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STUDIES ON HOST-PARASITE RELATIONSHIPS IN THE  
TICK-TRANSMITTED RODENT FILARIAE, ACKERTIA GLOBULOSA  
MULLER AND NELSON, 1975 AND DIPETALONEMA VITEAE  
(KREPKOGORSKAYA, 1933)

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by

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This thesis is dedicated to my dear wife Lesley  
and to my late mother Esmé Bianco



ABSTRACT

Studies on host-parasite relationships in the tick-transmitted rodent filariae, Ackertia globulosa Muller and Nelson, 1975 and Dipetalonema viteae (Krepkogorskaya, 1933)

by

Albert Edward Bianco

Studies have been made in the field and in the laboratory on the life-cycle, maintenance and host-parasite relationships of Ackertia globulosa, a filarial parasite of African rodents.

In natural habitats in Kenya the parasite was found in 4 species of rodents, with a high prevalence of infection in Lemniscomys striatus striatus (the striped mouse). The adult worms are highly site-specific to the pulmonary arteries and release microfilariae that travel via the blood to the skin where they concentrate principally in the ears. This is a good adaptation to the feeding habits of the tick Haemaphysalis leachii leachii, which was identified as a natural vector of A. globulosa in Kenya and attaches preferentially to the head region on striped mice. A histological examination of tick attachment sites revealed that the ingestion of microfilariae may be further enhanced by their accumulation beneath the mouthparts during the 3 day feeding period.

In H. leachii the microfilariae of A. globulosa develop to infective-stage larvae in 14-18 days. Studies made by dissection and histological techniques revealed that development is intracellular and occurs for the first 9 days in the epidermis and for the next 5 days in fat cells. Third-stage larvae then enter the haemocoel and are transmitted to rodents when the ticks next feed.

A. globulosa was established in the laboratory and maintained in Mongolian jirds (Meriones unguiculatus) and laboratory bred striped mice. In experimentally infected rodents the microfilarial densities were low although 63% developed patent infections. The prepatent period is 74-88 days and the adult worms survive for up to 500 days. The development of A. globulosa was studied in experimentally infected jirds. Microfilariae and adult worms caused no serious pathology in the vertebrate host although a few microfilariae were found in the eyes of heavily infected striped mice. The treatment of experimentally infected rodents with diethylcarbamazine resulted in the destruction of 80-100% of the microfilariae in skin but appeared to be ineffective against the adult worms. Skin responses seen in animals after drug treatment suggest that a Mazzotti-type reaction may have been elicited. The potential of A. globulosa in rodents as a laboratory model infection and primary chemotherapeutic screen for human onchocerciasis and streptocerciasis is discussed.

An investigation was also made of possible relationships between tick-bite hypersensitivity in Mongolian jirds and the transmission of Dipetalonema viteae by its soft tick vector. Jirds sensitised to the bites of Ornithodoros moubata produced vigorous immediate and delayed hypersensitivity reactions but remained fully susceptible to all stages of the arthropod and to infection with filarial larvae transmitted by infected ticks. Although the uptake of microfilariae by ticks fed on hypersensitive animals was relatively greater than on normal hosts, the difference was not sufficient to identify hypersensitivity to tick bites as a significant influence on the transmission of D. viteae. The relevance of these findings to the epidemiology of human filarial infections is discussed.

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KEY TO ABBREVIATIONS USED IN THE FIGURES

a	anus	oe	oesophagus
ab	atrial bulb	ov	ovary
av	anal vesicle	pm	protractor muscle
bc	buccal capsule	pt	pharyngeal thread
c	cloaca	r	rectum
cc	cap cell	R1	R1 cell
cs	cephalic space	R2	R2 cell
ec	excretory cell	R3	R3 cell
go	glandular oesophagus	R4	R4 cell
gp	genital primordium	rgc	rectal gland cell
gt	genital tube	rp	rectal plug
h	hook	s	spicule
i	intestine	sp	spicular primordium
ib	inner body	t	testis
m	mouth	u	uterus
mf	microfilaria	v	vagina
mo	muscular oesophagus	vd	vas deferens
nr	nerve ring	vu	vagina uterina
od	oviduct	vul	vulva
		vv	vagina vera

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GENERAL INTRODUCTION

This thesis includes studies on two tick-transmitted rodent filariae, Ackertia globulosa Muller and Nelson, 1975 and Dipetalonema viteae (Krepkogorskya, 1933).

