larvae which can be supported through each larval stage, irrespective of the number of microfilariae initially ingested, which suggests that mosquitoes which survive can tolerate only very low Onchocerca infections.

This is most evident at the beginning and end of the development period. During the transition between microfilariae and first stage larvae, and between second stage larvae and infective stages; these correspond with the periods of the highest insect mortality.

2. Intra-thoracic injection experiments :

Two trials were conducted in which microfilariae were inoculated directly into the thorax using an injection device to semi-automate the process (Bianco et al., in preparation). Females of A. aegypti were immobilised with carbon dioxide gas on a modified microscope stage and inoculated in the membraneous area below the parategite with a glass pipette (see page 143), having a terminal diameter of 60 - 80 μm.

In the first trial, a total of 353 mosquitoes were injected with approximately 40 microfilariae each suspended in Tyrode's solution containing 20% bovine serum. Eleven per cent of the mosquitoes survived the 12 days development period and 80% were infected with a mean of 3.0 (range 1 - 10) third stage larvae each.

In order to improve the survival of infected insects, a second trial was performed with 6 groups of 50 mosquitoes which were infected with 20 microfilariae each suspended in various media (Table 23). Mosquitoes in all six groups survived better than in the first injection trial, presumably due to the smaller number of microfilariae given. Nevertheless, there was only a slight drop in the average yield of third stage larvae. Although the difference between groups was not great, the best results were obtained using medium RPM1 1640 which combined high survival of infected mosquitoes and relatively high infection rate with the greatest recovery of third stage larvae. Mosquitoes injected with media which did not contain parasites, or which had been sham-operated by inserting the needle without inoculating liquid, survived equally as well as the untreated mosquitoes (86 - 98% survival).

This provided good evidence that the actual procedure of injection did not cause any appreciable increase in the death rate of mosquitoes, although the introduction of Onchocerca microfilariae produced a significant mortality.

Third stage larvae from A. aegypti were recovered

mainly from the head (51%) and thorax (46%), with very few in the abdomen (3%). Most larvae appeared normal and when fixed in 70% alcohol measured 474 (426 - 573) microns long and 17.9 (16.2 - 21.2) microns wide.

In S. ornatum, which is the natural vector (Steward, 1937), the larvae are similar in size, although most migrate to the mouth parts. Eighty to ninety per cent of the third stage larvae in S. ornatum are normally recovered from the head at 12 days after infection, and measured a mean of 484 (406 - 633) microns long and 17.8 (16.3 - 20.4) microns wide.

However, in S. ornatum, the recovery of infective larvae and survival of heavily infected flies is far greater than in A. aegypti after infection by intra-thoracic injection. The mean recovery of larvae is 10 - 20 per fly (maximum 53) and 30 - 70% of flies survive the development period.

Low survival of A. aegypti infected with O. volvulus was also reported by Zielke (1977) as were very low worm recoveries. In contrast, Townson (1975) obtained good survival (85%) of A. aegypti injected with microfilariae of Brugia pahangi to which the strain of mosquitoes used had been adapted.

Until a similar selection is made of strains to accept Onchocerca infections, it seems unlikely that substantial numbers of O. gutturosa third stage larvae can be produced in A. aegypti.

Table 21.

INFECTION OF AEDES AEGYPTI WITH O. GUTTUROSA BY MEMBRANE FEEDING USING VARIOUS CONCENTRATIONS OF MICROFILARIAE (mf) IN BLOOD

Concentration mf/ml	No. of insects fed	% survival	% with mf	Mean no. mf/range	% with L3 *	Mean No. L3 * range
2500	112	62	100	3 (2-14)	41	1.0 (1-2)
5000	324	41	100	5 (4-11)	54	1.1 (1-2)
10000	110	20	100	17 (14-20)	100	1.6 (1-2)
15000	112	19	100	21 (12-25)	95	1.2 (1-3)

* L3 = third stage larvae)

Table 22.

MEAN (RANGE IN PARENTHESIS) NUMBER OF LARVAE OF O. GUTTUROSA IN AEDES AEGYPTI AT FOUR TIME INTERVALS AFTER FEEDING ON VARIOUS CONCENTRATIONS OF MICROFILARIAE IN BLOOD

Concentration mf/ml	Days after infection (larval stage)			
	0 (mf)	3 (L1)*	7 (L2)**	12 (L3)***
2,500	3 (2-14)	1.6 (1-2)	1.3 (1-2)	1.0 (1-2)
5,000	5 (4-11)	2.3 (2-3)	2.5 (2-3)	1.1 (1-2)
10,000	17 (14-20)	3.7 (2-5)	4.0 (1-8)	1.6 (1-2)
15,000	21 (12-25)	7.6 (3-9)	3.6 (1-5)	1.2 (1-3)

* L1 = first stage larvae

** L2 = second stage larvae

*** L3 = third stage larvae

Table 23.

INFECTION OF *AEDES AEGYPTI* BY INTRA-THORACIC INJECTION WITH MICROFILARIAE OF *O. GUTTUROSA* IN VARIOUS MEDIA. ALL MEDIA ADJUSTED TO pH 7.2

Medium	No. of insects infected	Percentage survival	Percentage infected	Mean recovery of infective larvae (range)
TC medium RPM1 1640	50	72	45	2.6 (1 - 5)
TC medium 199	50	78	20	2.3 (1 - 4)
TC medium NCTC	50	52	25	1.0 (1 - 3)
Tyrode's saline	50	62	33	2.4 (1 - 4)
Tyrode's 20% serum	50	66	20	2.0 (1 - 3)
Hayes saline	50	48	30	1.8 (1 - 6)

v. Behaviour of C. kingi in relation to transmission of O. gutturosa

This section is concerned only with the factors affecting transmission and behaviour of O. gutturosa in Culicoides kingi. It is not proposed to deal with the bionomics and taxonomy of the vector, these aspects having been extensively reviewed by Edward et al. (1939), Kettle and Lawson (1953), Khamala and Kettle (1971).

Macfie (1947) reported the species of Culicoides known in the Sudan up to that time, and these did not include Culicoides kingi. Kirk (1957) gives an account of filarial disease in the Sudan transmitted by Culicoides and again C. kingi is not mentioned.

Culicoides and Simulium, because of their manner of feeding, are well adapted to the ingestion of skin microfilariae; the short scarifying proboscis is ideally designed for this purpose, it penetrates just deeply enough to pick up the microfilariae from beneath the epidermis.

In each geographical region the local vectors are adapted to the local strain of O. gutturosa. So S. ornatum, which are predominantly low biters, are well adapted for picking up microfilariae of the British strain of parasite. Steward (1937) and Eichler (1970) have studied this aspect of transmission. In the same way, C. kingi is a high biter; the observations by Bain (, 1979) have shown that Culicoides is well adapted to transmit O. gutturosa.

Time of biting :

During 1979 catches of C. kingi were made throughout a whole day using a bait animal. The flies nearly always bite in the hump and neck and occasionally attack the eyes, ears and nose. It was noticed that C. kingi will bite anywhere, outdoors, indoors, under a shed, but the flies bite more in an open shed.

The present observations were noticed while the animal was in an open shed. Observations were made at various hours of the day in order to ascertain at what hours the fly was prepared to feed in the farm, and whether any particular hours were preferred by it. It was found that individuals were not biting earlier than 5.00 and that only the latest stragglers were biting as late as 19.00. Before the flies attacked the animals most flies were seen to be moving in groups near the floor in wet places of the stable, especially near excreta and heaps of animal waste.

Days in July were chosen to ensure the maximum catch. The bait cow was 7 years old and was kept in a shelter a few yards away from the waste of the animals (breeding site). At about 6.00 the midge collection was started and midges were collected throughout the day until 19.00. Two persons were involved in the collection, one every half hour. All caught insects were transferred to waxed card boxes to await identification.

The results recorded in Table 24 and Figure 39 , show that there are morning and evening peaks of C. kingi activity.

The evening peak occurred just before sunset and was twice as great as the morning peak. The collection was stopped at 19.00 because of the difficulty of seeing the midges at that time, and it was also noticed that midges are very rare in the animal shed at night.

Combined catches were made on 10 separate days using two different bait cows as the number of biting C. kingi often varies from day to day. Steward (1933) in Britain considered that the moderate temperature, calm air and low bariometric pressure encourage the flies to bite.

The attractiveness of the hump surface of the host is probably due to a combination of lack of hair and a quiet place for blood feeding away from the head and from tail switching.

Mellor (1971) demonstrated that C. nubeculosus bites predominantly on the ventral surface of horses because of illumination and arrangement of hair.

Culicoides kingi was found to prefer the dorsal area of the bait cow and landed either on the hump or the face. Few midges were seen to land on the chest, back or ears, while none were feeding or biting on the ventral surface and the number of flies landing on those areas was quite small. This may be because the movement of the head and tail brushes off all flies and the only peaceful place for feeding was the hump region.

The biting rate of Culicoides is considerably more difficult to determine than for S. ornatum, because the

Figure 39.

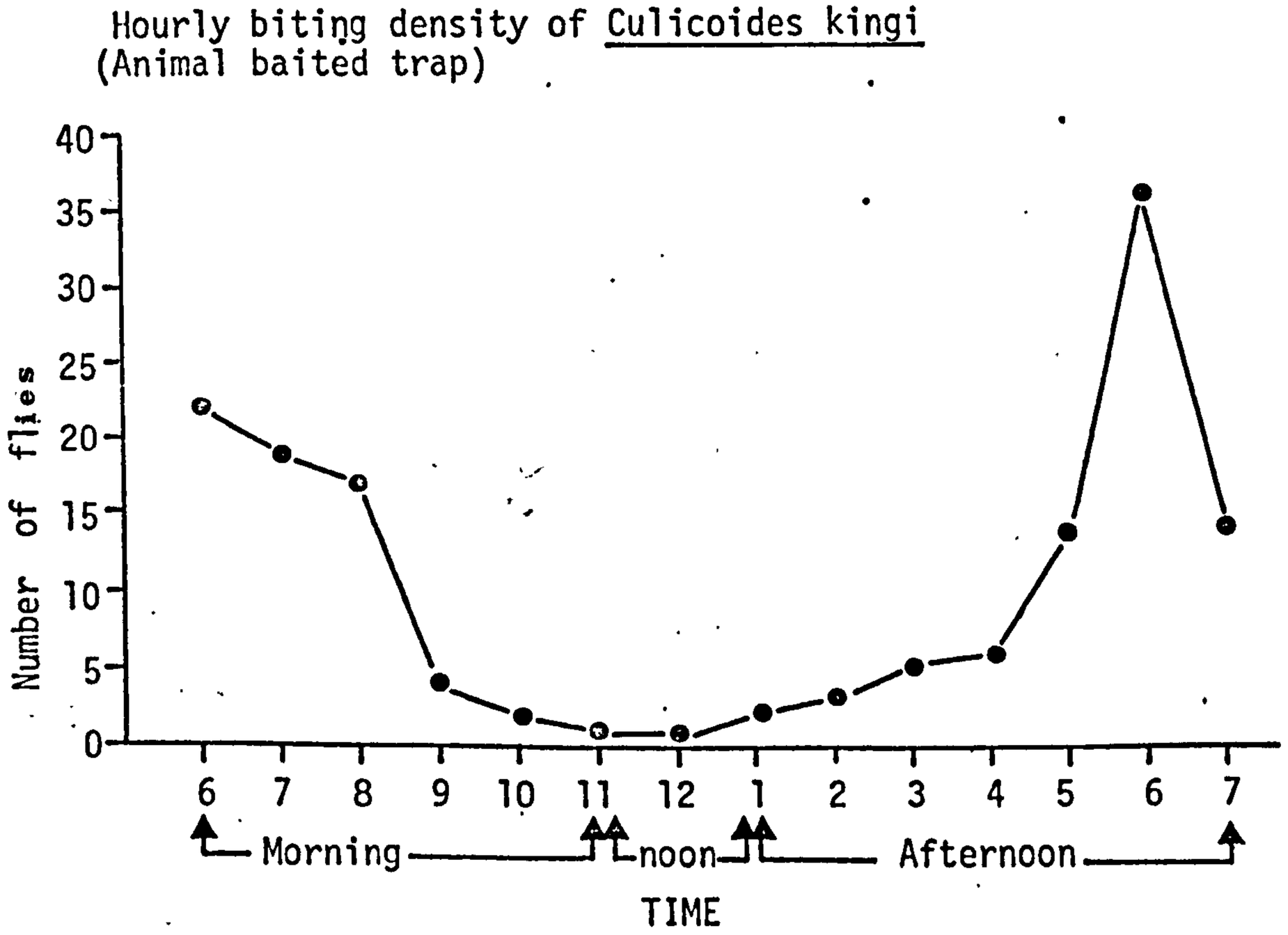

Hourly biting density of Culicoides kingi
(Animal baited trap)
Number of flies
0
5
10
15
20
25
30
35
40
6 7 8 9 10 11 12 1 2 3 4 5 6 7
Morning
noon
Afternoon
TIME

Table 24.

HOURLY CATCHING DENSITY OF C. KINGI FROM A BAIT ANIMAL

	Time in day hours														
	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14	14-15	15-16	16-17	17-18	18-19	19-20
Total no. of flies collected	207	175	164	35	19	12	14	19	31	45	55	128	322	126	30
Mean	23	19	18	4	2	1	1	2	3	5	6	14	36	14	3
Range	20-25	10-25	15-21	3-6	1-3	1-3	1-3	1-4	1-6	2-8	3-9	10-20	25-45	3-22	1-4
Total no. of blood fed flies	14	11	5	3	2	1	0	0	Nil	4	2	8	47	13	3
Percentage of blood fed flies	6.76	6.28	3.03	8.57	10.5	8.33	Nil	0	0	8.8	3.63	6.25	14.6	10.3	10.0
Mean	2	1.2	0.5	0.3	0.2	0.1	Nil	Nil	Nil	0.4	0.1	1.0	5.0	1.4	0.3
Range	0-3	0-2	0-2	0-1	0-1	0-1	Nil	Nil	Nil	0-1	0-1	0-2	4-8	0-4	0-1

midge is a smaller insect, it bites on a confined area of the host and is most active during the evening and early morning when failing light hinders observation.

The time required by a fly to feed to repletion was noticed on several occasions. The midges were usually first observed flying in circles above the cow's neck and head. Sometimes this behaviour continued for 2 - 3 minutes and there was a considerable interval after the fly alighted until it disappeared from view. Engorgement took from 2 - 3 minutes, during which time the flies remained invisible. The host often attempted to rid itself of the flies by shaking its withers.

Throughout the work it was clear that landing activity was markedly reduced or absent on windy days or those with heavy rain.

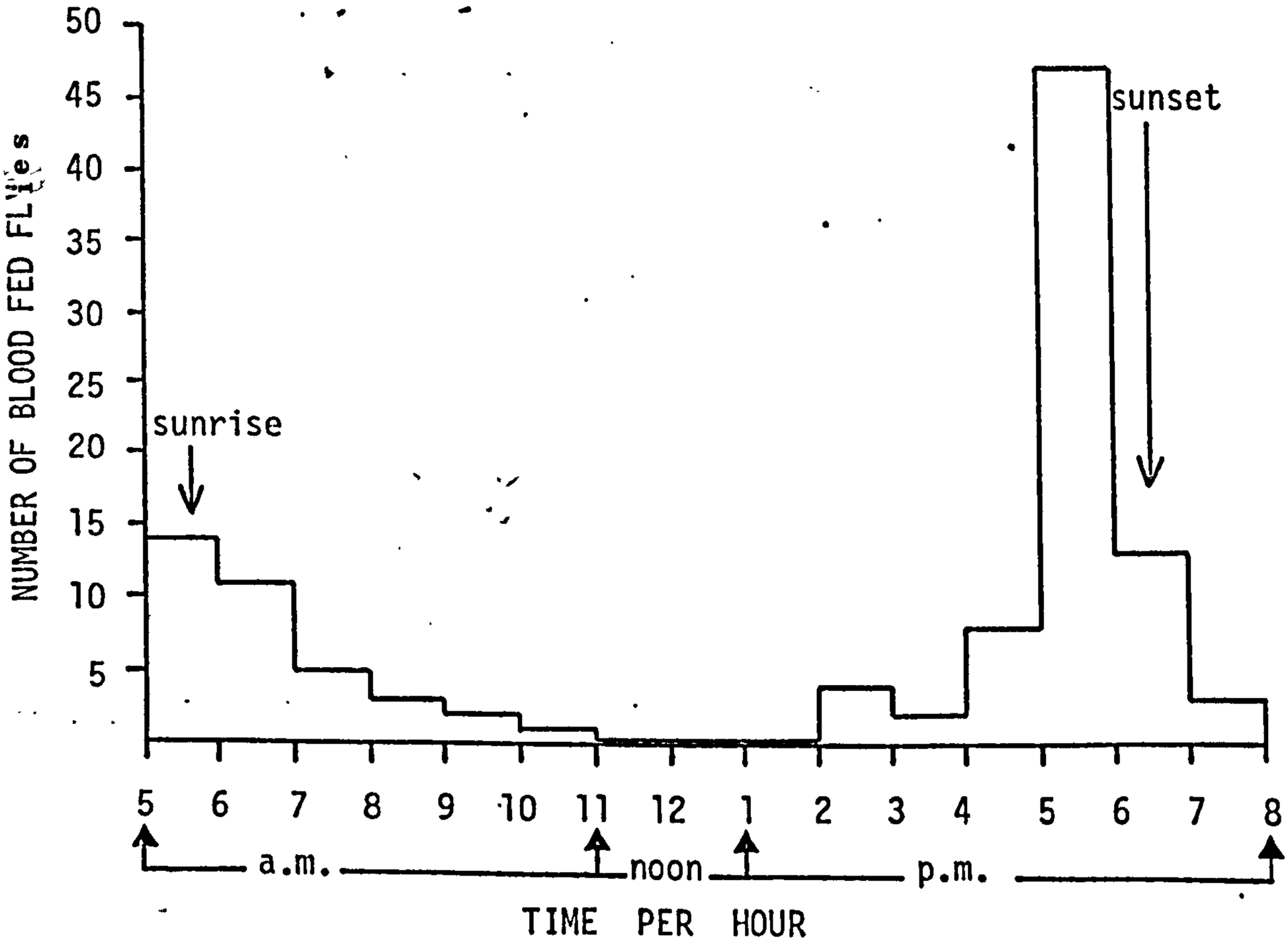
Those midges which did succeed in obtaining a blood meal re-emerged from between the hairs of the withers and flew away almost immediately.

The collected flies were divided into blood fed and blood unfed flies. The results show that the highest number of flies with a blood meal were collected from the evening peak, and in the middle of the day the flies would not go far in order to bite (Figure 40).

Eichler and Nelson (1971) noticed that 90% of microfilariae of O. gutturosa concentrated around the umbilicus of the cow. They also found that 80% of the vectors bite in the same area. This observation was confirmed by Mellor

Figure 40.

Hourly blood fed density of Culicoides kingi showing times of sunset and sunrise



(1974) for O. cervicalis in British horses, by Buckley (1938) for O. gibsoni and by Weinmann et al. (1973) for O. cervipedis (Wehrdikmansia cervipedis) in black tail deer in California. This is a remarkable adaptation of the parasite to accommodate its vectors' feeding habits.

This project has shown that a similar situation also exists between the bovine parasite O. gutturosa and its vector C. kingi; 90% of the microfilariae of O. gutturosa occurred in the skin of the hump of the host in an area bitten by 85% of the vectors.

Length of life in captivity :

At first it was difficult to keep C. kingi in captivity. At room temperature (25 - 27°C) on 10% sugar meals without the necessary humidity, flies failed to survive for more than 7 days.

Various experiments were made in order to discover some means of keeping wild flies alive in captivity for as long a time as required for the larvae in infected flies to develop to the third stage.

In the present work, one effect of relative humidity in the survival of flies was studied in flies captured while just commencing to feed which were placed in waxed card boxes (4 x 3 in), each having 50 flies in one cage. To keep the flies off the bottom of the cage a long piece of filter paper was placed in each cage. Eight different relative humidities were used with the saturated solutions

of Winston and Bates (1960) as shown in Table 25.

Each cage was placed in an airtight container with a particular relative humidity (R.H.) after being supplied with 10% sugar, and the death of the flies was checked daily.