Part I deals with a newly described species of filarial worm (Ackertia globulosa) in which microfilariae are confined to the skin. Studies have been made on the life-cycle, maintenance and host-parasite relationships of this filaria in rodents. Its potential as a laboratory model for studies on the chemotherapy and pathology of skin microfilariae in human onchocerciasis and streptocerciasis has also been examined.

In Part II, D. viteae in the jird Meriones unguiculatus has been used to study the effects of tick-bite hypersensitivity in a rodent host on the dynamics of filarial transmission by a tick vector.

PART I

STUDIES ON ACKERTIA GLOBULOSA

CHAPTER 1INTRODUCTION

Filarial worms (Superfamily Filarioidea) are thread-like nematodes which are obligate parasites of vertebrates and arthropods. All of the known filarioids of man, and many of those of his domesticated animals, belong to a single family, the Onchocercidae (Nelson, 1966). In the life-cycle of these helminths, adult worms in the vertebrate host liberate microfilariae that reach the skin or blood: These remain within the body without further development until they are eventually ingested by a haematophagous arthropod. Within susceptible arthropods, the parasites develop to infective (third-stage) larvae which are transmitted to other vertebrates when the vectors next feed. The third-stage larvae then migrate into the tissues and finally mature to become adult worms.

There is an inherent interest in the specialised life-cycles of filarial worms as many species exhibit highly-evolved host-parasite relationships to ensure their survival and transmission. Moreover, research in this field of parasitology has been greatly intensified by the knowledge that several of these parasites are the cause of serious ill-health. In man, filariasis (caused by Wuchereria bancrofti or Brugia malayi) and onchocerciasis (caused by Onchocerca volvulus) are widespread and debilitating diseases that affect many millions of people throughout the tropical world. Disease syndromes associated with filariasis are generally attributed to the presence of adult worms in the lymphatic vessels (Nelson, 1966): In onchocerciasis, they



are mainly related to the presence of microfilariae in the skin, which may often invade the eyes (Nelson, 1970). Streptocerciasis (caused by Dipetalonema streptocerca) was long regarded as a harmless infection, but recent evidence suggests that the skin microfilariae of this parasite are responsible for dermal lesions in man that are similar, if milder, to those caused by the microfilariae of O. volvulus (Meyers et al., 1972).

While in recent years the study of experimentally infected animals has greatly facilitated our understanding of lymphatic filariasis, research into filarial parasites with skin-dwelling microfilariae has been severely restricted by the lack of a convenient laboratory host. Most of the studies of onchocerciasis have therefore been made based on natural infections; and in the field of chemotherapy, reliance has had to be placed on the primary screening models of lymphatic filariasis (which have blood-borne microfilariae) in the search for new drugs for onchocerciasis and streptocerciasis.

Progress towards the development of a laboratory model for onchocerciasis has been impeded by three main factors. The first is the apparent strict host specificity of the human parasite, O. volvulus. Present evidence suggests that this is almost exclusively restricted to man as natural infections of O. volvulus have only been found in gorillas (Van den Berghe et al., 1958a, 1964). Furthermore, attempts to infect other vertebrate hosts have only been successful with chimpanzees (Duke, 1962, 1968c). Unfortunately, these animals are rare and costly which limits their use for experimental purposes.

A second problem in developing an Onchocerca model is that many species are transmitted by simuliid flies which cannot be colonised in the laboratory. After much effort to achieve the cyclical maintenance of various Simulium species it seems now that this may shortly be

realized (Raybould and Grunewald, 1975). However, whether these will be strains and species able to support the development of Onchocerca parasites has yet to be assessed (Wenk and Raybould, 1972).