The experiment showed that the longest period of survival in the total of 400 flies was 12 days, but the majority were dead within 4 days. On the whole it may be said that 85% relative humidity gave better results than did the other seven different relative humidities.

Table 25.

SATURATED SOLUTION FOR CONTROL OF HUMIDITY

Winston and Bates (1960) Ecology 41 No 1

Chemical	R.H. at 27°C	Amount per gram needed for saturation 100 cc water
Calcium chloride	33%	300 gms
Potassium carbonate	44%	125 gms
Calcium nitrate	54%	200 gms
Sodium nitrate	65%	100 gms
Sodium chloride	76%	40 gms
Potassium chloride	85%	40 gms
Sodium carbonate	92%	15 gms
Potassium sulphate	97%	15 gms

Seasonal factors governing population of Culicoides kingi

The present work was carried out to provide quantitative information on the activity of C. kingi. Austen (1922) (Diptera = Ceratopogonidae) on cattle under various weather conditions and at different times of day.

A useful basis for this work was afforded by El Bihari (personal communication) who examined the species of Culicoides and breeding sites in Khartoum. Culicoides are proven or suspected vectors of a number of animal and human parasites (Steward, 1933; Buckley, 1938; Kirk, 1957; Kettle, 1965; Spratt et al., 1978), and the finding in the present study of the role of C. kingi as vector of the nematode O. gutturosa (Neumann, 1910) parasitic in cattle provided an incentive to study the fly in more detail.

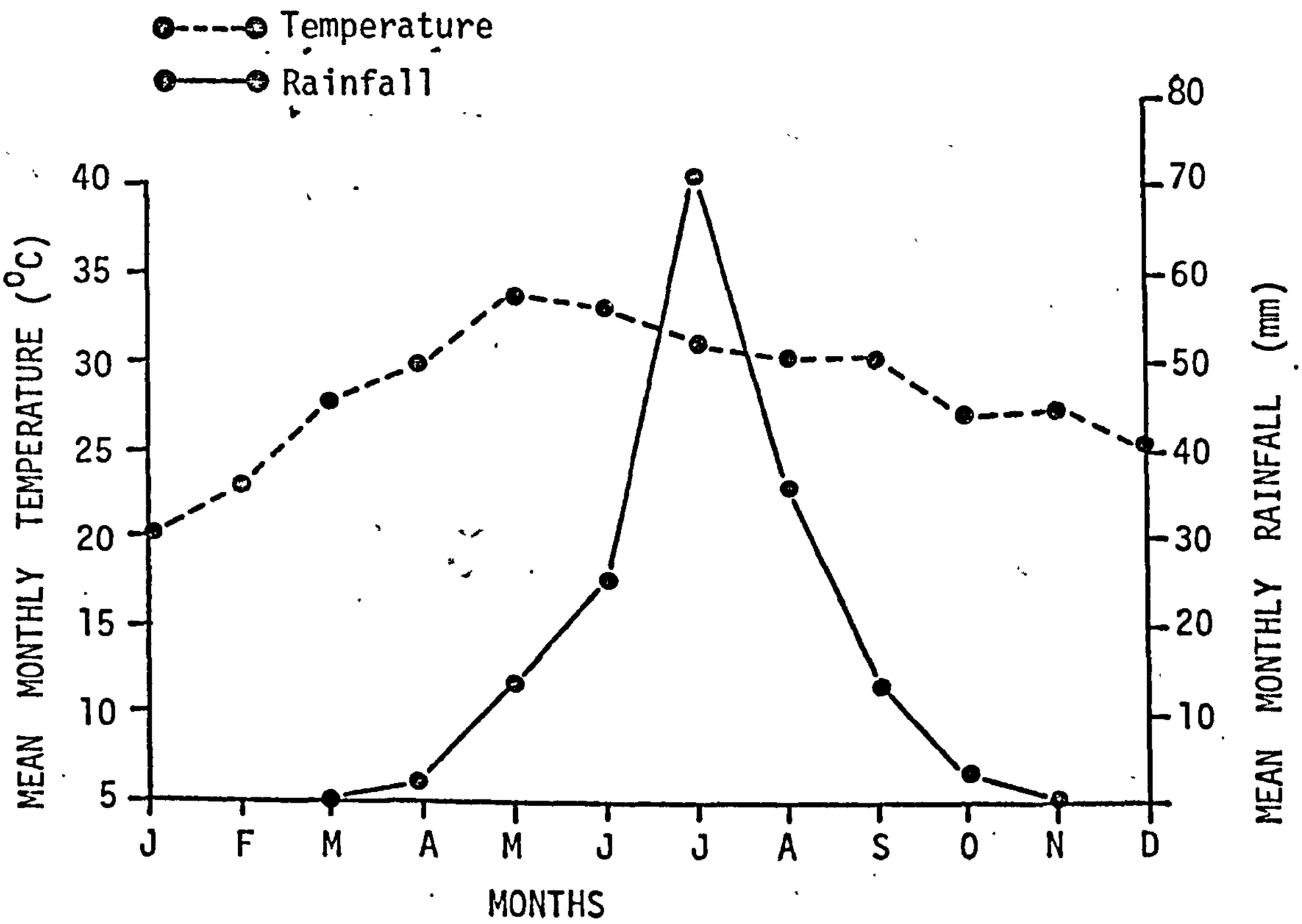
Culicoides kingi was the only species caught from animals and from the light in the animal house during the period from October 1978 to September 1979 in Shambat area.

Adult midges were collected from the animals and from the light in the animal sheds (Figure 31) using suction collector methods, which are some of the most efficient for this purpose. At the same time meteorological records for the period of investigation were obtained for Khartoum showing the temperature and rainfall (Figure 41).

In view of the abundance of C. kingi in the animal pens, samples were collected regularly twice a week. Observations were made each 30 minutes, starting one hour

Figure 41.

Monthly mean temperature and rainfall during 1978 - 1979



before sunset and ending two hours after sunset, in order to ascertain what was the best time for collection from the light.

It was found that flies were most abundant 30 minutes after sunset and that they disappeared one hour after sunset (Figure 42).

Figure 43 shows the mean of monthly variation in the light trap catches between 18.00 and 19.00 hours. The curve of C. kingi indicates a continuous increase from late June onwards, with a peak in late July, although high numbers were collected during February. The number of flies had dropped to a low level by late April and May (Figure 43). The time during which C. kingi was most abundant coincides with the rainy season and a drop in temperature in the area, namely from June until October (Figure 41).

No definite correlation was observed between the weather conditions and the prevalence of the flies. Temperatures are relatively high throughout the greater part of the year and highest during April and May (Figure 41), but as a general rule rain during the night meant a good collection the following day, while rain during the day greatly reduced the number of flies collected.

Daily factors governing biting activity

With practice, it was found that one person could collect from one cow each fly as it landed. C. kingi

Figure 42.

Average catches of adult Culicoides kingi
After sunset from the light plotted against time

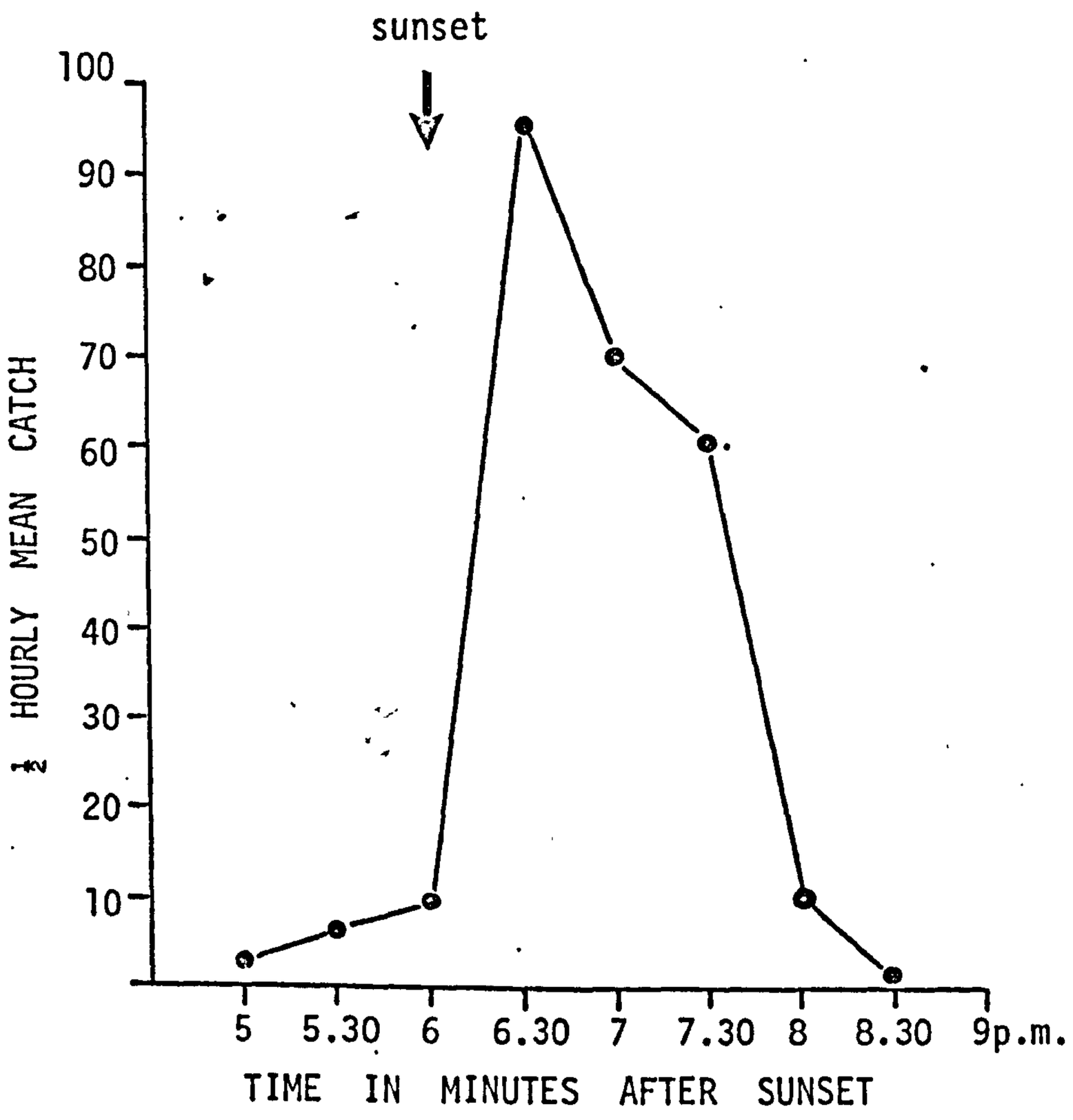
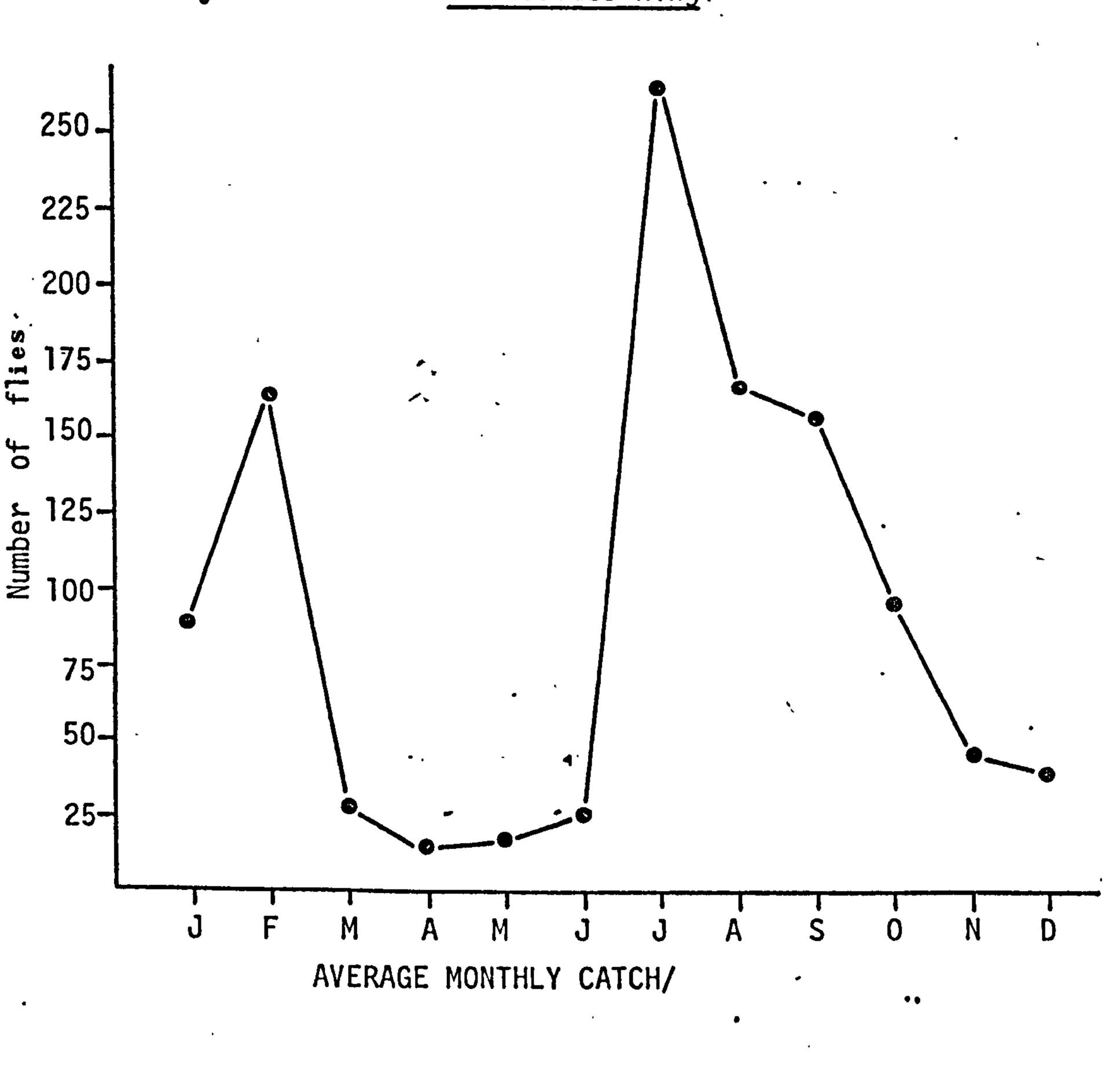


Figure 43.


Monthly abundance of Culicoides kingi
Number of flies
250
225
200
175
150
125
100
75
50
25
J
F
M
A
M
J
J
A
S
O
N
D
AVERAGE MONTHLY CATCH/

landed almost exclusively on the hump region of cattle and so a negligible number of flies were missed.

The numbers of C. kingi given in the following pages were obtained from 2 bait cows in sheds nearly one hundred yards from a heap of animal excreta, the best place for breeding El Bihari, personal communication

The collection was performed between 6.00 hours and 19.00 hours, the earliest and latest times at which it was light enough to see midges on the back of the animal.

In the early morning the midges began to arrive at the cows soon after dawn and increased in numbers after dawn to a small peak, varying in time from day to day, and then decreased again. This pattern was obtained on each of 10 mornings during observations in July when a high population of midges was available. In Figure 39 the density of flying adults, as determined by sucking tube trap, is plotted against the corresponding time of day. It is evident that C. kingi females followed a bi-phasic cycle of biting activity with the mean peak late in the afternoon, between 17.00 and 19.00 hours. There was also a subsidiary but more diffuse peak in the morning, between 06.00 and 07.00 hours, which was followed by a period of very low activity between 11.00 and 12.00 hours.

Direct sunlight, however, may be the explanation for short period of flight. Other factors might also limit flight to such a short period at midday. The duration of flight during the day was often clearly related to a

specific kind of behaviour. Downes (1950) related the peaks of activity to the two peaks of the males' swarming periods.

Discussion

Many factors are known to influence flight activity but, according to Khamala (1975) temperature and light intensity are the two most important. The present findings confirm Khamal's work, since the population peak of C. kingi occurred during the rainy season.

Flight and biting activities of C. kingi females appeared to be very much depressed, at least partly by higher temperatures and moderate wind speed. If the animals were in a stable, the flies could be found biting almost any hour of the day, except in actual rain or immediately after heavy rain or in the dark (Downes, 1950).

It was observed that two peak periods, one in the morning and the second toward the end of the day, existed during which the females became exceptionally active and this peak activity could be correlated with the two peak periods of male swarming. This was confirmed by finding that most flies caught during the day contained small ovaries with under-developed oocytes and no evidence of a blood meal (Downes, 1950).

vi. Seasonality of microfilariae in the skin

Eichler (1970) in his work on O. gutturosa in British cattle, reported that the number of microfilariae in the skin of infected cattle subjected to seasonal variations was reduced during the winter months.

A similar seasonal variation has been recorded for O. cervicalis microfilariae in Japan by Sasaki et al. (1955). They described seasonal fluctuation in the number of microfilariae in horse skin. No such reduction was observed by Mellor (1973), however, in 31 English horses infected with O. cervicalis; which is in agreement with Hussein et al. (1975) and with the present study for O. gutturosa following examination of almost 100 Sudanese cattle.

An investigation into the fluctuation of microfilariae in the skin of cattle was undertaken using two different approaches.

The first approach was by taking skin snips from 2 - 4 animals each month throughout the year. The skin snips were removed from the skin of the hump region of an infected animal, then divided into 3 layers: top layer 1 mm, middle layer 1 mm, the rest as bottom layer. Then the numbers of microfilariae which emerged from each layer within 24 hours were counted using the same technique described on page 22. The results are shown in Table 26.

The second approach was by taking skin snips every week. This skin was divided into two parts; one half fixed in 10% formal saline for histopathological study,

and the second part teased in Tyrode's plus 20% sheep serum. The numbers of microfilariae in the whole fluid were counted and the numbers of microfilariae per mg were calculated as shown in Table 27.

Density of microfilariae in the skin :

There was no significant reduction in the number of microfilariae per mg.of skin throughout the 12 months (Table 26). During the winter months (December and January) high concentrations were found in the top layer, coinciding with the winter peak in numbers of C. kingi.

The concentration in the superficial or top layer in March, April and May, the off-breeding time of C. kingi, was lowest regardless of the weather or temperature.

February and June were the peak biting periods. At the same time, the highest microfilarial concentrations were found in the top layer of the skin; so the behaviour of microfilariae is adapted to promote transmission by having the maximum numbers of microfilariae in the top layer at times when the arthropod vector is most likely to bite. Wegesa (1966a) in East Africa has shown that the microfilariae of O. volvulus were present in a much greater density at 8.0 and 10.0 a.m. in the morning and at 6.0 p.m. in the evening, coinciding with the peak biting activity of the local vector, S. woodi. Similar arrangements occur with D. immitis and D. repens in dogs in which the microfilariae are adapted to the biting habits of their vector

Table 26.

SEASONAL VARIATION IN THE DEPTH OF MICROFILARIAE IN THE HUMP SKIN OF THE COW

Month	No. of animals	Top layer mf/mg	Middle layer mf/mg	Bottom layer mf/mg
January	2	2.34	0.5	0.053
February	3	3.3	0.12	0.03
March	3	0.35	0.3	0.01
April	4	1.05	0.34	0.11
May	2	0.5	1.26	0.41
June	2	3.8	0.38	0.02
July	3	1.5	0.25	0.14
August	2	0.6	0.35	0.07
September	3	0.65	0.27	0.12
October	2	1.32	0.22.	0.06
November	3	1.66	0.30	0.08
December	4	2.74	0.89	0.02

Table 27.

SEASONAL VARIATION IN THE DISTRIBUTION OF MICROFILARIAE IN THE SKIN OF THE COW

Month	Combined weight of 3 snips (grams)	Total number of microfilariae	No. of microfilariae per mg
October	0.505	914	1.8
November	0.328	671	2.04
December	0.405	1956	4.83
January	0.418	1668	3.99
February	0.449	232	0.52
March	0.202	314	1.55
April	0.244	235	0.96
May	0.271	2464	9.09
June	0.254	505	1.99
July	0.258	235	0.91
August	0.116.	525	4.5
September	0.150	826	5.5

mosquitoes on an annual basis (Hawking, 1967).

Diurnal adaptations are quite clear in W. bancrofti in which the microfilariae are most numerous about midnight and are absent or rare during the day. (This worm is transmitted by night-biting mosquitoes.) In Loa loa, in which the microfilariae are numerous at midday and practically absent at night, diurnal adaptations are also clear, (This is transmitted by a day-biting fly, Chrysops).

The mechanisms producing such arrangements in the behaviour of microfilariae are largely unknown.

Depth distribution of microfilariae :

From histopathological examinations of sections of skin of infected cattle stained in haematoxyline and eosin, it was seen that there was a concentration of microfilariae immediately under the epidermis. However, microfilariae show an irregular distribution with a definite tendency to clumping together in a mass in certain spots, while not far distant there would be few or none.

This type of distribution explains the wide variation in the number of microfilariae ingested by individual vectors. Slides of skin snips containing microfilariae of O. gutturosa were selected over a 12 month period. The depth of all microfilariae in each positive slide in the 12 months were measured with the aid of an eyepiece micrometre, dividing the depth into three groups. Group A

included all microfilariae found between the surface to a depth of 200$^{\mu m}$, group B from 200 - 300$^{\mu m}$ and group C from 300 - 400$^{\mu m}$.

All fragments of microfilariae in each slide were measured at all depths and the results of a total of 200 microfilariae were:

44% (88 mf) were found at depth "A",

30% (60 mf) were found at depth "B",

11% (22 mf) were found at depth "C",

15% (30 mf) were found at depths deeper than 400 ^{um} from the surface.

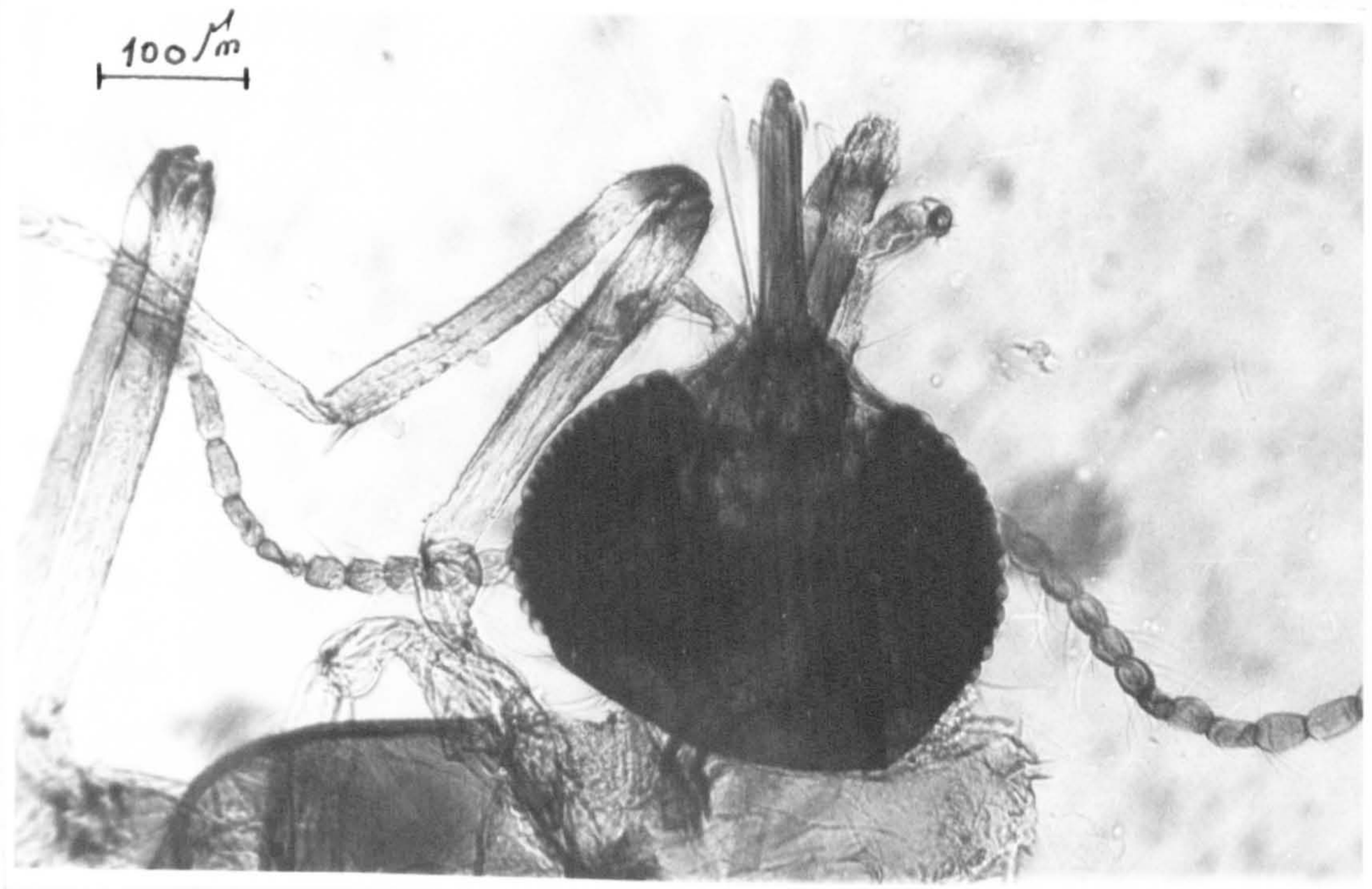
By measuring the proboscis of 20 C. kingi, the average length was found to be 165 $^{\mu m}$ (129 - 182 $^{\mu m}$), and the microfilariae in the top layer were within this range (Fig.44).

During the investigation of the vector of O. gutturosa in the Sudan, some flies were found to contain microfilariae in their blood meal; this was during January and February 1979, which is the winter-time in the Sudan. This means that microfilariae were within the range of the length of the proboscis of C. kingi, not in the deeper layers as reported by Sasaki and Sano (1955) in Japan and Eichler (1970) in England.

Thus it appears that the seasonal variation in the microfilariae densities in the skin reported in English cattle are absent from Sudan, or, if they are present, the fluctuations must be very small.

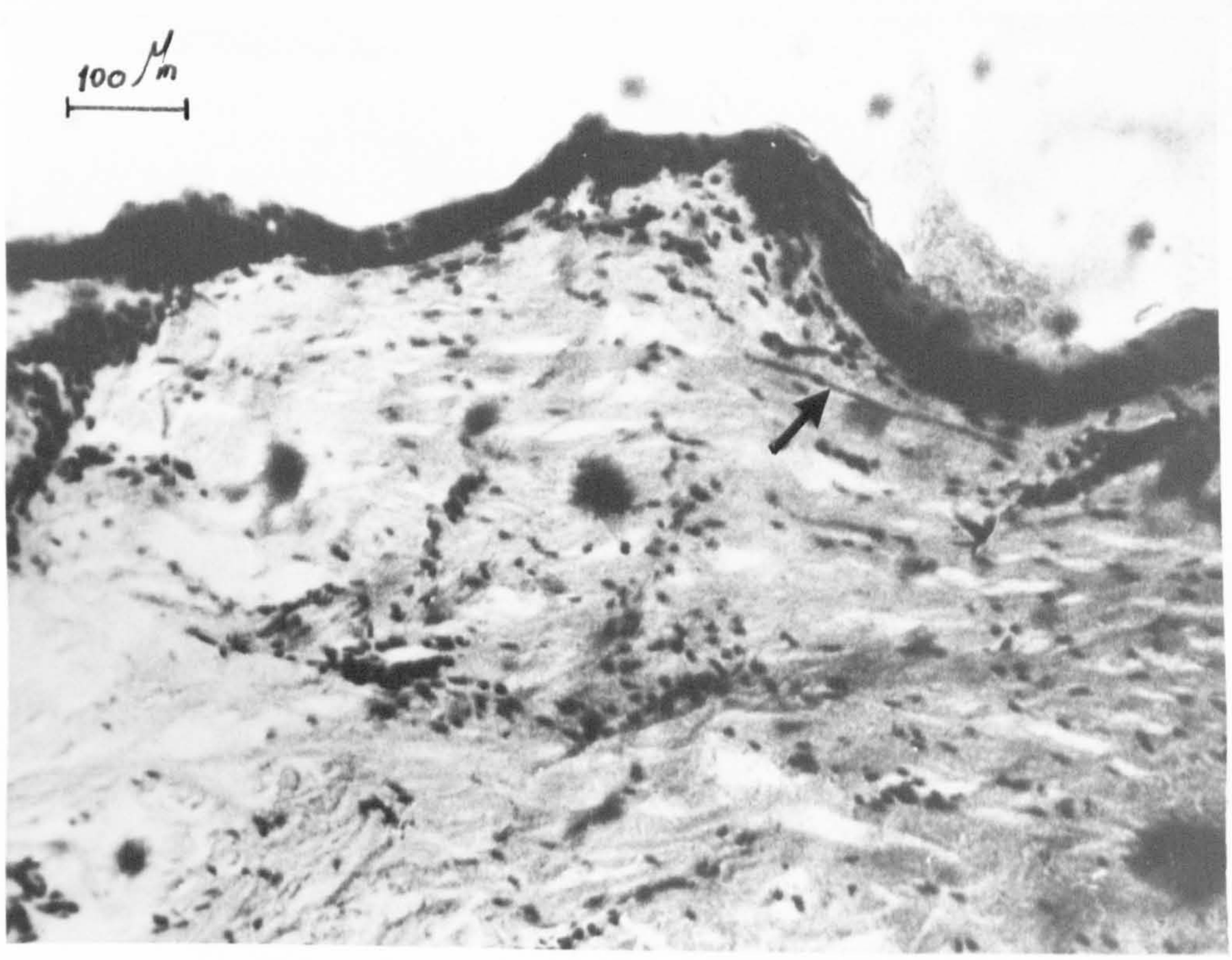
In Sudanese cattle the majority of the microfilariae of O. gutturosa were found just under the epidermis during most of the year (Figure 45), but sometimes in deeper layers. If there is a genuine migration of microfilariae, then it is probably connected with the biting cycle of the vector rather than the external temperature.

Figure 44.



The proboscis of C. kingi is well adapted for picking up microfilariae from the skin.

Figure 45.



Microfilariae in the top layer of the skin during the winter; this depth can be easily reached by proboscis of C. kingi.

Maintenance of O. gutturosa in laboratory animals and the application of this system to chemotherapy screening

A. Observations on experimental onchocerciasis in rodents

1. Studies on the microfilariae in Nile rats:

Experiment No. 1 :

An experiment was designed to investigate in detail the behaviour of uterine microfilariae of O. gutturosa in rodents and to assess its suitability as a system for testing drugs as microfilaricides and determine the type of reactions brought about by these compounds.

(a) Materials and methods

A total of 15 adult Nile rats (Arvicanthus nilotica) (Figure 46) were used in this experiment, most were males with an average weight of 135 gms.

Adult female O. gutturosa were removed from the ligamentum nuchae of freshly killed cows at Omdurman abattoir during the night and the ligaments kept at 4°C until morning.

The microfilariae used in these tests were all extracted from the anterior uterus of adult female worms and it was expected that they would be at a similar stage of development. Eichler (1970) indicated that posterior uterine microfilariae are too immature to migrate.

Figure 46.

The Nile rat (Arvicanthus nilotica) used for maintenance of O. gutturosa microfilariae and chemotherapy screening

In each case the worms were left in Tyrode's salt solution and 20% sheep serum, and cut under a dissecting microscope with a scalpel into small lengths from the anterior (one cm). They were then left for about 30 minutes to allow the microfiliarae to emerge.

The microfilariae were separated from broken adult tissue by sieving, then the suspension was centrifuged and 0.3 ml samples were injected intra-peritoneally into each of 15 rats in concentrations of 16,000 mf/rat.

The rats were killed by ether vapour, one daily for 12 days and two rats were left for a month and one killed on day 23.

The entire skin was removed, divided into 12 parts according to anatomical region, and each of these parts was cut up and placed in small bijou bottles and allowed to stand for 12 hours in Tyrode's solution plus 20% sheep serum at room temperature (25 - 27°C). The tissue was then removed and the total number of microfilariae in each part were counted.

(b) Results

The summary of microfilariae distribution in the Nile rats at 1 - 12 days post-inoculation is present in Table 28. From the Table it can be seen that the highest concentration of microfilariae was recovered from the ears, scrotum and tail, in a descending rate, and the highest number of microfilariae appeared on day seven in these regions.

Table 28.

SUMMARY OF MICROFILARIAE DISTRIBUTION IN THE NILE RAT

Region	Post-inoculation days and no. of mfs. found in each region											
	1	2	3	4	5	6	7	8	9	10	11	12
Lips	-	-	-	-	-	-	-	-	-	-	-	-
Ears	-	-	-	8	10	14	22	18	8	10	9	10
Nose	-	-	-	-	-	4	2	3	-	-	-	-
Cheeks	-	-	-	-	-	2	3	-	-	-	-	-
Top of head	-	-	-	-	-	-	2	-	-	2	-	-
Neck & shoulder	-	-	-	-	-	-	2	-	-	-	-	-
Front legs	-	-	-	-	-	2	-	-	-	-	-	-
Hind legs	-	-	-	-	-	3	-	-	-	-	-	-
Abdomen	3	-	-	-	-	-	-	-	-	-	-	-
Tail	-	-	-	6	6	8	10	5	5	6	4	2
Scrotum	-	4	6	12	4	12	14	4	3	6	4	2
Back	-	-	-	-	4	2	-	-	-	-	-	-

The results indicated that there is a wide range of variation in the ability of the microfilariae to migrate. Most microfilariae migrated to the ears, scrotum and tail and the rest were distributed along the whole body surface. This result was similar to the observations of El Bihari and Hussein (1975) who recovered microfilariae of O. armillata from the tail and ears of albino rats after three weeks. On the first day microfilariae were recovered from the skin of the abdomen, then no microfilariae were recovered until 12 days. This could be escaped during intra-peritoneal injection (I/P) of microfilariae and some microfilariae stayed in the skin.

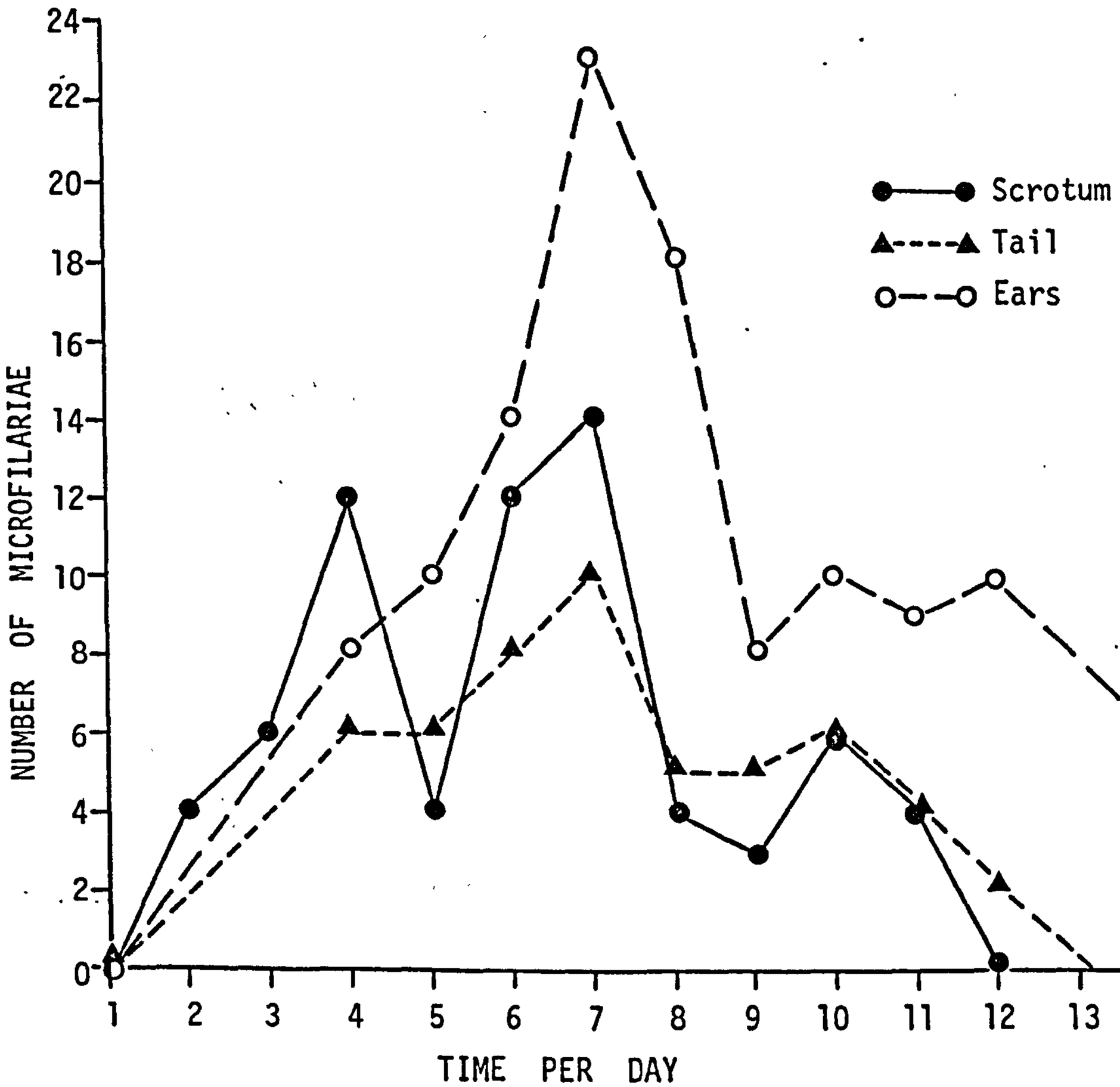
It is apparent that uterine microfilariae reach the skin of the scrotum within 48 hours, and ears and tail after 4 days. This is not as efficient as skin microfilariae which reach the skin in 24 hours (Al Zubaidy, 1973). Uterine microfilariae make their first appearance in most parts of the rat's body within 6 days, and their maximum numbers are reached 7 days after injection; then numbers started to decline. No microfilariae were found in the blood.

The numbers of microfilariae in the nose of the rat were few if compared with the number recovered from mice reported by Eichler (1970) or Al Zubaidy (1973) from the British strain of O. gutturosa.

The great majority of the microfilariae go to the ears, scrotum and tail (Figure 47). It is not known why

Figure 47.

Distribution of uterine microfilariae of
O. gutturosa in main 3 regions of the Nile rat



these sites were chosen. Eichler (1970) suggested that these sites (tail and nose) have the lowest surface skin temperature and the microfilariae are stimulated to move down a temperature gradient. From measuring the temperature of the tail, scrotum and ears it was found that they were nearly the same temperature (34°C) which was about the lowest temperature in the rat skin.

In mice Nelson et al. (1966) recovered microfilariae after 120 days and El Bihari and Hussein (1975) after 21 days, but in this study no microfilariae were found in the skin of the rats after 23 days, which is similar to Eichler's (1970) study in mice infected with microfilariae of the British strain of O. gutturosa.

2. Studies on microfilariae of O. gutturosa in mice

In the current study the first experiment was an attempt to determine the distribution of skin microfilariae of O. gutturosa in mice; the second experiment determined the better route for inoculating microfilariae, while the third experiment determined the maximum time for microfilariae to remain in a proxy host.

Eichler (1970), when studying the migration of uterine microfilariae of O. gutturosa, found that a certain proportion were immature. The proportion varied in relation to the part of the uterus from which they had been extracted. The proportion of microfilariae in one batch of mice could not, therefore, be reproduced in another batch unless they had both been injected with the same microfilariae suspension. In this study the difficulties experienced by Eichler were overcome by the use of mature skin microfilariae.

(a) Materials and methods :

Microfilariae of O. gutturosa were obtained from the umbilical skin of freshly slaughtered British cattle, from Reading abattoir. The microfilariae were extracted using aseptic techniques as previously described (Bianco et al., 1980; see page 163). The numbers of microfilariae in suspension were estimated by calculating the mean of the three 0.01 ml aliquots, and then multiplying up to find the number in the total volume of the suspension. Then, according to the number of microfilariae required for injecting each mouse, the suspension was divided equally in a dose of 0.2 ml per mouse.

i. Microfilarial distribution in the skin of the mice

A total of 5 mice were used; 5000 microfilariae were injected subcutaneously (nape of neck) into each mouse. The mice were killed after 6 days. At this time the higher number of microfilariae should be recovered from the ears and nose (Eichler, 1970; Mellor, 1971; Al Zubaidy, 1973). Their ears, nose, skin of the back, skin of the belly, foot pads and tail were all placed separately in clean universal bottles containing sterile Tyrode's solution supplemented with 20% serum, and chopped into small pieces with scissors, then left for 24 hours at 25 - 27°C (Bianco et al., 1980). All microfilariae which had emerged into the solution were counted in each region separately.