The third problem in achieving a convenient laboratory model for onchocerciasis is illustrated by Table 1. This lists most of the known Onchocerca species together with other filarial parasites which have skin-dwelling microfilariae. Without exception, all of the authenticated species of Onchocerca occur in large, ungulate hosts. Therefore, any model employing an Onchocerca parasite in its natural host is likely to be an expensive one to maintain.

While efforts continue towards the development of experimental onchocerciasis in convenient hosts, it would be of great value if any filarial species with skin microfilariae could be established in the laboratory. Of those known at present and listed in Table 1, a newly described species Ackertia globulosa was examined in this study as a possible laboratory model. Aspects of the life-cycle elucidated by Muller and Nelson (1975) suggested that this species might be particularly amenable to laboratory maintenance and experimental study, as it was shown that the parasite occurs naturally in rodents and hard ticks serve as intermediate hosts. The study of A. globulosa also provided the opportunity to learn more of host-parasite relationships in a genus of filarioids that has received little attention.

Because Ackertia is a relatively unfamiliar genus, this is made the subject of the following review.

TABLE 1

A synopsis of the known species of Onchocerca and other filarial parasites with skin-dwelling microfilariae

<u>HOST</u>	<u>FILARIAL SPECIES</u>	<u>VECTOR</u>	<u>SITE OF ADULT</u>	<u>SELECTED REFERENCES</u>
Man	<u>Dipetalonema streptocerca</u>	Ceratopogonid midge	intra-dermal	Duke, 1954; Meyers <u>et al.</u> , 1972
	* <u>Mansonella ozzardi</u>	Ceratopogonids/ Simuliids	peritoneal cavity	Nelson, 1966; Moraes, 1976; Nelson and Davies, 1976
	<u>Onchocerca volvulus</u> ( = <u>O.caecutiens</u> )	Simuliid flies	subcutaneous and deep tissue nodules	Nelson, 1970; Duke, 1971
Primates (sub-human)	<u>Dipetalonema streptocerca</u>	Ceratopogonid midge	intra-dermal	Peel and Chardome, 1946 Van den Berghe <u>et al.</u> , 1958b
	<u>Dipetalonema rodhaini</u>	?	intra-dermal	Peel and Chardome, 1946; 1947
	<u>Onchocerca volvulus</u>	Simuliid flies	subcutaneous	Caballero and Barrera, 1958; Van den Berghe <u>et al.</u> , 1964
	<u>Microfilaria binucleata</u>	?	?	Peel and Chardome, 1946

TABLE 1 - continued

<u>HOST</u>	<u>FILARIAL SPECIES</u>	<u>VECTOR</u>	<u>SITE OF ADULT</u>	<u>SELECTED REFERENCES</u>
Porpoise	? <u>Onchocerca fulleborni</u>	?	intermuscular connective tissue	Lopez-Neyra, 1956
Camel	<u>Onchocerca fasciata</u>	?	subcutaneous	Raillet and Henry, 1910
Horse/ Donkey	<u>Onchocerca cervicalis</u> (= <u>O. reticulata</u> )	Ceratopogonid midge	ligamentum nuchae and tendons in leg	Neumann, 1892; Steward, 1933; Mellor, 1973a
	<u>Onchocerca railletii</u>	?	subcutaneous	Bain <u>et al.</u> , 1976
Cow/ Buffalo Antelope	<u>Onchocerca armillata</u>	?	wall of aorta	El Bihari and Hussein, 1975
	<u>Onchocerca dukei</u>	?	subcutaneous	Bain <u>et al.</u> , 1974
	<u>Onchocerca gibsoni</u> (= <u>O. cebei</u> )	Ceratopogonid midge	subcutaneous/ intramuscular	Buckley, 1938