Table 29.

THE DISTRIBUTION OF SKIN MICROFILARIAE OF O.GUTTUROSA IN THE SKIN OF MICE

Days after infection	No. of mice used	Mean number of microfilariae per mg skin					
		Ears	Nose	Back	Belly	Foot pad	Tail
6	5	0.44	0.75	0.08	0.0085	0.08	0.015

Table 30.

THE NUMBER OF MICROFILARIAE RECOVERED FROM THE EARS AND NOSE AFTER DIFFERENT ROUTES OF INOCULATION

Route of injection	No. of mff per mouse	No. of mice	No. of microfilariae recovered			
			Ears		Nose	
			Mean	S.D.±	Mean	S.D.±
Subcutaneous	2000	5	64	55	5.4	2.9
Intravenous	2000	5	3.0	4.16	0.3	0.84
Subcutaneous	2500	5	11.3	1.2	2.3	0.6
Intramuscular	2500	5	0.2	-	0.4	0.6
Subcutaneous	5000	5	63.4	23.8	11.0	7.0
Intraperitoneal	5000	5	6.0	4.5	1.8	1.6

The results were similar to those obtained by Nelson et al.(1966), Eichler (1970), Mellor (1971) and Al Zubaidy (1973)(Table 29).

Almost all microfilariae which migrated moved either to the ears or to the nose, while the rest of the microfilariae were distributed over the other regions of the body as shown in Table 29.

ii. Experiment to compare methods of administration of microfilariae into mouse skin

The trial was carried out to assess the best method for inoculating the microfilariae into the mice.

Three groups of mice were used, 10 mice per group, and the group sub-divided into two sub-groups of 5 each.

Group one : 5 mice were injected with 2500 skin microfilariae of O. gutturosa each intramuscularly and another 5 subcutaneously.

Group two : 5 mice were injected subcutaneously and the other 5 intravenously (tail vein) with 2000 microfilariae each.

Group three : 5 mice were injected intraperitoneally and another 5 subcutaneously with 5000 microfilariae each.

It was hoped that, by injecting the same number of microfilariae into each mouse in each group, approximately the same number would migrate by each route.

The mice were sacrificed 6 days after injection (Nelson et al, 1966; Eichler, 1970; Mellor, 1971).

The previous trials with microfilarial distribution in the skin of mice suggested that most microfilariae concentrate in the ears and nose, so microfilariae recovered from the ears and nose only were counted (Table 30).

The results indicate that there is a wide range of variation in the ability of microfilariae to migrate to the ears and nose according to the route of infection (Table.30). The preferential distribution pattern in the skin of the mice is not necessarily determined by the routes of inoculation. In all routes the highest numbers of microfilariae were recovered from the ears and nose with variations in number. The subcutaneous route proved to be the best route for inoculating microfilariae of O. gutturosa for study in a proxy host, because high percentage (3.2%) from inoculated microfilariae migrated to the ears.

iii. Maintenance of skin microfilariae of O. gutturosa in (T.O.) mice

In this experiment all 75 mice were each injected subcutaneously between the shoulders with 2000 microfilariae extracted by the same techniques already described before. The mice were killed, 5 mice at a time, after intervals of 24 hours, 3 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 12 days, 14 days, 16 days, 18 days, 20 days, 22 days and 25 days. Their ears were removed at their bases as well as the nose from each mouse separately and placed

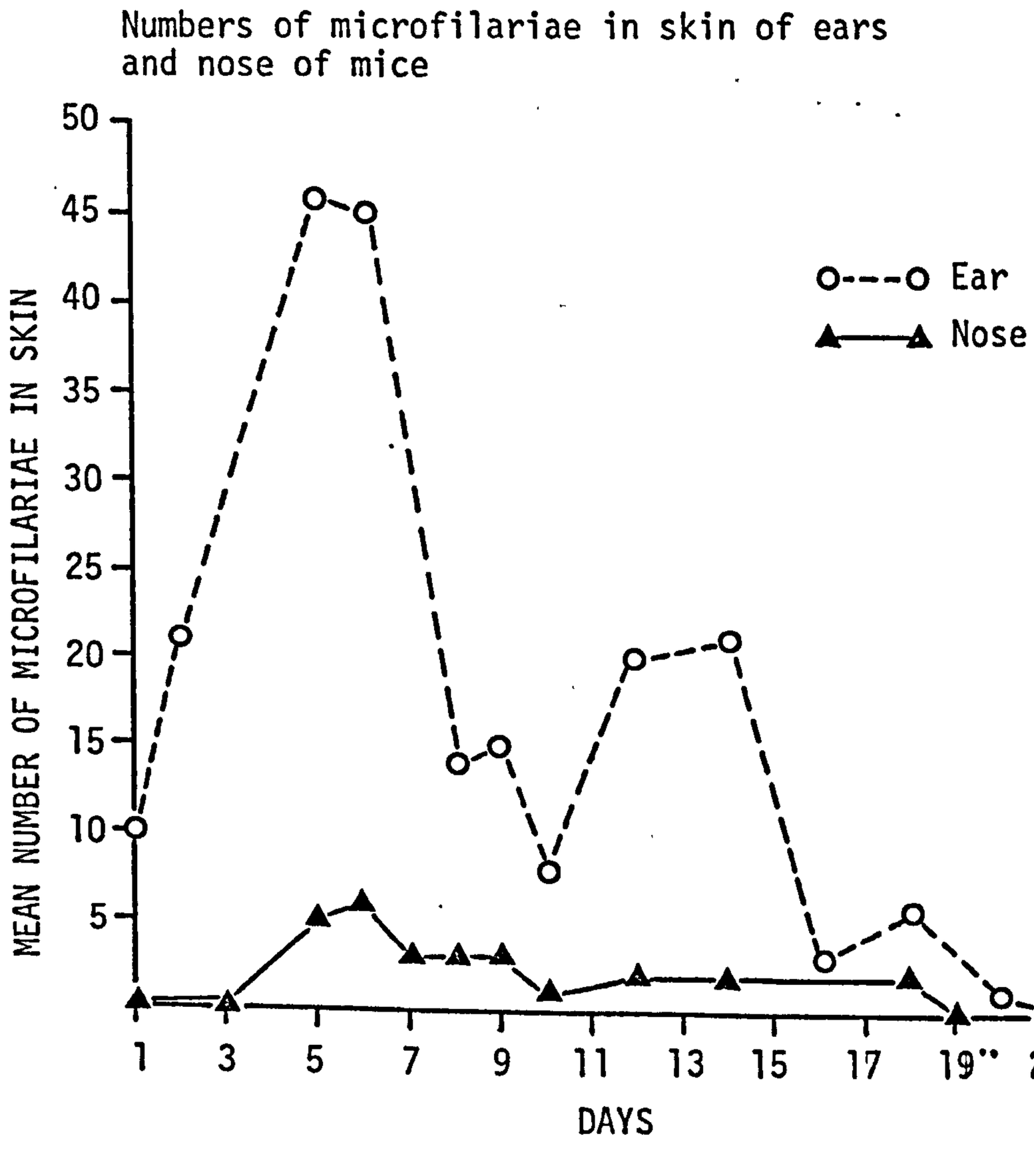
in Tyrode's solution containing 20% serum; chopped into small portions with a pair of scissors, then left for 24 hours at 27°C. All microfilariae which had emerged in the solution were counted for each mouse, and the mean calculated.

The results are expressed in Figure 48, which indicate the rates of migration of microfilariae to the ears and nose during the 25 days of the experiment.

The results were similar to those obtained by Eichler (1970), Mellor (1971) and Al Zubaidy (1973). Peak numbers were recovered in the nose after 6 days (mean of 6 mff) and in the ears after 5 days (mean of 46.6 mff). Then the numbers rapidly decreased and by 18 days very few microfilariae were found in the ears and nose, and by 22 days, none. This fluctuation in the number of microfilariae in mouse skin is plotted in Figure 48.

Two thousand microfilariae had been injected into each of 75 mice used in this experiment, but a mean maximum value of only 46 microfilariae (2.3%) were recovered from each of the five mice killed on day five. Graph 48 shows a rapid migration of microfilariae to the ears and nose. It is not known why the ears and nose are chosen sites to which microfilariae move. Eichler (1970) suggested that microfilariae move down a temperature gradient.

Figure 48.


Numbers of microfilariae in skin of ears and nose of mice
MEAN NUMBER OF MICROFILARIAE IN SKIN
50
45
40
35
30
25
20
15
10
5
1
3
5
7
9
11
13
15
17
19**
21
23
DAYS
Ear
Nose

3. Preliminary studies on transplantation of adult O. gutturosa into laboratory rodents

Filarial worms are mostly very specific to their vertebrate hosts, but accidental and laboratory infection may occur. O. gutturosa, a common parasite of cattle, has been reported from horses (Ottley and Moorhouse, 1978) and from sheep (Bain and Muller, 1978). Onchocerca armillata of cattle has been reported from camels (Schillhorn van Veen et al., 1976) and from sheep (Bhatia, 1960).

Duke (1962) succeeded in infecting chimpanzees with O. volvulus, but the chimpanzee is not very suitable as a model for laboratory studies. Complete development of adult worms of onchocerciasis in other laboratory animals has not yet been reported, although Suswillo et al. (1977) tried to inject jirds with O. volvulus, but no adult filarial parasites were recovered.

The failure of the laboratory animals to harbour this worm could be due to their resistance to infection

or to the failure of worms that penetrated the skin to find the environment suitable for maturation.

Many filarial worm transplants have been achieved successfully in laboratory animals. Wharton (1946) transplanted adult filarial worms of L.carinii into new cotton rats; Williams (1955) succeeded in transplanting Setaria cervi from cattle to the rabbit and Nelson (1962) transplanted Setaria labiatopapillosa into rabbits and monkeys; Ansari (1964) succeeded in transplanting Setaria cervi into white rats; Zielke (1977b) transplanted adult D. immitis, the heart filaria of dogs, into laboratory rodents. Butts and Rabalais (1974) transplanted juvenile (LSS) and Suswillo and Denham (1977) transplanted adult Brugia pahangi into jirds. These successes led people to hope that this technique would be suitable for studying onchocerciasis because the experimental study of filariasis is handicapped by the prolonged period of development of filarial worms in man and animals.

An attempt has been made to transplant adult worms of Onchocerca gutturosa of cattle into laboratory rodents and to study

its behaviour in such abnormal hosts, with the following purposes in mind:

1. evaluation of any possible sterilizing effect of drugs upon adult worms;
2. obtaining quantitative estimation of the number of microfilariae produced for a definite period of time by single fertilized female worms.

(a) Materials and methods

As a first step it was necessary to extract complete live adult worms from the connective tissue of cervical ligaments.

Live male and female O. gutturosa were obtained from the ligamentum nuchae of recently killed animals using collagenase digestion technique (Schulz-Key et al. (1977) see page 20).

Fully active females were recovered after 2 - 24 hours digestion. After long digestion times some worms were found to be adversely affected, therefore complete active worms were selected for worm transplants.

After complete digestion of connective tissues, the released worms were placed in artificial media of Tyrode's solution plus 20% cow serum in cover petri dishes. There was usually $\frac{1}{2}$ - 1 hour before they were transplanted into experimental animals. Kershaw (1949) showed that with Litomosoides carinii, transplants were most effective if the transfer was done rapidly.

The recipient rodent is anaesthetized, (using ether vapour), the abdominal fur removed with electric clippers and the skin wiped with alcohol, and tied on its back to a cork board. A 1 cm incision was made in the lateral abdominal wall of the posterior abdomen and the worms (mean length 43 cms) placed directly onto exposed viscera in the abdominal cavity with gentle manipulation, using fine forceps, and pushed inside with a blunt instrument. The incision was then closed with a Michel clip; no special aseptic technique needed to be applied.

In most cases female worms were implanted without males. Webber (1954) demonstrated that it is probably not necessary to transplant male worms; she found that L. carinii transplanted females produced microfilarial densities equal to those produced by males and females. On some occasions recovered active male worms were re-transplanted again; the transfer from donor to recipient was rapid.

Because of difficulties in obtaining complete adult females, without using collagenase, and the cost of collagenase was expensive, these difficulties have been largely overcome by developing a new technique: trans-planting adult worms in the connective tissue, by selecting lightly infected ligament, and removing the connective tissue containing worms from the ligament, and placing in a petri dish containing Tyrode's solution plus 20% cow serum, under a dissecting microscope. All tissue around

the worms was removed very carefully, leaving a very small amount of connective tissue with the worms embedded in it.

By using the technique described above, adult worms in a small amount of tissue were placed in the peritoneal cavity of mice.

The third method used was transplanting adult males only extracted by gentle dissection of the ligamentum nuchae under a dissecting microscope.

(b) Autopsy of infected animals

Dead animals were eviscerated and the abdominal and peritoneal cavities and thorax were thoroughly searched for worms.

The organs were then removed and placed in petri dishes containing Tyrode's solution and the peritoneal cavity was washed with Tyrode's solution, and adult worms were recovered under a dissecting microscope. Live active worms recovered were placed in warm Tyrode's solution and then re-transplanted to uninfected mice.

(c) Survival of adult males in vitro, in a variety of simple media :

Media used included distilled water, normal saline, Tyrode's solution, Tyrode's solution supplemented with 20% bovine serum, and tissue culture medium 199 (Wellcome). The worms were dissected from freshly collected ligaments, maintained in covered petri dishes as 23°C and examined

daily to assess their mobility.

Results obtained from six trials indicated that Tyrode's solution plus 20% bovine serum was the best medium of those tested, supporting active worms for up to 15 days. This period of survival might well be improved by the elimination of bacteria from the culture medium.

4. <u>The transplantation of adult worms into laboratory rodents</u>

Three methods were employed:

1. Intra-peritoneal transplant of male worms isolated from cow tissue by gentle dissection
2. Intra-peritoneal transplant of female worms isolated from the cow tissue by collagenase digestion
3. Intra-peritoneal transplants of female worms contained within the cow connective tissues removed from fresh cervical ligament.

(a) <u>Intra-peritoneal transplant of adult male worms</u>

Large numbers of active adult males, up to 7, can easily be obtained from the cervical ligaments of infected cows by careful dissection under a dissecting microscope. By pulling the anterior part of an adult female, a wriggling male will be seen coiled in the connective tissue. Using fine forceps the males were picked up and placed in Tyrode's solution plus 20% serum.

All males of 2 - 3 halves of <u>ligamentum nuchae</u> can be

extracted within one hour. Extracted males remained active in Tyrode's solution plus serum at 23°C for at least 24 hours.

In an attempt to determine how long the adult male worms will survive in an abnormal host, transplants of male worms were made in 25 laboratory rodents. Eight of these died, and two were used for chemotherapy. The remaining rodents survived and autopsies were carried out. The animals were killed to recover worms in various periods of time and worm recovery data is shown in Table 3.1.

The above experiments have clearly demonstrated the ability of the transplanted worms to survive in rodents. However, the most significant feature of the experiment was the survival of the worms in a new host. Thirteen living worms (30%) were recovered from rodents autopsied within various time periods (6 - 133 days). All worms recovered were found to be still active and in good condition; however CBA mice proved to be the most suitable rodent host for experimental purposes.

(b) Intra-peritoneal transplant of adult females isolated from cow tissue by collagenase enzyme

It was possible to obtain live complete adult females of O. gutturosa by collagenase digestion technique (Schulz-Key et al., 1977) within 2 - 24 hours of digestion depending upon the amount of connective tissue in which the adult worms were embedded (see page 20).

It was uncertain whether the collagenase digestion caused any damage to adult worms, because many of them failed to survive implantation. Even when the worms survived transplantation for several days, their reproduction was adversely affected by the procedure, and the microfilariae were absent from the skin, even when the female worms were living. This observation was in agreement with Webber (1954).

Of 16 animals into which adult female worms were transplanted, no microfilariae were found in their ears, except for one animal which showed microfilariae after 5 days post-infection, which persisted for 3 days only.

Live worms were recovered from only two mice with 10 days maximum survival time. Rodents were autopsied for examining the state of worms originally transplanted. Details of this observation are given in Tables 31,32.33.

(c) <u>Intra-peritoneal transplant of female worms contained within cow connective tissue</u>

Because of the difficulties of obtaining complete live worms without collagenase digestion, which is very expensive, a new technique of transplanting adult worms with a small amount of connective tissue was used. The technique requires much practice until isolated complete worms in very small host tissues can be obtained.

Five laboratory animals were transplanted. Two of them died within 24 hours post-infection; the remaining

Table 31.

RECOVERY OF TRANSPLANTED ADULT MALE WORMS IN LABORATORY MICE

Recipient mouse strain	No. of male worms transplanted	Duration of transplant (days)	No. of worms recovered	Condition of recovered worms
Shasha	5	6	5	All in good condition
Shasha	2	6	0	
Shasha	2	13	0	
CBA	1	7	0	
CBA	2	7	1	In good condition
CBA	2	48	2	Live and active
CBA	2	133	1	In very good condition
CBA	3	180	0	
CBA	3	64	1	Live and in good condition
CBA	3	88	1	Live and re-transplanted
CBA	2	60	1	Live from re-transplant
Shasha	4	90	4	All dead
CBA	3	104	1	Dead
CBA	3	104	0	
CBA	5	104	1	Live

Table 32.

DATA ON ADULT WORMS TRANSPLANTED WITH CONNECTIVE TISSUE

Recipient mouse strain	Total no. of mff. per mg in each day after transplant								Duration of transplant (days)	Condition of recovered worms
	1	2	3	4	5	6	7	8		
Shasha	3.3	1.05	12.2	2.5	3.75	17.2	0.17	0.37	50	Worms recently dead
Shasha	-	-	-	-	-	-	-	-	15	Live male and dead female
Shasha	0.13	5.0	0.2	-	-	-	-	-	66	Live male and female

Table 33.

DETAILS OF ADULT WORMS OBTAINED BY DIGESTION TECHNIQUE AND TRANSPLANTED INTO LABORATORY RODENTS

Recipient mouse strain	Digestion time in hours	No. of worms transplanted	First appearance of microfilariae (days)	Duration of microfilariae (days)	Maximum no. of microfilariae	Duration of transplant (days)	Condition of recovered worms
Shasha	20	1 female	-	-	-	71	No worms recovered
CBA	2	1 "	-	-	-	66	"
CBA	13	1 "	-	-	-	90	"
CBA	13	1 "	-	-	-	3	Worm in good condition
Shasha	6	1 "	-	-	-	1	Worm in good condition and re-transplanted
Shasha	6	1 "	-	-	-	84	Dead worm
CBA	18	1 "	-	-	-	83	"
CBA	18	1 "	Mouse died no autopsy was carried out				
Shasha	Re-transplant	1 "	-	-	-	83	No worm recovered
CBA	16	1 "	-	--	-	10	Worm dead
Shasha	16	1 "	Both died no autopsy				
Shasha	16	1 "					
T.O.	5	1 " 3 males	Animal found dead				
T.O.	5	1 female 1 male	-	-	-	26	No worm was recovered
T.O.	28	1 female	Mouse died no autopsy - after two days				
T.O.	6	1 female 4 males	15	3	8	14	3 live males, female not recovered

three Shasha mice survived and regained full health.

Two of them exhibited microfilariae in the skin of the ears; they were repeatedly examined each day until no microfilariae were seen. The figures of these counts are given in Table 32.

In the first mouse (No. 1) detectable microfilariae were evident 3 days post-transplant, and in the second mouse (No. 3) after 9 days. The fluctuation in the number of microfilariae recovered continued in the first mouse for 37 days and in the second for 14 days (Figure 49).

The peak of microfilariae in both mice occurred on Day 17 and Day 15 respectively.

Because of the successful survival of adult female worms for 66 days in a proxy host, this easy technique can be used to transport adult worms in a small suitable host over long distances.

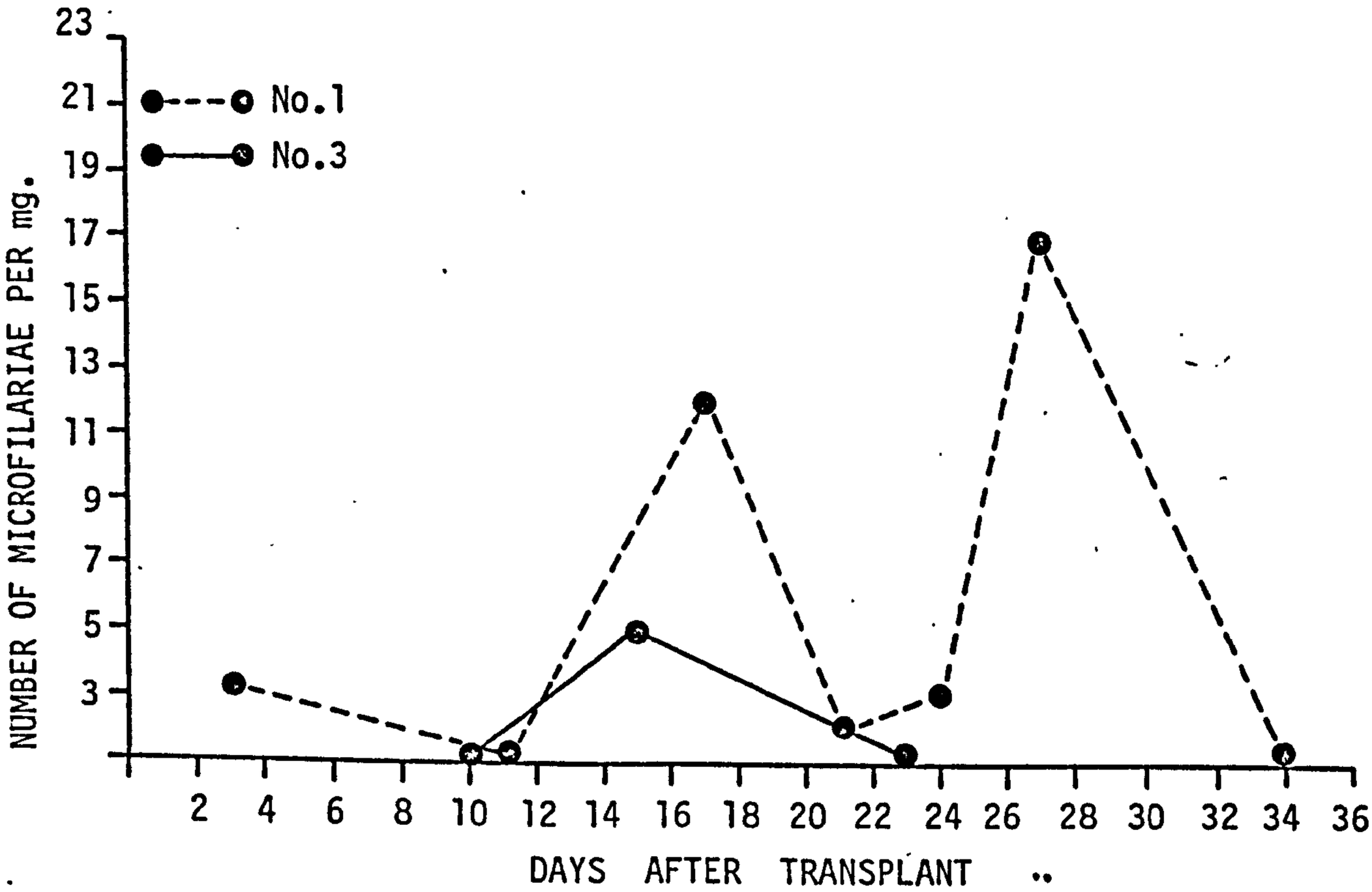
(d) Discussion

The above experiments have clearly demonstrated the ability of the transplanted worms to produce microfilariae in mice. Johnston et al. (1974) and Beaver et al. (1974) followed the microfilariae of recipient jirds infected with transplanted Dipetalonema viteae for up to 2 years.

In the present work in which large numbers of adult O. gutturosa were transplanted to different mouse strains, the longest time interval between worm transplant and host necropsy of adult worms recovered (regardless of

Figure 49.

Graph showing the fluctuation of microfilariae in mice infected with transplanted O.gutturosa



worms' sex) was 133 days.

A low level of microfilariae for a short duration have been reported for other filarial species transplanted into abnormal hosts (Williams, 1955; Nelson, 1962; Ansari, 1964; Spratt, 1972).

The few microfilariae detected for a short time in the skin of the ears of mice after transplantation of adult worms presumably reach the skin of the ears before the new host can mount its response to the parasite.

Wharton (1946) reported that male worms often survive attacks by defence mechanisms of the host which destroy the females. This could be the reason why more adult males were recovered alive.

Kershaw (1949) used a technique in which adults of Litomosoides carinii were transferred very rapidly from donor to pleural cavity of recipient. Nearly all the worms survived the transplantation, and this success was attributed to the rapidity of the transfer.

In these experiments on the transplantation of adult worms, the worms had been in the digestion enzymes for various periods of time, varying from 2-20 hours, and the live worms transferred to artificial media waiting for transplant. Although they were alive during this procedure for at least 18 hours, many of them failed to survive implantation.

It is obvious from these experiments that mice

did not support adult worms as well as micro-filariae for a long period. However, they proved to be fairly suitable hosts for short term experimental purposes. Sen and Bhattacharya (1964) transplanted adult worms of Dipetalonema viteae into rats. They were, however, unable to obtain any worms on autopsy and they postulated that these worms died soon after transplantation and the debris was phagocytosed.

5. Efficacy of diethyl carbamazine (Hetrazan) in the Nile rat

(a) Methods :

A suspension of uterine microfilariae of O. gutturosa was prepared in Tyrode's medium plus 20% sheep serum as already described (page 196) and divided equally between 10 rats, 20,000 microfilariae to each. Four days after the intra-peritoneal introduction of microfilariae they had already appeared in the ears, tail and scrotum, but not at its highest figure (Table 34, Figure 50). Half the rats were each injected intra-peritoneally with 200 mg/kg body weight of DEC aqueous solution, and the other half received equal doses of normal saline.

On day seven from the introduction of the microfilariae, the total number of microfilariae recovered from ears, tail and scrotum of each rat was counted after processing in the same way as described earlier (page 198).

(b) Results and discussion :

The results are summarized in Table 34 and show a substantial reduction in the number of microfilariae recovered from the treated group.

The present investigation has been limited to a small number of animals, but the results show that the differences are highly significant. The efficacy of diethyl carbamazine in this sytem can be judged from the high reduction in the

numbers of microfilariae.

Microfilariae can be maintained alive in the skin of laboratory animals up to 120 days (Nelson, 1966). So this method can be applied for detailed study of the activity of microfilaricidal compounds, although this is far from ideal.

Table 34.

EFFICACY OF A SINGLE DOSE OF DEC AGAINST MICROFILARIAE OF O. GUTTUROSA

Control			DEC Group		
No. of microfilariae recovered			No. of microfilariae recovered		
Ears	Tail	Scrotum	Ears	Tail	Scrotum
6	5	13	Nil	1	3
10	4	12	Nil	2	2
15	7	10	1	1	3
7	7	8	Nil	Nil	2
12	8	10	Nil	2	2

6. Action of DEC in Natural Host

Since Duke (1962) showed that O. volvulus could be transmitted experimentally to chimpanzees, this animal has remained the only experimental host in which drugs against the human disease could be tested.

Chimpanzees obviously cannot be developed into models for routine screening, as it is unacceptable to exploit this vanishing species when alternative systems are available for development.

Denham and Mellor (1976) and Copeman (1979) proposed that cattle infected with O. gutturosa or O. gibsoni are relevant to human onchocerciasis and that much is to be learned by a comparative study of this system.

Of the 34 species which have been assigned to the genus, most infect herbivores (Muller, 1979). Surprisingly few studies on chemotherapy of animals have been reported, a possible reason for neglect of these parasite may be their relative unimportance as a cause of ill health in animals.

Thomas (1958) has had some success with the treatment in the case of skin lesion in a horse with onchocerciasis; Thienpont and Bichwe (1957) claimed to have cured two cows with dermal onchocerciasis; Sasaki et al. (1957) treated skin lesions in horses by DEC; Patnaik (1962) treated two bulls suffering from acute attacks of fits and acute ocular onchocerciasis with vitamin A injected intramuscularly,

supplemented by oral administration of Hetrazan.

Denham and Mellor (1976) treated three cows infected with O. gutturosa by Compound E (Friedheim).

Many drugs have been used for human onchocerciasis treatment but are either ineffective or too toxic (Rodger, 1958; Duke, 1977). According to reports by Hawking (1958); Nelson (1970); Duke (1972); and Gibson et al. (1977) at the present time only two drugs are recommended against O. volvulus. The first of these is diethyl carbamazine citrate (Banocide, Hetrazan, Notezine, Carbilazine, Carbicide and 3799RP) which appears to lead to the death of microfilariae by unmasking them, so that they are recognized as foreign bodies to be destroyed by the host's cellular defence; it has no effect on adult worms (Duke, 1972). Treatment with this drug causes severe and unpleasant reactions (Adams, 1953; Hawking, 1958; Nelson, 1970; Duke, 1972).

The second drug is Suramin (Antrypol, Bayer 205, Belgamyl, Fornean 309, Germanin, Moranyle, Naganin, Naganol and Napharide). In addition to its effect on adult worms, Suramin can cause serious toxic reaction in infected or non-infected hosts (Gibson et al. 1977), and has little effect on microfilariae (Duke, 1972).

The aim of this study was to compare the effect of the two drugs on O. gutturosa in the natural host and at the same time to determine whether this parasite system shows

promise as a tertiary testing screen for onchocerciasis drugs.

(a) Materials and Methods :

Three Zebu naturally infected cows were purchased from Omdurman local market. The animals, apart from having onchocerciasis, were all in average good health. The three animals were between 7 - 10 years old, because by this time the concentration of microfilariae will be reasonably abundant in the skin and will be reasonably constant.

In order clinically to determine the number of microfilariae in the skin of each animals, pieces of skin were removed from the hump regiou of each cow using snippers (see page 14). The snipping was always done in the morning between 08.30 hours and 12.30 hours. These skin snips were weighed and then teased in Tyrode's solution plus 20% sheep serum. After three hours the microfilariae which had emerged were counted and the number of microfilariae per milligram of skin of each examination calculated.

The drugs were assessed for microfilarial concentration in the skin and on the condition of the adult worms and uterine microfilariae by dissecting ligamentum nuchae after treatment. In parallel with this experiment a histological examination was made; a skin snip being divided into two halves, one fixed in 10% formal saline for histopathological study, and the other half for assessment of the number of microfilariae.

The three cows were each treated separately. Cow No.3 was used as a control and treated with normal saline.

The drugs and normal saline were injected intravenously into each cow after assessment of the proper dose. The drugs were dissolved or suspended in distilled water.

In order to study the effect of the treatment on adult worms, autopsies were carried out on the three cows 20 days from the first dose. The ligamentum nuchae were removed and attempts were made to extract the whole worms from the connective tissue using an enzymatic digestion method (Schulz-Key et al., 1977).

Complete unbroken worms were obtained in each case; the uteri of the female worms were dissected and the viability of microfilariae judged by mobility.

To study the depth distribution of microfilariae after treatment, a 2 cm square of skin was removed from the skin at autopsy and sliced with a scalpel into three layers. The first surface layer and the next layers were all about 870$^{\mu m}$ in thickness. Each layer was weighed and processed in the manner already described to extract the microfilariae. The number of microfilariae per milligram of skin in each of the three layers are shown in Tables 36, 38 and 40.

(b) Results :

Cow No.1 : A 10 year old cow weighing 250kg and in good health was treated with DEC (Hetrazan) in a concentration of 2 mg per kg body weight. Hetrazan was in tablet form of 50 mg.

Fresh preparations were made up in distilled water, the solution containing 500 mg per 20 cc distilled water. The drug was administered by intravenous infusion on 4 occasions on days 1, 2, 3 and 5, making a total dosage of 8 mg per kg body weight. Each infusion lasted about 10 minutes.

The cow showed no sign of distress after the injections but there was swelling at the site of the injection. There were no subsequent changes in the skin, except small rashes diffusely distributed over the back of the animal; the rashes were transitory and lasted only two days. There were no severe reactions as reported by Thomas (1958) after his injection in the horse or by Denham and Mellor (1976) when treating cows with Compound E.

Skin snips were collected before, during and after treatment; each time half of the skin snip was fixed in 10% formal saline and prepared for histological examination in the usual way, and the other half used for counting the number of microfilariae (Table 35).

The cow was killed 20 days after the first dose. One complete alive female and 3 males were extracted by the digestion method from the ligamentum nuchae. Others were extracted by dissection; all females were alive, although few males were dead. Other adult females were calcified which is usually found in this age of animal.

The microfilariae in the uterus of the adult female worms were alive and wriggling and all stages of development

Table 35.

THE NUMBER OF MICROFILARIAE RECOVERED FROM THE SKIN OF A TREATED COW FOLLOWING **DEC** TREATMENT

Date	Treatment and amount	Weight of skin sample (gm)	No. of microfilariae recovered	No. of microfilariae per mg
10.9.1979	No treatment	0.150	1030	6.8
11.9.	5 gms	-	-	-
12.9.	5 gms	0.047	54	1.15
13.9	5 gms	0.115	31	0.27
15.9.	5 gms	0.113	19	0.17
17.9	No treatment	0.131	Nil	Nil
19.9	No treatment	0.117	Nil	Nil
21.9.	No treatment	0.056	Nil	Nil
25.9.	No treatment	0.165	Nil	Nil
30.9.	No treatment	0.241	25	0.12

were found. There was no malformation in the eggs as reported in the treatment of cows with Compound E (Denham and Mellor, 1976).

(c) Study of the depth distribution of microfilariae :

Microfilariae which emerged from each depth after 24 hours in Tyrode's solution plus 20% sheep serum, at room temperature of 27°C are shown in Table 36.

Analysis of the variations in the microfilarial count in the three layers are shown in Table 36. There was a significant increase in the microfilarial count in the deep layers than in the surface layer. As shown in the previous work supported by histological examination, most microfilariae were normally principally in the upper layer, thus ensuring that all microfilariae in the skin were killed by the effect of the drug. However, after 15 days microfilariae concentration began to build up again (Duke, 1957), newly emerged microfilariae taking 15 days to appear in the skin snip after the last dose of DEC.

This is evidence that DEC killed all microfilariae of O. gutturosa in the skin of the infected animal, but the drug had no lethal or sterilizing action on adult worms. This is similar to the action of the drug in treatment of O. volvulus (Hawking, 1958; Adams, 1953; Duke, 1968) and recently of O. gutturosa (Copeman, 1979).

Table 36.

CONCENTRATION OF MICROFILARIAE IN THE THREE LAYERS OF SKIN AFTER TREATMENT WITH HETRAZAN

Skin layer	Thickness in micron	No. of microfilariae	No. of microfilariae per mg
Superficial	820	98	0.15
Middle	870	320	0.54
Deep	Remainder	210	0.61

7. Action of Suramin in natural host

(a) Cow No.2 : A 10 year old cow weighing 255 kg which was in good health when treated with 2.5 gm doses of Suramin in powder form.

A freshly prepared solution of 2.5 gm of Suramin in 20 cc distilled water was given intravenously on two occasions on day one and day eight, making the total dosage 5 gm over 8 days. Each infusion lasted 10 minutes.

No apparent adverse effect was observed and the animal remained in good health although the injection site was rather swollen. Skin snips were collected before, during and after treatment; each time half of the snip was fixed in formal saline for histopathology, and the other half used for microfilarial assessment.

The result of the microfilarial count in each snip is shown in Table 37.

Nelson (1970) indicated that Suramin has an effect on both adults and microfilariae of O. volvulus; however Rodger (1958) found Suramin less satisfactory.

In the present experiment within 24 hours of administration of the first dose of Suramin, there was a slight decrease in the number of microfilariae (Table 37), but it was not so high as was seen in DEC treatment (Figure 50).

No clinical symptoms were seen after administration of the drug, as was seen in a chimpanzee with O. volvulus by Gibson et al. (1977).

In order to study the effect of the treatment on adult worms, autopsy was carried out in the animal 20 days from the first dose. Two complete live adult females and two dead males were extracted by the digestive technique from the ligamentum nuchae and another two males were extracted by using a dissection microscope from the other half of the ligamentum nuchae not used in the digestion method.

A possible reason for the death of the adult males may be their response to antifilarial treatment. The microfilariae in the uterus of adult female worms were alive and survived in Tyrode's solution plus 20% serum of sheep for 8 hours at room temperature (25 - 27°C).

(b) Study of the depth distribution of microfilariae :

Two square cm of skin was removed from the hump of the

Figure 50.

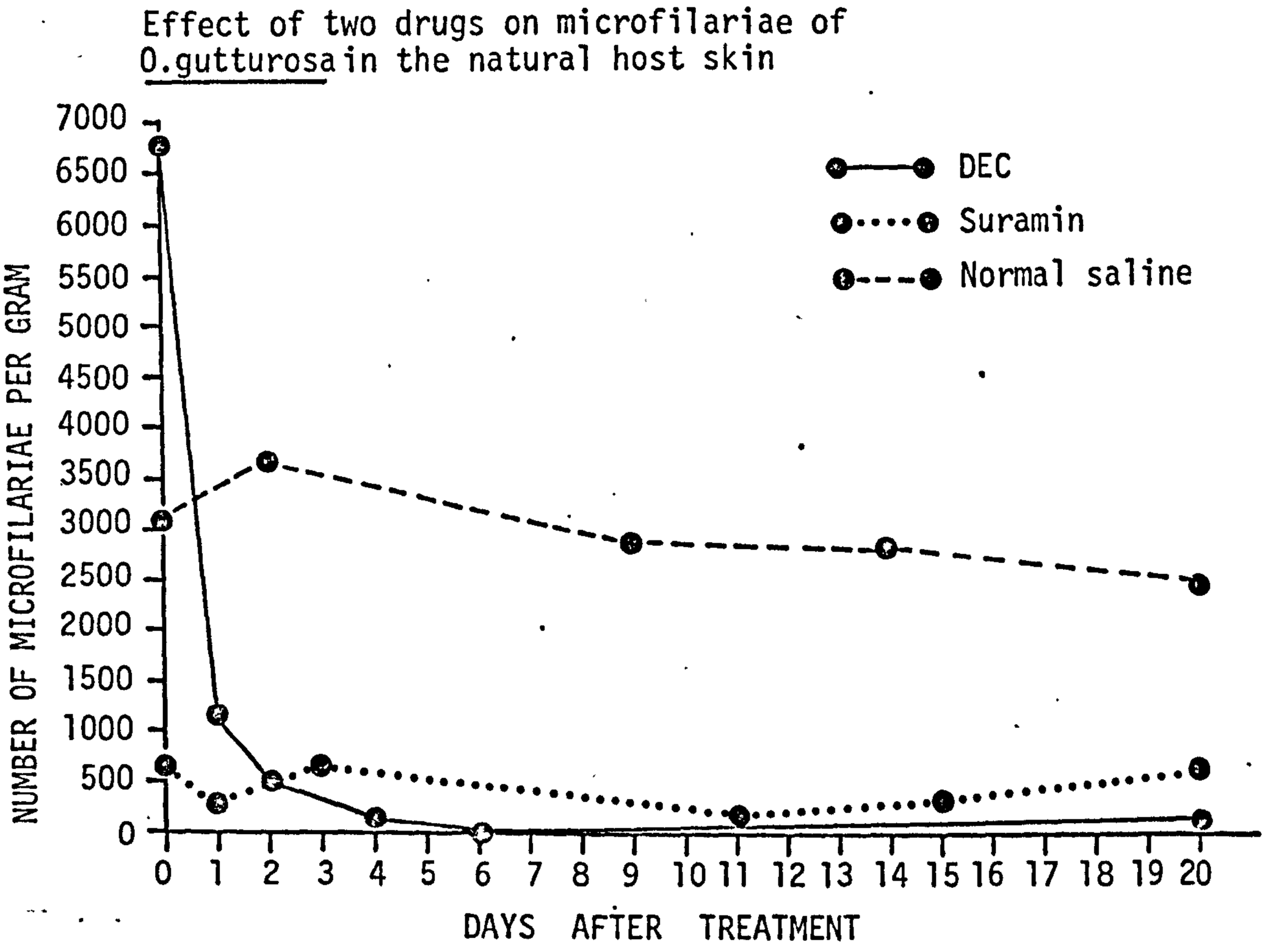

Effect of two drugs on microfilariae of O.gutturosa in the natural host skin
DEC
Suramin
Normal saline
NUMBER OF MICROFILARIAE PER GRAM
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
DAYS AFTER TREATMENT

Table 37.