TABLE 1 - continued

<u>HOST</u>	<u>FILARIAL SPECIES</u>	<u>VECTOR</u>	<u>SITE OF ADULT</u>	<u>SELECTED REFERENCES</u>
Cow/ Buffalo Antelope ..continued	<u>Onchocerca gutturosa</u> ( = <u>O. bovis</u> ) ( = <u>O. lienalis</u> )	Simuliid flies	Connective tissue of ligamentum nuchae & gastro/splenic ligament	Steward, 1937; Eichler and Nelson, 1971
	<u>Onchocerca indica</u>	?	Connective tissue	Steward, 1946
	<u>Onchocerca ochengi</u> ( = <u>O. dermati</u> )	?	intra-dermal	Bwangmoi, 1969; Bain et al., 1974; Bain et al., 1977a
	<u>Onchocerca stilesi</u>	?	Stifle joint	Eberhard, 1977
	<u>Onchocerca sweetae</u>	Ceratopogonid midge	intra-dermal	Spratt and Moorhouse, 1971; Spratt et al., 1978
	<u>Onchocerca synceri</u>	?	ligamentum nuchae & gastro/splenic ligament	Lopez-Meyra, 1956
Deer	<u>Cutifilaria venki</u>	?	intra-dermal	Bain and Schulz-Key, 1974a
	* <u>Elasophora schneideri</u>	Tabanid flies	arteries	Hibler et al., 1969; Weinmann et al., 1973

TABLE 1 - continued

<u>HOST</u>	<u>FILARIAL SPECIES</u>	<u>VECTOR</u>	<u>SITE OF ADULT</u>	<u>SELECTED REFERENCES</u>
Deer ...continued (= <u>Wehrdikmansia cervipedis</u> )	<u>Onchocerca cervipedis</u>	Simuliid flies	subcutaneous	Hibler, 1965; Weinmann <u>et al.</u> , 1973 Bain and Schulz-Key, 1974b
	<u>Onchocerca flexuosa</u>	Simuliid flies	subcutaneous	Schulz-Key, 1975a and b
	<u>Onchocerca garmsi</u>	?	subcutaneous	Schulz-Key <u>et al.</u> , 1975
	<u>Onchocerca tarsicola</u>	?	connective tissue of legs	Bain and Schulz-Key, 1974b; Schulz-Key, 1975b
	<u>Onchocerca tubingensis</u>	?	subcutaneous	Bain and Schulz-Key, 1974b; Schulz-Key, 1975b
	<u>Wehrdikmansia rugosicauda</u>	? Ixodid ticks	subcutaneous	Schulz-Key, 1975c
Goat	? <u>Onchocerca caprae</u>	?	intramuscular (tongue)	Steward, 1946

TABLE 1 - continued

<u>HOST</u>	<u>FILARIAL SPECIES</u>	<u>VECTOR</u>	<u>SITE OF ADULT</u>	<u>SELECTED REFERENCES</u>
Pig	<u>Onchocerca</u> sp.	?	aorta	Remanujachari and Alvar, 1953
	<u>Onchocerca</u> <i>aff.</i>	?	?	Remachandran and Tam, 1967
	<u>Onchocerca</u> <i>devittiei</i>	?	tendons and subcutaneous	Bain <i>et al.</i> , 1977b
Kangaroo	? <u>Onchocerca</u> <i>websteri</i>	?	knee-joint	Supperer, 1966
Phalanger	<u>Microfilaria</u> from <u>Schoinobates</u> <i>volans</i>	? Ixodid tick	?	Moorhouse, 1969
	<u>Dipetalonema</u> <i>grassii</i>	Ixodid tick	subcutaneous	Noè, 1908; Nelson, 1966
Dog	<u>Dipetalonema</u> sp.	?	subcutaneous	Heisch <i>et al.</i> , 1959
Armadillo	<i>aff.</i> from <u>Dasyurus</u>	?	?	Convit, 1976 (unpublished)