MICROFILARIAE COUNTS IN SKIN SNIPS FROM COW NO.2 BEFORE, DURING AND AFTER TREATMENT WITH SURAMIN AT 2.5 GM PER DOSE

Date	Treatment and amount	Weight in gm	Number of microfilariae	No. of microfilariae per mg
10.9.79.	Before treatment	0.185	122	0.65
11.9.	2.5 gm	-	-	-
12.9.	-	0.231	75	0.32
13.9.	-	0.179	121	0.68
19.9.	2.5 gm	-	-	-
21.9.	-	0.285	60	0.21
25.9.	-	0.360	13	0.369
30.9.	-	0.244	143	0.59

animal during autopsy and divided into three layers as described on page .

The number of emerged microfilariae from each depth after 24 hours in Tyrode's solution plus 20% sheep serum at room temperature (25 - 27°C) was as recorded in Table 38.

The superficial layer contained the highest number of microfilariae, which is the usual pattern of microfilarial distribution in the skin of untreated animals.

Table 38.

THE MICROFILARIAL COUNT IN THE SKIN OF EACH DEPTH FROM COW NO.2 AFTER TREATMENT WITH SURAMIN AT 5 GM PER COW

Skin layer	Thickness in microns	No. of microfilariae	No. of mff per mg
Superficial	830	182	0.85
Middle	870	72	0.29
Deep	Remainder	20	0.06

8. Control

(a) Cow No.3 : A 7 year old cow weighing 225 kg was used as a control, no drug being given to this animal. Twenty cc of normal saline were injected intravenously three times on days 1, 7 and 12; the animal remained in good health.

Skin snips from the hump region were collected before, during and after administration of normal saline, and the microfilarial count was as shown in Table 39.

Kershaw et al. (1954) showed that the concentration of microfilariae in adult men is fairly constant on repeated examination over short intervals. The concentration of microfilariae in cow No.3 was slightly different on repeated examinations in samples taken over short intervals, and this difference was due to irregular distribution of microfilariae in the skin (Buckley, 1938; Gibson, 1952; Nelson, 1970; Mellor, 1973; Anthony and Cello, 1975).

The cow was sacrificed 20 days after the first dose of normal saline; the ligamentum nuchae were removed and examined for adult worms. One live adult female and three live males were obtained by the enzymatic digestion technique, and two live males were obtained by dissection. Mostly anterior parts of other females were alive, the uterus of the female worms being dissected and all uterine microfilariae which emerged were wriggling and survived in Tyrode's solution plus 20% sheep serum for 20 hours at room temperature (25 - 27°C).

Table 39.

MICROFILARIAE COUNTS IN THE SKIN SNIPS FROM COW NO.3 BEFORE, DURING AND AFTER TREATMENT WITH SALINE

Date	Dose and treatment	Weight in gm	Number of microfilariae	No. of Microfilariae per mg
10.9.79.	Before treatment	0.405	1270	3.14
11.9.	20 cc N.S.	-	-	-
12.9.	-	0.418	1525	3.650
18.9.	20 cc N.S.	-	-	-
19.9.	-	0.505	1464	2.899
23.9.	20 cc N.S.	-	-	-
24.9.	-	0.328	918	2.8
30.9.	-	0.320	784	2.45

(b) Studies of the depth distribution of microfilariae :

The number of microfilariae obtained from each depth was similar in all three cows after 24 hours in Tyrode's solution plus 20% sheep serum, as shown in Table 40, which gives the total number of microfilariae per mg of skin from cow No.3, which represents the normal distribution of the microfilariae in the infected skin.

Table 40.

MICROFILARIAE COUNTS IN THE SKIN OF EACH DEPTH FROM COW NO.3 (CONTROL)

Skin layer	Thickness in microns	No. of microfilariae	Microfilariae per mg
Superficial	800	480	2.25
Middle	885	54	0.18
Deep	Remainder	10	0.08

9. Histopathology

Specimens of skin were taken from the first and second cows before, during and after treatment.

a) Before treatment

Untreated skin of both cows was similar as to its histological aspects.

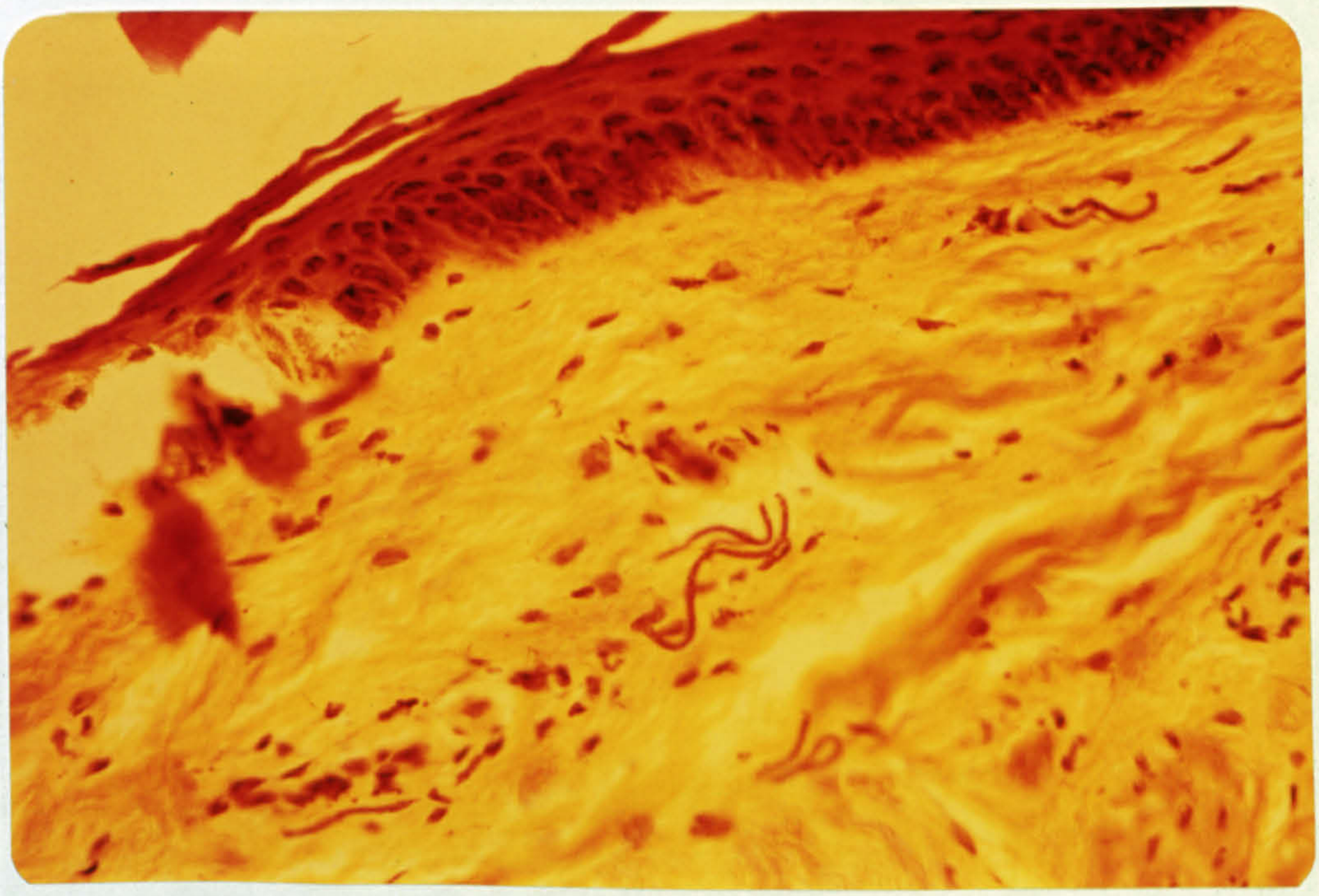
Examination showed considerable variation in the degree and types of cellular infiltration, although there was probably an increase in the cellular infiltration in dermis, particularly the perivascular regions. The cells were often gathered around small blood vessels, but some were also scattered diffusely through the tissue (Figs.51 & 52). The cells are monocytes, eosinophils and plasmacytes. The $\frac{E}{m}$ ratio is approximately $\frac{15}{48}$ and in areas may be higher or lower. Most specimens contained microfilariae in superficial layer free of cells and looking healthy. There was no evidence of severe inflammatory reactions in the immediate vicinity of the parasite.

b) <u>After treatment</u>

Histological examination of the skin taken from the cow treated with Suramin in most sections showed normal histological appearance, apart from a few inflammatory cells. In some sections there were a few lymphocytes and eosinophils which could have been due to minor reactions caused by drug therapy. This picture is not highly different from the pre-drug situation. The microfilariae in the superficial layer free of cells can be compared to the histological picture of the skin of the cow treated with DEC (Hetrazan).

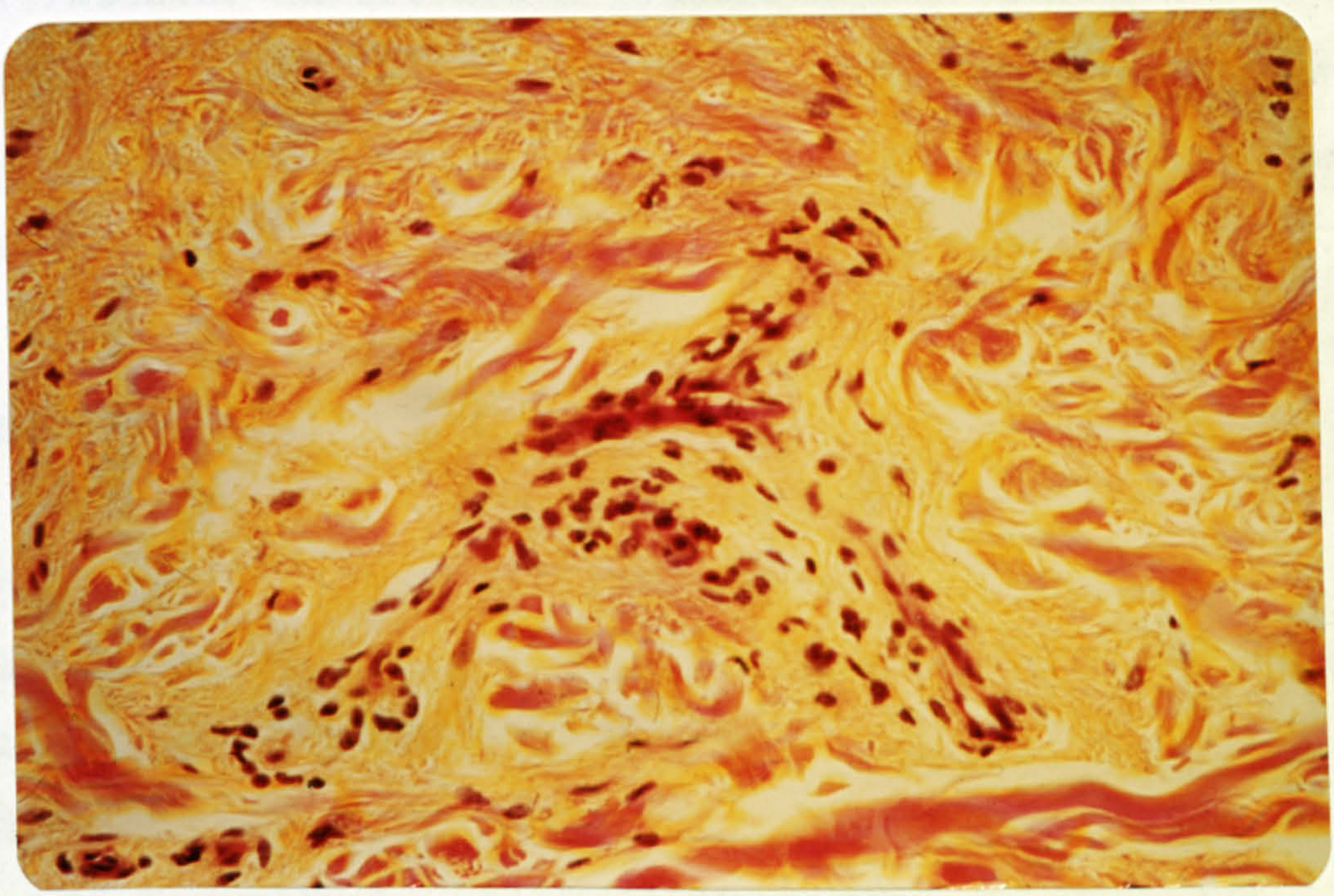
In the specimens of skin taken 24 hours after the first dose there was an increase in eosinophils in the

Figure 51.



Very little perivascular accumulation of cells and no accumulations around the healthy microfilariae

Figure 52.



Mild perivascular accumulation of cells in the tissue before treatment

cellular infiltration, and mast cells showed a degranulation response; most of these cells are normally present in an inflammatory reaction.

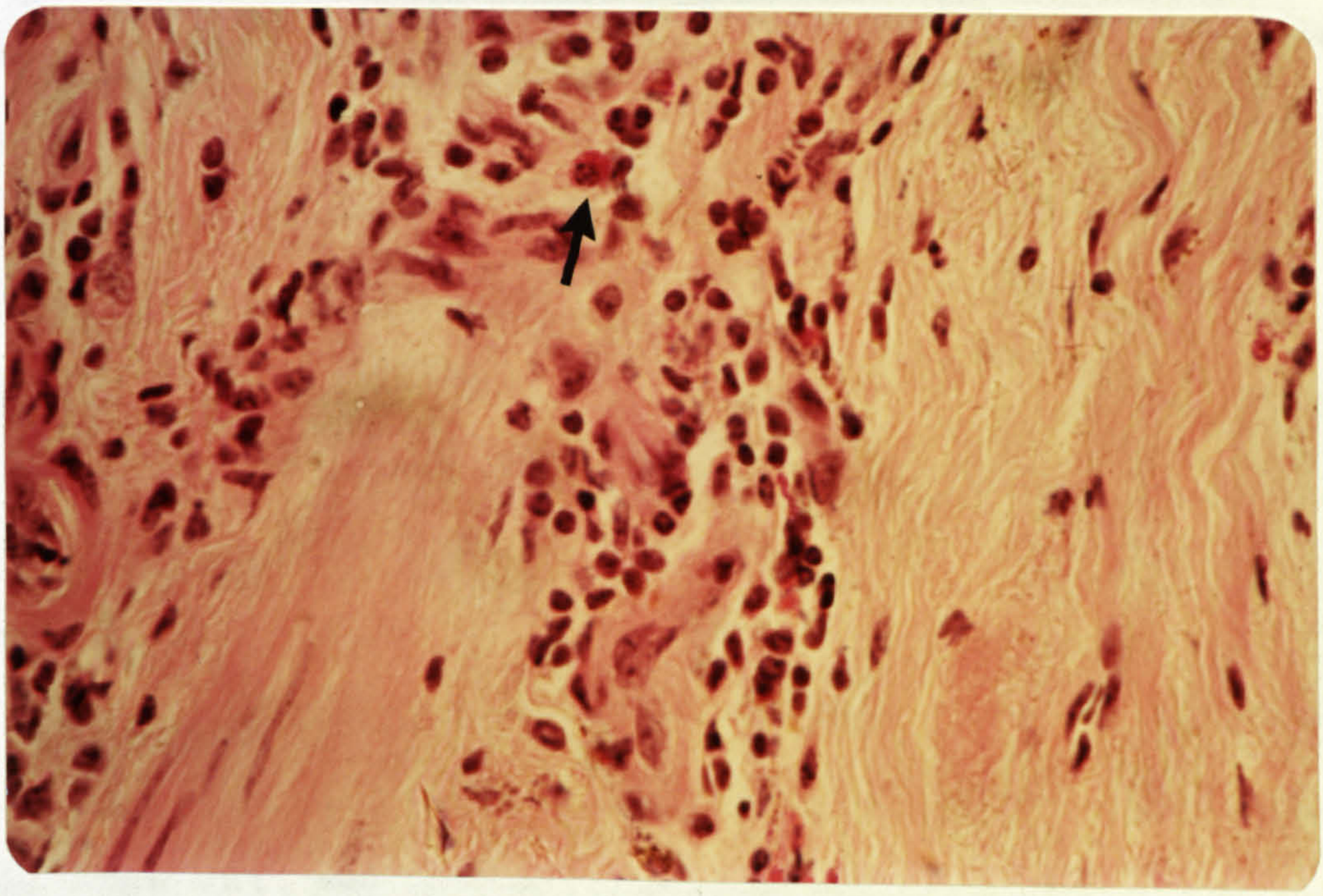
After eight days, severe inflammatory foci consisted mainly of neutrophils and eosinophils, polymorphs and fibroblasts were also common, while lymphocytes and plasma cells were few. There may also be an increase in degranulation in mast cells and it would seem that DEC causes peri-vascular infiltration (Figure 53). Some sections of microfilariae were often, but not always, present in these inflammatory foci, phagocytic cells being clustered near them.

10. Discussion

It was not possible in this study to examine the mobilization of microfilariae into animals body fluids following the administration of DEC (Fuglsang and Anderson, 1974; Kale, 1979) or the toxicity of Suramin to the host (Gibson et al., 1977).

The studies were required to determine the effect of the drugs on the microfilariae and adult worms. Thienpont and Bichwe (1957), Sasaki et al. (1957) and Thomas (1958) reported encouraging results in the treatment of animal onchocerciasis with diethylcarbamazine. Our finding confirmed the efficacy of DEC in eliminating skin microfilariae and, as far as is possible in a single experiment, it

Figure 53.



High power photomicrograph of perivascular accumulation of eosinophils (arrow) and mononuclear cells after treatment of infected animal with diethylcarbamazine

demonstrated yet again the importance of using repeated course of DEC in all cases in which skin symptoms are associated with Onchocerca microfilariae (Underwood, 1934; Datta, 1939; Dikmans, 1948).

The main conclusions which have been drawn from this study are that Hetrazin is microfilaricidal against O. gutturosa (as well as O. armillata; Sasaki et al., 1957; Patnaik, 1962) but does not act on adult worms.

Adams (1953) and Murgatroyd and Woodruff (1949) showed that treatment with DEC killed adult Loa loa. Adams explained this action by the parasite wondering in the tissue, the drug therefore being in more intimate contact than is possible with adult worms of O. volvulus or probably O. gutturosa.

From the examination of cow No.1 and No.3, there is a slight suggestion that Suramin had killed some adult male worms in cow No.2. But perhaps the interval of 20 days between treatment and slaughtering the animal in this trial was insufficient for the effects of the drug on O. gutturosa to become apparent, and the lack of effect may have been because the dose was too small.

The phenomenon of the delayed killing of filarial worms has previously been reported with Suramin against L. carinii in cotton rats (Worms and Hawking, 1978) and in cattle (Copeman, 1979).

Further information on the toxicity of Suramin is obviously needed, so it can be used with greater safety

in patients with onchocerciasis. Denham and Mellor (1976) who tested Compound E against O. gutturosa in cattle, showed that its action on them is very similar to that reported by Duke (1977) on chimpanzees infected with O. volvulus.

The effect of DEC on microfilariae of O. gutturosa reported by Copeman (1979) is supported by this study and has many similarities to the action of DEC on microfilariae of O. volvulus. In common is the disappearance of microfilariae from the skin after administration (Fuglsang and Anderson, 1974; Duke, 1972).

This suggests that Onchocerca infection in cattle may constitute a suitable model for tertiary screening of new filaricides for potential action against O. volvulus in man.

CHAPTER 5

GENERAL CONCLUSIONS AND SUMMARY OF THE MAIN OBSERVATIONS

In the last decade animal onchocerciasis has received a considerable amount of interest, stimulated by the West African campaign to control human onchocerciasis and the desire to find an animal model for Onchocerca. This is demonstrated by the fact that twelve new species have been recently described (Muller, 1979).

Additional interest in animal onchocerciasis has been due to the reports of human infections with Onchocerca of animal origin (Azarova et al., 1965; Siegerthaler and Gubler, 1965; Beaver et al., 1974; Khan, 1977). O. gutturosa (Neumann, 1910) is of special importance because it is probably a good model for onchocerciasis problem studies (Nelson et al., 1968; Denham and Mellor, 1977; Copeman, 1979).

The adult worms of O. gutturosa have a number of anatomical features that, when taken together, enable one to make a specific identification. The most important of these features are: size of the worms, the structure of the cuticle and the appearance of the anterior "guttural" dilation.

From the present study there is no doubt that O. gutturosa is a common parasite of old cattle in the

Sudan and Britain, the adult worms being localised mainly in the ligamentum nuchae; interestingly, adult worms were also found in the stifle joint of Sudanese cattle.

In all the ligaments of the cow, the cuticle was relatively thick in the female worm with pronounced outer ridges with two distinct layers, three to four striae between each two adjacent ridges (Johnston, 1921; Strong et al., 1934; Eichler, 1973; Beaver et al., 1974; Eberhard, 1977; Bain et al., 1978). All the studies indicated that O. gutturosa can provide invaluable material for observation.

The development of O. gutturosa in Culicoides, with the advantage of early establishment of a laboratory colony of Culicoides species, could prove of great value in studying aspects of transmission and host parasite inter-relationship. Further work would be useful on the possibility of colonising C. kingi in the laboratory.

The trials for testing drugs against onchocerciasis in cattle gave results similar to those observed in humans. This observation supports similar results obtained by Denham and Mellor (1976) and Copeman (1979).

In the present studies, the successful survival of adult O. gutturosa in proxy hosts up to 133 days increases the importance of this model for onchocerciasis studies.

On the basis of the present study of the morphology of the British and Sudanese worms removed from the ligamentum

nuchae, parasites from these two geographical areas appear to be similar. No worms were found in the gastro-splenic ligaments of 100 Sudanese cattle examined, suggesting that O. lienalis is not present in Sudanese cattle. This supports previous studies of African cattle by Clarkson (1964); Bwangamoi (1970) and Hussein et al., (1975).

The absence of O. lienalis from Sudanese cattle helps to solve the problem of the confusion surrounding tht taxonomy of O. gutturosa (Neumann, 1910) from the ligamentum nuchae and O. lienalis (Johnston, 1921) from the gastro-splenic ligament of British cattle. Steward (1937) and Eichler and Nelson (1971) suggested that they are synonymous. But the fact that only one occurs in the Sudan indicates that they are distinct. Isoenzyme technique proves to be a reliable indicator for identifying and differentiating between the two forms while showing that O. gutturosa in the Sudan and Britain is identical (H. Flockhart, personal communication).

1. In this study in a sample of 500 cows from the Sudan, 410 (82%) were infected. Infection was more common in older cows and occurred in both sexes, but was a bit higher in females than in males.

For comparison, examining autopsy material from Reading abattoir (U.K.), out of 239 animals examined, 168 (70.3%) contained adult worms in the cervical ligament.

In a survey study in living animals based on the presence of microfilariae in the skin, 541 animals, ranging in age from newborn to 10 years old, were examined; 56% of these animals contained microfilariae and the highest infection rate was 95% among animals aged 8 years old, and the lowest infection rate was 9% among animals ranging from newborn to one year old.

In Shambat farm, where infection rates reached 70%, there is considerable circumstantial evidence suggesting that animals develop a well-marked resistance to superinfection. There is an increase in infection with increasing age, yet many older animals show low infection rates despite continuous transmission.

The immune response may result in the failure of infective stages of O. gutturosa to find an environment suitable for maturation on penetrating the skin, resulting in a reduction of the development of new infection, or to a decline in the number of microfilariae in the skin, or to the death of some adult worms.

2. Studies that have been made on the morphology of adult worms were initially made on adult worms from the ligamentum nuchae and from the stifle joint. No difference in either measurements or cuticular morphology were detected. Both were found to be identical and the guttural dilatation was marked on both. Morphological studies on specimens from

British and Sudanese cattle from the ligamentum nuchae, with special reference to the male tail and cuticular ornamentation of the female, have been made from a large number of worms, but no real differences were found and the average length of complete adult females in both countries was the same (43 cm mean, 26 - 59 cm range).

3. The number of adult worms in cervical ligaments of individual animals as detected by enzymatic digestion of connective tissue removed from the surface of infected ligament has been determined. Examination of 19 old British cows showed the number of females per animal was between 3 - 18, while on examining 23 Sudanese cattle the number of female worms per animal was between 3 - 47. In all cases the male to female ratio was nearly 1.2 - 1, there were always more males.

In general the finding in regard to the infection rate and the number of adult worms recovered from the ligamentum nuchae of individual animals suggests that the British cattle are more resistant to infection than Sudanese cattle, or are infected less.

In regard to the worm burden, it is very difficult to make a true assessment of the number of adult worms in the body of an infected animal if we consider that the ligamentum nuchae is not the only predilection site of the adult worms. Ottley and Moorhouse (1978a) showed that

adult worms can be recovered from seven different anatomical sites in an infected animal.

4. The pathological effect of adult worms of O. gutturosa on the ligementum nuchae of infected animals was mild, but for the first time in Sudan serious pathological lesions were found in the ligamentum nuchae due to the presence of adult worms.

Histopathological examinations confirmed that the lesion was due to the presence of adult O. gutturosa by finding severe tissue reactions.

5. During the assessment of the rate of emergence of the microfilariae of O. gutturosa from the skin of infected animals during 24 hours at room temperature (25 - 27°C), the number of emerged microfilariae were counted in each of the 4 media used at 0.5, 1.5, 3, 7, 9, 22 and 24 hours. It was found that the number which emerged within 3 hours represented 85% of the total emerged microfilariae in 24 hours. This rate was equal in the 4 different media used, but Tyrode's solution plus 20% serum was the best for keeping the microfilariae alive for the longest time.

It is concluded that there is no exact time limit for total emergence of microfilariae. Collins(1973) found microfilariae in the skin after 30 hours. However, 3 hours is a long enough time to obtain 85% of the active microfilariae (El Bihari and Hussein, 1976; Bianco et al.,

1980). To leave them for 24 hours besides being time-consuming may expose the medium to yeast and bacteria which will reduce the activity of microfilariae and thus their ability to emerge.

6. The most interesting finding in the present study is the pattern of the skin distribution of the microfilariae which contrasts sharply with the distribution reported for English and European cattle. The microfilariae were found in the upper part of the animal's body (the hump), while none were found in the umbilical region. In fact, when the hump was examined in detail it was found that more than 80% of the microfilariae were concentrated in an area 4 cm posterior to the central part of the hump.

The difference in the microfilariae distribution is probably related to the biting habits of the local vector. The microfilariae are always concentrated in the hump irrespective of whether there were adult worms in either or both of the neck and stifle joint. No adult worms were found in the gastro-splenic ligament, most microfilariae in the hump were those of O. gutturosa with very few microfilariae of O. armillata in mixed infections (the two can be easily differentiated).

It has been shown that the number of microfilariae in the skin of infected animals is built up by an increase in the numbers of adult worms up to a certain level. Then the numbers of microfilariae stop increasing or start to

decline in animals with more adult worms. This supports the previous suggestion that immunity builds up gradually, as there is an increase in the number of adult worms.

7. Detailed pathological studies on the skin of infected animals due to the presence of microfilariae of O. gutturosa were carried out. However, no obvious skin or eye lesions were seen which could be attributed to the presence of the microfilariae. In heavily infected animals, small hairless patches were found in the hump, probably due to a hypersensitivity reaction to the bites of C. kingi. When sections were prepared from the hump skin of infected cows a generalized but mild perivascular infiltration of round cells and eosinophils was usually seen. The inflammatory cells, however, were never found concentrated around microfilariae, even in heavy infections.

8. There were distinct morphological differences between microfilariae emerging from the uterus of adult female worms and those found in the skin of infected cows. Two types were found in the uterus, types "A" and "B". Skin microfilariae were similar to the type "B" but longer. The majority of type "B" uterine microfilariae were recovered from the vaginal end of the uterus.

It is concluded that the microfilariae in the uterus of the female worms have not yet acquired the capacity to develop further in the intermediate host. Apparently,

this property has been gained by the time that the parasites have migrated from the uterus to the skin.

It has been shown in the present study that the large type "A" microfilariae found in the uterus is an immature form which shortens as it moves towards the anterior part of the uterus. This supports the observation by Eichler (1970) when studying the migration of uterine microfilariae in mice, that a certain proportion were immature depending on the part of the uterus.

9. Cattle and donkeys in Shambat area (Sudan) are infected with sheathless microfilariae which inhabit the dermal layer of the skin and do not circulate in the blood. These microfilariae belong to the species O. gutturosa (Neumann, 1910), O. armillata (Raillet and Henry, 1909) in cattle and O. raillieti (Bain et al., 1976) in donkeys. The microfilariae of these three species can be distinguished morphologically on the basis of their form and size, on the arrangement of caudal and cephalic nuclei, and on the number of nuclei between the nerve ring and cephalic space. Of the many measurements made, only the difference between the gut and the mean body length were significant.

10. The problem of the vector of O. gutturosa was investigated in the Sudan at Shambat area where the parasite is endemic and a high percentage of cattle are infected. Two

species of blood-sucking flies were suspected, Culicoides kingi and Simulium griseicolle, in the area of investigation, and it seemed likely that one of these two must be the normal vector. Because of experimental and epidemiological evidence, S. griseicolle was excluded as the natural vector and studies were concentrated on C. kingi.

Culicoides kingi which had fed on microfilariae carriers of O. gutturosa as well as infected artificially by intrathoracic injection technique, were dissected at intervals thereafter.

All flies which survived for seven days or more after the infection contained infective larvae in the head and proboscis. It is concluded, therefore, from the results obtained and from the morphological similarity of the mature larvae found in the present study with those obtained by other workers, that C. kingi is the most likely natural vector of O. gutturosa in the Sudan.

The ability of C. kingi to support the development of O. gutturosa should prove of great value in studying aspects of transmission and host/parasite inter-relations. Thus attention should be paid to the possibility of colonizing C. kingi in the laboratory.

11. Females of Aedes aegypti were infected with microfilariae of O. gutturosa in an attempt to infect a surrogate

insect host which can easily be bred under laboratory conditions, using different laboratory techniques for infection. After infection the mosquitoes were maintained in an insectary at 23°C on 10% sugar meals. Despite a high mortality, most survived for 12 days and contained third stage larvae similar in morphology to those from the natural host.

The successful completion of development of O. gutturosa in A. aegypti reported here indicates an alternative approach. A. aegypti mates well in the laboratory and all basic stages involved in colonization have been completed in the laboratory. Further investigations to increase the recovery of third stage larvae and improvements in the survival of infected mosquitoes are needed.

12. The present study is the first in which the complete development of O. gutturosa from microfilaria to infective larva has been obtained in the Sudan. The description of the development of microfilariae of O. gutturosa in Culicoides kingi was described in detail from wild caught flies infected in the laboratory. A technique has been developed to facilitate the artificial infection of these midges with O. gutturosa.

The infected flies were maintained at room temperature (25 - 27°C) under humidity control of 85% on 10% sugar meals. At 27°C O. gutturosa take seven days to develop from microfilariae to infective stage. A moult occurred

between each stage and finally the infective stage was reached.

The technique used for extracting large numbers of mature microfilariae of O. gutturosa (Bianco et al., 1980) in addition to the advantages of the easy establishment of a laboratory colony of Culicoides with intrathoracic techniques for infecting the flies could be a great help for vaccine studies, which are badly needed due to lack of satisfactory drugs.

13. Investigation of O. gutturosa (Neumann, 1910) in cattle has been investigated in the Shambat area. The vector in the Sudan was found to be C. kingi which bites mainly on the hump in the region where the microfilariae predominate, indicating a high degree of adaptation by the parasite to the biting behaviour of its vector.

Ninety per cent of the midges attacking bait cows landed within the hump region. Twenty-five per cent of midges landing on the hump region of a bait cow also succeeded in obtaining a blood meal, the remainder were either dislodged or squashed by the movement of the host; but none were found feeding in the umbilical region.

A series of observations confirmed the suggestion that C. kingi lands on the back of the animal (hump) mainly because of two factors: there is less amount of hair and it is a quiet peaceful place for blood feeding away from the head and tail switching.

14. The seasonal flight activity of C. kingi was determined at Shambat farm by means of a suction trap from the light inside the animal pens, from September 1978 through until August 1979, to determine seasonal environmental influences on the population fluctuation. Records of temperature and rainfall were made during the study.

The results showed a monthly variation in the light trap catch, the greatest number were caught during July although appreciable numbers were collected during February. The time during which C. kingi is most abundant coincides with the rainy season in this area, with a fall in air temperature and a rise in air humidity. The biting activity was determined by means of a bait animal trap method. Day-long catches of midges showed that there are morning and evening peaks of activity. The evening peak was found to occur just before sunset and was twice as great as the morning peak.

It appears, therefore, that for the biting activity of C. kingi, air temperature and air humidity may be limiting factors and may directly influence the daily, as well as the annual, biting cycle.

15. The seasonal variation in the number of microfilariae in the cow skin as has been reported in Japanese and British cattle was not observed in the present study. No evidence was found to suggest that the microfilariae in Sudanese cattle migrate into deeper layers in winter; it is concluded that there is genuine migration of the microfilarine

in the infected skin, probably connected with the biting cycle of the vector, rather than the extrenal temperature.

In histological sections of the infected cow skin 44% of the microfilariae appeared to concentrate in the superficial layers to a depth of 200$^{\mu m}$ for most of the year. In the present study this depth was found to be within the range of the proboscis of C. kingi.

The only method of diagnosis so far is by finding the microfilariae in skin snips. It has been proved that the microfilariae in the skin have very uneven distribution in the dermis (Buckley, 1938; Mellor, 1973; Anthony and Cello 1975), so a false negative diagnosis is possible, also young animals with prepatent infections give negative results.

More investigations are needed to discover a reliable method of diagnosing early and prepatent infections.

16. Studies were carried out on the behaviour of the microfilariae of O. gutturosa in laboratory animals, as shown below.

1. In the Nile rat :

In a suspension prepared from the anterior part of the uterus of adult worms, microfilariae recovered from several regions of an infected rat; predominantly in the ears, scrotum and tail. Peak numbers of microfilariae were found in the ears, scrotum and

tail after seven days from the intra-peritoneal injections, but the numbers decrease rapidly during the following two weeks.

The Nile rat/O. gutturosa model was used to study the effect of a single dose of DEC on the micro-filariae in the ears, scrotum and tail, but it was concluded that a single dose is not sufficient for eliminating all the microfilariae.

II. In mice :

Three experiments were carried out in an attempt to determine the distribution of skin microfilariae in mice, a better route for inoculating micro-filariae into laboratory animals, and for maintaining the microfilariae in the skin of mice.

The highest numbers of microfilariae migrate to the ears and nose, peak numbers were recovered after five and six days respectively. Then the microfilariae rapidly decreased and by 22 days no microfilariae were recovered from either the ears or nose.

Four routes were used for inoculating microfilariae into mice (intra-peritoneal, intra-muscular, intra-venous and subcutaneous) and the results showed a wide range in the ability of the microfilariae to migrate and the subcutaneous route gave the highest

number of migrating microfilariae in the ears and nose.

17. Transplantation of adult worms into laboratory animals - three methods were used.

Collagenase digestion was used for isolating intact adult live females and males free from the host tissue for worm transplants; males were also extracted by dissection, while some worms were transplanted within the connective tissue of the host.

Adult O.gutturosa were transplanted into various strains of mice, either free from host tissue or within the host tissue.

The results of this work so far indicate that dissected out male worms can survive for up to 133 days in the peritoneal cavity of a proxy host, and the females transplanted within the host tissue can survive for 66 days; while those worms extracted by collagenase technique survived only for 14 days.

These results show that the transplantation of adult O. gutturosa in mice is a workable procedure which will have application in laboratories involved with onchocerciasis research for the evaluation of any possible sterilizing effect of a drug upon the adult worms found alive in treated animals at autopsy, and for obtaining a quantitative estimation of the number of microfilariae produced for a definitive period of time by a single fertilized female worm.

The collagenase technique is the only available method for extracting intact living worms for transplantation so far, but its effect on the adult worms is not yet known. Preliminary E.M. studies, however, indicate that collagenase causes some damage both to the cuticle and interior structures of adult worms (David Ellis, personal communication).

Thus the technique needs more detailed study for improvement. The method allows the maintenance of adult living stock in the laboratory for a considerable time, but the laboratory source for their microfilariae needs further investigation also.

18. Trials were carried out to see the effect of two drugs, diethylcarbamazine (hetrazan) and suramin (antrypol), in bovine onchocerciasis.

Hetrazan was successful in destroying the entire microfilarial population of the skin after repeated courses, but two weeks after treatment the microfilarial population began to build up again, and when the animal was sacrificed, the drug was found to have no lethal or sterilizing action on the adult worms.

Further efforts should be made to find a satisfactory chemotherapeutic agent, because no drug has been found to date that is entirely effective in the treatment of onchocerciasis, and when planning chemotherapeutic trials it must be remembered that onchocerciasis is never fatal, and that the tragedy of a patient dying from toxic reactions to a drug must be avoided at all costs.

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phorylation by host cell thymidine kinase, this enzyme apparently being absent from the parasite, as demonstrated by absence of ^{3}H-thymidine incorporation. A K_i for FdUMP against deoxyuridine monophosphate of $7{\cdot}3 \times 10^{-9}$ M was obtained in short-term experiments, which demonstrated typical competitive kinetics similar to those obtained by other authors using different sources of material.

These results confirm that thymidylate synthase is present in *E. tenella* and is a suitable target for chemotherapy.

SESSION 2B

Helminth biology

Successful Development of *Onchocerca* from Cattle in *Aedes*, *Anopheles*, *Culex* and *Culicoides* sp.

By A. E. Bianco, S. Townson, K. El Sinnary and G. S. Nelson (*Winches Farm Field Station, London School of Hygiene and Tropical Medicine*)

Onchocerca microfilariae, from the umbilical skin of cattle slaughtered in England, have been inoculated into various species of laboratory-reared arthropods to test their susceptibility to infection.

Complete development of microfilariae to third-stage larvae, which has previously been reported in *Simulium ornatum* (Steward (1937), *Parasitology* 29, 212; Eichler (1973), *Journal of Helminthology* 47, 73), by this technique could also be obtained in several other insects, including *Aedes aegypti*, *Anopheles labranchiae atroparvus*, *Culex molestus* and *Culicoides nubeculosus*. Partial development of microfilariae to first-stage larvae was observed in *Anopheles stephensi* and *Musca domestica*, but no development occurred in *Culex pipiens fatigans*, *Calliphora vomitoria*, *Locusta migratoria*, and *Ornithodoros moubata*.

The time required for larvae to complete development was the same in each of the arthropod species susceptible to infection, although in all cases this was determined by the ambient temperature. Microfilariae developed to third-stage larvae in 9 days at 25 °C, 8 days at 26 °C and 7 days at 27 °C.

Third-stage larvae from *Aedes*, *Anopheles* and *Culex* resembled those from *Simulium* described as *Onchocerca gutturosa* (Eichler, 1973). These worms were a mean of 474 (426–573) μm long in contrast with the larvae from *Culicoides* which measured 785 (726–833) μm long. This difference in size suggests that the two types of third-stage larvae may be separate species, and from work which is in progress and will be published subsequently it appears that the longer type from *Culicoides* are probably of *Onchocerca lienalis*.

Studies on the Life-Cycle of *Maritrema linguilla* (Jäg.) (Digenea: Microphallidae).

By C. R. Newell, I. Popiel and B. L. James (*University College, Swansea*)

Abstract not received from authors.

Environmental Correlates of Variations in Digenean Infections of *Littorina neritoides* (Gastropoda) from Plymouth Breakwater.

By J. T. Davey (*Natural Environment Research Council, Institute for Marine Environmental Research, The Hoe, Plymouth, Devon*)

The Plymouth Breakwater represents a topographically uniform, exposed shoreline without a splash zone except where this is provided by the walls of shelter blocks. *Littorina neritoides*, characteristic of the splash zone of exposed rocky shores, can be found living permanently submerged in pools on top of the breakwater, as well as on the walls of the shelter blocks.

The present study of the ecology of digenean relationships in these snails suggests that a former interpretation of gigantism due to a cercarial infection is suspect, while the incidence of an unidentified metacercaria exhibits a fascinating correlation with microhabitat.