TABLE 1 - continued

<u>HOST</u>	<u>PILARIAL SPECIES</u>	<u>VECTOR</u>	<u>SITE OF ADULT</u>	<u>SELECTED REFERENCES</u>
Bat	<u>Litomosia filaria</u>	?	peritoneal cavity	Nelson, 1964
Rodent	<u>Ackertia globulosa</u>	Ixodid tick	pulmonary arteries	Muller and Nelson, 1975
	<u>Ackertia marmotae</u>	Ixodid tick	lymphatics	Ko, 1972b
	<u>Ackertia nilotica</u>	? Ixodid tick	heart	El Bihari et al., 1977
	<u>Dipetalonema johnstoni</u>	?	subcutaneous	Spratt and Varughese, 1975
Bird	<u>Eufilaria cyrseii</u>	Mallophagan louse	connective tissue of neck	Nelson, 1962
	? <u>Onchocerca bambusicola</u>	?	abdominal cavity	Supperer, 1966

\* Microfilariae concentrated in dermal capillaries rather than the skin itself

Synonymised species based on Supperer, 1966



A review of the genus Ackertia Vaz, 1934

Vaz (1934) examined filarial parasites from the abdominal cavity of Cavia aperea in Brazil and decided that they were synonymous with worms from Cavia pamparum in Argentina designated by De La Barrera (1926) as Litomosa burgosi. He erected a new genus, Ackertia, to contain them because of the presence of a chitinous ring at the base of the buccal capsule which differentiated the new genus from Litomosa. At present, the genus Ackertia contains 6 species including A. burgosi (De La Barrera, 1926), A. marmotae Webster, 1967, A. dorsti (Mazza and Fiora, 1932), A. afromuris Fain, 1974, A. globulosa Muller and Nelson, 1975 and A. nilotica El Bihari, Hussein and Muller, 1977.

Webster (1967) described Ackertia marmotae from the connective tissue of the gall bladder and bile ducts of Canadian groundhogs (Marmota monax) and discussed the generic differences separating Ackertia from Wuchereria, Brugia and Dipetalonema. He regarded these genera as closely related and noted that the pattern of cephalic papillae is essentially similar in all four genera, consisting of an outer and inner ring of four papillae each. However, the presence of a peribuccal shield was thought to be an important feature distinguishing these genera.

The following year, Bain and Hocquet (1968) described another species of Ackertia from the abdominal cavity of Lagidium peruanum in Peru, which they named A. dorsti. These authors regarded the structure of the buccal capsule as similar to that of Breinlia (Yorke and Maplestone, 1926) and Dipetalonema (Chabaud and Anderson, 1959), but differentiated A. dorsti from Breinlia on the absence of a gubernaculum in the male, and from Dipetalonema on the characteristics of the microfilariae.

In a later paper, Bain (1973) synonymised A. dorsti with Dipetalonema finlayi (Mazza and Flora, 1932) originating from the peritoneal cavity of Lagidium tacumanus in Argentina. This species was redescribed by Buckley (1973) based on worms collected from L. peruanum in Peru. Buckley (1973) was of the opinion that his specimens closely resembled Litomosa but differentiated them on the presence of a circumoral cephalic shield, prominent cuticular bosses on the male tail, and host preference. Muller and Nelson (1975) concluded that the position of D. finlayi in the genus Ackertia is correct after examining the material of Bain and Hocquet (1968) and Buckley (1973).

Fain (1974) examined microfilariae from the blood of 2 species of Muridae in Zaire and designated them "Ackertia afromuris". While a full description was given of the microfilaria, no adult worms were examined from the hosts Dasymys incommis and Thomomys surdaster. In the absence of a description of the adult worms, the status of the species A. afromuris requires clarification.

Ackertia globulosa, with which the present study is concerned, was described by Muller and Nelson (1975) based on worms collected from the pulmonary arteries of Lemniscomys striatus striatus. Four genera of rodents were found to harbour the parasite in Kenya. Muller and Nelson (1975) noted that the cuticular thickening of the wall of the buccal capsule in A. globulosa was less pronounced than in other members of the genus. They also pointed out that some heterogeneity was evident in morphological characters between A. marmotae and A. globulosa on the one hand, and A. burgosi and A. finlayi on the other. These included a larger funnel-shaped buccal capsule and longer microfilariae in the former pair. However, a comparison of the size of adult females revealed that A. marmotae and A. dorsti are considerably longer (78

and 75.5 mm respectively) than those of A. burgosi (30 - 45 mm) and A. globulosa (14.3 mm).