The cercaria, believed to be that of *Microphallus similis*, is shown to be typical of the snails that live in the pools. Incidence is slightly higher in females, and increases with host size, though not exceeding 10% in even the largest size classes. However, a non-seasonal increase in incidence of the parasite from the western to the eastern end of the breakwater correlates with a decreasing degree of exposure to wave action and an increasing exposure to the faeces of resting seagulls; factors that would facilitate the transmission of microphallid eggs from

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ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE

Demonstrations

Laboratory Meeting

London School of Hygiene and Tropical Medicine, Winches Farm Field Station, St. Albans
3rd May, 1979

Warble fly—control or eradication?

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Cattle warble flies (*Hypoderma lineatum* and *H. bovis*) produce larvae which severely damage hides of cattle in Britain, an over-all 10·0% of all hides being affected in 1978.

A 5-year Ministry of Agriculture Eradication Programme was launched in October 1978 and is now taking effect. Over 6·7 million doses of systemically active organophosphorus insecticides were used in autumn 1978 and further treatments have been given this spring.

Two insecticides are being employed—Tiguvon (fenthion: Bayer) and Poron (phosmet: Youngs), costing about 40p to 65p per treatment. Both are used as a dressing applied to the back, usually before the larvae emerge. Autumn dressing is encouraged, but "spring" dressing of any visibly warbled cattle is compulsory between 15th March and 31st July. Treated cattle may not go for slaughter for 14 to 21 days.

Problems can arise from occasional OP poisoning or from the death of larvae next to the spinal cord, and this aspect of the eradication campaign is under active investigation.

It remains to be seen if 100% eradication or (as in Eire) 98 to 99·5% "control" will be achieved. In spring 1979, fewer cattle were seen to be infected, and they had fewer warble larvae than in the previous year, e.g. 1·0% in March 1979 against 4·1% in March 1978. Numbers of larvae in the backs have fallen from a typical 25 to 50 to one to 10.

Ocular complications of onchocerciasis: the search for an animal model

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Laboratory research into the pathogenesis of ocular onchocerciasis is hampered by the specificity of *Onchocerca volvulus* for its human host and its restriction to tropical or sub-tropical countries. Consequently we have been examining the capacity of other species of *Onchocerca* indigenous to temperate zones to produce eye disease in animals commonly used in laboratory experiments.

Primarily, we have tested the ability f *Onchocerca* sp. from bovines and equines to cause corneal damage in rabbits and guinea-pigs. Microfilariae of *O. gutturosa* from the skin of English cattle were recovered freshly after slaughter in Tyrode's solution supplemented with 20% newborn calf serum. These were washed in normal saline, concentrated by centrifugation, and inoculated in batches of 5,000 into the perilimbal bulbar conjunctiva of 8 rabbits and 8 guinea-pigs. However, only in one rabbit and 3 guinea-pigs was there significant corneal oedema and vascularization and in none was there any residual scarring.

Microfilariae of *O. cervicalis* from the skin of horses have given slightly more encouraging results in experimentally infected animals. Using inoculates of either fresh or cryopreserved microfilariae, 3 of 8 guinea-pigs and 2 of 4 rabbits developed transient but substantial corneal induration and vascularization although in none was there much permanent opacification. Repeating the procedure at intervals of several weeks on 3 or 4 occasions appeared to have little cumulative effect. Our most recent experience, however, suggests that the low level of corneal pathogenicity may be related to technical limitations since the inoculation of only fresh microfilariae and not those which have been cryopreserved has produced extensive anterior segment inflammation in each of 6 guinea-pigs.

Although these studies are still in progress, our impression that *O. cervicalis* is more pathogenic for ocular tissues than is *O. gutturosa* recalls the veterinary experience that eye disease due to onchocerciasis is seen occasionally in horses but not in cattle.

Large-scale recovery of *Onchocerca* microfilariae from naturally infected cattle and horses

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Large numbers of microfilariae of *Onchocerca gutturosa* and *O. cervicalis*, for use in the production of infective larvae and immunological and *in vitro* studies, have been obtained in our laboratory on a routine basis by their recovery from skin under aseptic conditions.

Skin from the umbilicus of cattle infected with *O. gutturosa*, and from the belly of horses harbouring

O. cervicalis, is collected at local abattoirs from freshly slaughtered animals for rapid examination in the laboratory. To identify skin samples that contain microfilariae, a standard disc of tissue 9 mm in diameter is taken from each piece with a cork-borer powered by an electric drill. The skin biopsies are placed in the wells of a tissue culture plate (Linbro) containing Tyrode's solution with 20% newborn calf serum and incubated for one hour at 37°C to encourage the rapid emergence of microfilariae. The discs of tissue are then removed and the wells examined for parasites on a stereo microscope.

Infected skins are washed, shaved, pinned to a cork board and surface-sterilized with absolute alcohol in a laminar flow cabinet. Observing usual aseptic procedures, large slivers of skin, which are not of full thickness, are taken from all over the surface using a needle and scalpel. The skin snips are placed in disposable universal containers (Sterilin) filled with Tyrode's and serum .with penicillin and streptomycin (200 units per ml) and incubated overnight at 25-27°C. Microfilariae are recovered from the tubes by Pasteur pipette, resuspended in fresh medium by gentle shaking and passed through a filter of sponge ("Spontex") held in the barrel of a syringe to remove any particles of host material.

With relatively few infected skins from either cattle or horses, between 100,000 and two million microfilariae may be obtained in good condition using the described techniques. The suspension of parasites is free of host particulate material and of any significant bacterial contamination. It is believed that this technique, which is both quick and simple, offers considerable advantages over methods previously described (e.g. EICHLER, D. A. & NELSON, G. S., 1971; *J. Helminth.*, 45, 245-258; MELLOR, P. S., 1971; *Trans. R. Soc. trop. Med. Hyg.*, 65, 199-201).

Cryptosporidial and viral enteritis in immunodeficient animals and man

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Electronmicrographs on display: Illustrated life-cycle stages of a *Cryptosporidium* spp. invading intestinal epithelial cells of an immunodeficient patient with concomitant viral infection.

Other human cases of cryptosporidial enteritis have been reported: one in which overwhelming diarrhoea distressing an immunosuppressed patient ceased after stopping' cyclophosphamide therapy and coincided with disappearance of the parasite (MEISE, J. L. *et al.*, 1976; *Gastroenterology*, 70, 1156-1160). The second, a 3-year-old female child who suffered a self-limiting acute attack of enterocolitis, while the third, a child with congenital hypogammaglobulinaemia had an attack of enterocolitis which improved with pyrimethamine and sulphadiazine treatment but relapsed on treatment withdrawal. Two further cases have been brought to our attention by personal communication, making 6 known cases in all.

The first animal infection with cryptosporidium (*C. muris*) was described by Tyzer in 1910 (TYZER, E. E., 1910; *J. med. Res.*, 23, 487-509). The list of animal hosts has since grown to include guinea-pigs, rabbits, pigs, lambs, calves, horses, rhesus monkeys, the Indian Jungle cat, snakes, chickens and turkeys. Enteritis has not occurred with all these infections. However, SNYDER, S. P. *et al.*, 1978 (*Vet. Path.*, 15, 12-17) recorded cryptosporidiosis in Arabian foals with combined B and T lymphocyte immunodeficiency. All the foals died, but the effect of the cryptosporidial infection could not be assessed in the presence of a concomitant adenovirus enteritis. Elsewhere it has been suggested that cryptosporidiosis is a probable factor in neonatal diarrhoea of calves.

The real pathogenic nature of *Cryptosporidium* is not known. It may well be an opportunist appearing where abnormal conditions in the host permit it to thrive and add to the host's discomfort.

In vitro erythrocyte sickling in mammals

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Although erythrocyte sickling is apparently pathogenic only in man, the occurrence of sickled erythrocytes in the blood of other mammalian species is of interest because of its contribution to the understanding of erythrocyte physiology and pathology and of the basic mechanisms of haemoglobin polymerization which underlie the disc to sickle transformation.

A survey of the distribution of *in vitro* erythrocyte sickling in 29 species of mammals, often found in zoological collections, showed that sickling was common in most species of Cervidae and several species of Caprinae and Viverridae. On the basis of the conditions producing sickling in these species, two distinct categories were distinguished, both of which however were dependent on oxygenation. Intra-species haemoglobin polymorphisms were studied by isoelectric focusing and sickling was always associated with certain haemoglobin types of combinations. The biophysical properties of these sickling haemoglobins from several species indicated different molecular mechanisms in the two types of sickling and that both differed from human HbS polymerisation.

No clinical or haematological abnormalities could be associated with the presence of sickling in these animals. The marked difference in pathogeneity of sickle cells in man and other mammals has been attributed to differences in the physical properties of the sickled cells and to the conditions under which they occur. It is unlikely that the conditions necessary for *in vitro* sickling in these species would prevail physiologically and sickling is usually considered an *in vitro* phenomenon.

This study was presented as an example of the potential usefulness of comparative haematology to veterinary and medical science. (BUTCHER, P. D. & HAWKEY, C. M., 1977; *Comp. Biochem. Physiol.*, *57A*, 391-398; 1978; *Proc. Symp. Comp. Path. Zoo Anim., Washington, D.C.*).

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RESEARCH NOTE

Infection of *Aedes aegypti* with *Onchocerca gutturosa*

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The production of infective, third-stage larvae of several *Onchocerca* species has been hampered by difficulties in maintaining the *Simulium* vectors in the laboratory. For this reason, attempts have been made to infect a variety of surrogate (unnatural) insect hosts by either membrane feeding or intrathoracic injection. Development of a few microfilariae to third-stage larvae has been reported for *Onchocerca volvulus* in *Aedes aegypti* (Zielke *et al.*, 1977; Zielke, 1977) and recently for *Onchocerca gutturosa* in this, and 2 other mosquito species (Bianco *et al.*, 1979). This paper describes further work carried out in our laboratory to determine whether large numbers of infective larvae of *O. gutturosa* can be produced in *A. aegypti* by manipulation of the infection procedures.

Microfilariae of *O. gutturosa* were obtained from the umbilical skin of freshly slaughtered cattle using an aseptic technique which has been previously described (Bianco *et al.*, 1980). These were concentrated by centrifugation and either resuspended in blood for membrane feeding, or in one of a variety of media for intrathoracic injection. Females of *A. aegypti* of the selected Liverpool strain (Macdonald, 1962) were obtained from a colony maintained in our Department. Infected insects were held in an insectary at 23°C and 85% relative humidity and fed on 10% sugar/water solution. Daily checks were made of insect mortality and dead or moribund mosquitoes were dissected immediately. Each mosquito was divided into head, thorax and abdomen and examined in medium 199 for developing larvae. The remaining insects were examined 12 days after infection, when microfilariae had developed to third-stage larvae.

1. Membrane feeding experiments

Using standard techniques of membrane feeding (Rutledge *et al.*, 1964), three trials were conducted in which recently emerged females of *A. aegypti* were fed through chick skin membranes on suspensions of microfilariae in heparinised cow blood at 37°C. Four suspensions were used containing 2500, 5000, 10 000 or 15 000 microfilariae per ml of blood (Table 1). The majority of insects fed within 30 minutes during which time microfilariae remained active in the blood. Dissection of 10 mosquitoes from each group 1 hour after feeding revealed that all had taken up microfilariae, of which 17% had reached the thorax. The number of microfilariae ingested by each group of mosquitoes

TABLE 1

Infection of *Aedes aegypti* with *Onchocerca gutturosa* by membrane feeding using various concentrations of microfilariae (mf) in blood (L3 = third stage larvae)

Concn. (mf/ml)	No. insects fed	% survival	% with mf	Mean no. mf (range)	% with L3	Mean no. L3 (range)
2500	112	62	100	3 (2 – 14)	41	1.0 (1 – 2)
5000	324	41	100	5 (4 – 11)	54	1.1 (1 – 2)
10 000	110	20	100	17 (14 – 20)	100	1.6 (1 – 2)
15 000	112	19	100	21 (12 – 25)	95	1.2 (1 – 3)

TABLE 2

Mean (range in parenthesis) number of larvae of *Onchocerca gutturosa* in *Aedes aegypti* at four time intervals after feeding on various concentrations of microfilariae in blood (L1 = first stage larvae; L2 = second stage larvae; L3 = third stage larvae)

Concentration of microfilariae (mf/ml)	Days after infection (larval stage) 0 (mf)	3 (L1)	7 (L2)	12 (L3)
2500	3 (2 – 14)	1.6 (1 – 2)	1.3 (1 – 2)	1.0 (1 – 2)
5000	5 (4 – 11)	2.3 (2 – 3)	2.5 (2 – 3)	1.1 (1 – 2)
10 000	17 (14 – 20)	3.7 (2 – 5)	4.0 (1 – 8)	1.6 (1 – 2)
15 000	21 (12 – 25)	7.6 (3 – 9)	3.6 (1 – 5)	1.2 (1 – 3)

increased with increasing concentration of the blood-parasite suspension as did the mortality in insects subsequent to the infective blood meal (Table 1). However, the yield of third-stage larvae, 12 days after infection, was no greater in groups of mosquitoes fed on the highest concentration of microfilariae. From Table 2 it can be seen that this appears to be due to a significant reduction in the number of larvae which can be supported through each larval stage, irrespective of the number of microfilariae initially ingested, which suggests that mosquitoes which survive can tolerate only very low *Onchocerca* infections. This is most evident at the beginning and end of the developmental period, during the transition of microfilaria to first-stage larva and second-stage larva to the infective stage, which correspond with periods of the highest insect mortality.

2. Intrathoracic injection experiments

Two trials were conducted in which microfilariae were inoculated directly into the thorax using an injection device to semi-automate the process (Bianco *et al.*, in preparation). Recently emerged females of *A. aegypti* were immobilised with carbon-dioxide gas on a modified microscope stage and inoculated in the membraneous area below the parategite with 0.5 μl of the parasite suspension using a fine glass needle. In the first trial, a total of 353 mosquitoes were infected with approximately 40 microfilariae each suspended in Tyrodes saline containing 20% bovine serum. 11% of the mosquitoes survived the 12 day development period and 80% were infected with a mean of 3.0 (range 1 – 10) third-stage larvae each.

In order to improve the survival of infected insects, a second trial was performed with 6 groups of 50 mosquitoes which were infected with 20 microfilariae each suspended in

TABLE 3

Infection of *Aedes aegypti* by intrathoracic injection with microfilariae of *Onchocerca gutturosa* in various media (all media adjusted to pH 7.2)

Medium	No. insects injected	% survival	% infected	Mean recovery of infective larvae (range)
TC medium RPMI 1640	50	72	45	2.6 (1 – 5)
TC medium 199	50	78	20	2.3 (1 – 4)
TC medium NCTC	50	52	25	1.0 (1 – 3)
Tyrodes saline	50	62	33	2.4 (1 – 4)
Tyrodes + 20% serum	50	66	20	2.0 (1 – 3)
Hayes saline	50	48	30	1.8 (1 – 6)

various media (Table 3). Mosquitoes in all 6 groups survived better than in the first injection trial, presumably due to the smaller number of microfilariae given. Nevertheless, there was only a slight drop in the average yield of third-stage larvae. Although the differences between groups were not great, the best results were obtained using medium RPMI 1640 which combined high survival of infected mosquitoes and relatively high infection rate with the greatest recovery of third-stage larvae. Mosquitoes injected with media which did not contain parasites, or which had been sham-operated by inserting the needle without inoculating liquid, survived equally well as untreated mosquitoes (86–98% survival). This provides good evidence that the actual procedure of injection did not cause any appreciable increase in the death rate of mosquitoes, although the introduction of *Onchocerca* microfilariae produced a significant mortality.

Third-stage larvae from *A. aegypti* were recovered mainly from the head (51%) and thorax (46%) with very few in the abdomen (3%). Most larvae appeared normal and when fixed in 70% alcohol measured 474 (426–573) microns long and 17.1 (16.3–21.2) microns wide. In *Simulium ornatum*, which is the natural vector (Steward, 1937), the larvae are of similar size, although most migrate to the mouthparts. Eighty to 90% of third-stage larvae in *S. ornatum* are normally recovered from the head at 12 days after infection and measure 484 (406–633) microns long by 17.8 (16.3–20.4) microns wide. However, in *S. ornatum* the recovery of infective larvae and survival of heavily infected flies is far greater than in *A. aegypti* after infection by intrathoracic injection. The mean recovery of larvae is 10–20 per fly (maximum 53) and 30–70% of flies survive the development period.

Low survival of *A. aegypti* infected with *O. volvulus* was also reported by Zielke (1977), as were very low worm recoveries. In contrast, Townson (1975) obtained good survival (85%) of *A. aegypti* injected with microfilariae of *Brugia pahangi* to which the strain of mosquito used has been adapted. Until a similar selection is made of strains to accept *Onchocerca* infections, it seems unlikely that substantial numbers of *O. gutturosa* third-stage larvae can be produced in *A. aegypti*.

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SHORT COMMUNICATIONS

Culicoides kingi, Austen: a vector of *Onchocerca gutturosa* (Neumann, 1910) in the Sudan

Onchocerca gutturosa is a widespread parasite of Sudanese cattle, but its vector in the Sudan is not known (El Bihari and Hussein, 1978). Its vector in Britain is *Simulium ornatum* (Steward, 1937; Eichler, 1971). However, Bain *et al.* (1978) believe that *S. ornatum* is the vector of *Onchocerca lienalis*, and that *O. gutturosa* is more likely to have as its intermediate host some species of *Culicoides*. This suggestion is based on the observation that in some hosts the microfilariae of *O. lienalis* congregate in the skin of the umbilicus region (the preferred feeding site of *S. ornatum*), while the microfilariae of *O. gutturosa* are found mainly over the shoulders and head. In Sudanese cattle the microfilariae of *O. gutturosa* are similarly located (El Bihari and Hussein, 1976, 1978) and it was suggested that a species of *Culicoides*, not *Simulium*, is the likely vector of bovine onchocerciasis in the Sudan (El Bihari and Hussein, 1967).

The predominant species of *Simulium* in Shambat (northern suburb of Khartoum) is *S. griseicolle*, which commonly feeds on the umbilicus and perineum of cattle, while the predominant *Culicoides* species, *C. kingi*, prefers the hump region. Although Hussein *et al.* (1975) reported *O. gutturosa* microfilariae from the umbilicus, this was not confirmed in a more detailed study by El Bihari and Hussein (1978). The ability of both species to transmit *O. gutturosa* is now being investigated and preliminary results are presented here.

One hundred *C. kingi* and 50 *S. griseicolle* were collected from the University Farm at Shambat, where *O. gutturosa* is known to occur in cattle (El Bihari and Hussein, 1978). The flies were carefully dissected and examined for microfilariae and other developmental stages of *Onchocerca*. Only one *Simulium* was positive, harbouring a single microfilaria in its abdomen. Five microfilariae were found in the abdomens of four *Culicoides*; four other flies contained: two first stage larvae in the thoracic muscles of two flies, one second stage larva in the thoracic muscles of another fly and one third stage larva in the head of another fly. These larval stages all matched the descriptions of known *O. gutturosa* larvae.

Later, 80 *C. kingi* and 50 *S. griseicolle* were collected for studies on artificial infection. *O. gutturosa* microfilariae were obtained from a skin snip taken from the hump of an infected cow. They were placed in Tyrode's solution containing 20% bovine serum and ten of these microfilariae were infected into the thorax of each fly. Four flies of each species were then dissected every 48 hours.

During the first 48 hours, three first-stage larvae were seen in the thoracic muscles of each *Culicoides*, while the *Simulium* contained only microfilariae. During the second 48 hours two second-stage larvae were seen in each *Culicoides* and nothing in the *Simulium*. Two third-stage larvae were found in the heads of two *Culicoides* on the sixth day but the *Simulium* were negative. The larval stages were all typical of known *O. gutturosa*.

Our results show the suitability of *C. kingi*, but not *S. griseicolle*, as an intermediate host for *O. gutturosa*. The feeding site of each species also points to its suitability, or otherwise, as a vector for this parasite: *C. kingi* feeds on the hump, which has the highest concentration of *O. gutturosa* microfilariae, while *S. griseicolle* feeds on the dependent parts of the host where no microfilariae of *O. gutturosa* have been detected (El Bihari and Hussein, 1978).

Transmission experiments are currently under way in our laboratory using *C. kingi* to establish experimental *O. gutturosa* infections.

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