The most recent addition to the genus Ackertia is the newly discovered species, A. nilotica (El Bihari et al., 1977). This occurs in the heart of Arvicanthus niloticus and has been described from specimens collected in the Sudan. Although little is yet known of the life-cycle of this parasite, A. nilotica appears to bear a number of similarities, both in morphology and biology, to A. globulosa which have been discussed by El Bihari et al. (1977). However, the adults of A. nilotica are somewhat larger than those of A. globulosa.

While the characters of adult worms are of major significance in the classification of filarial nematodes, other features are necessary in determining their phylogenetic relationships. As our knowledge grows of filarial life-cycles, greater emphasis should be placed on such features as host specificity, habitat of the adult worms, larval characters, cycles of development in the intermediate hosts and other biological characteristics, as suggested by Nelson (1961). This, of course, would be in addition to the grouping of species based on adult morphology. Such features have been used by Webster (1967), Bain and Hocquet (1968) and Muller and Nelson (1975) to separate members of the genus Ackertia from other genera in the sub-family Onchocercinae.

In an unpublished thesis, Anteson (1968, Ph.D. thesis - University of Connecticut) suggested that A. marmotae should be placed in a new genus, Monanema, principally on a difference in the shape, size and habitat of the adults and microfilariae from that of the type species, A. burgosi. Muller and Nelson (1975) recognised that both A. marmotae and A. globulosa differ from A. dorsti and A. burgosi in the habitat

of the adults and the presence of microfilariae in the skin rather than the blood. Recently, Anderson and Bain (1976), in agreement with Anteson (1968), placed A. marmotae and A. globulosa in the genus Monanema. However, Muller and Nelson (1975) pointed out that the original type specimens of both De La Barrera (1926) and Vaz (1934) are no longer available for study and descriptions based on this material are inadequate. It might therefore be best, as concluded by Muller and Nelson (1975), not to divide the genus Ackertia until redescrptions are available of the type species.

#### Biological characteristics of the genus Ackertia

Relatively little is known of the biology of the genus Ackertia. Of the six described species, one occurs in Canada (A. marmotae), two in South America (A. burgosi and A. dorsti), and three in Africa (A. afromuris, A. globulosa and A. nilotica). All six are restricted to hosts of the order Rodentia, with the Canadian species in ground-hogs, the South American species in chincillas and the African species in rats and mice. The adult worms of both South American species occur in the peritoneal cavity, while A. globulosa and A. nilotica are restricted to the vascular system of the host. It was originally believed that A. marmotae lives in the connective tissues (Webster, 1967), but subsequent work by Anteson (1968, Ph.D. thesis, University of Connecticut) and Ko (1972b) revealed that the adults occur in the lymphatics.

Sheathed microfilariae have been described for all six species of Ackertia. The microfilariae of A. burgosi, A. dorsti and A. afromuris occur in the blood, whilst those of A. marmotae,

A. globulosa and A. nilotica appear principally in the skin. However, as the microfilariae of both A. marmotae and A. globulosa were first discovered by their presence in the blood before it was found that the site of predilection is the skin (Ko, 1972b; Muller and Nelson, 1975), it might be wise to verify that the so-called blood-dwelling forms of other Ackertia species are truly restricted to the vascular system.

The life-cycles of members of the genus Ackertia have received little attention. Only Anteson (1968, Ph.D. thesis, University of Connecticut) and Ko (1972b) working with A. marmotae, and Muller and Nelson (1975) with A. globulosa have attempted to identify the intermediate hosts. In both species, ixodid ticks have been implicated as vectors and a complete description of the development of A. marmotae in Ixodes cookei has been given by Ko (1972b). Similarities in the life-cycles of A. marmotae and A. globulosa were noted by Muller and Nelson (1975). Both have skin-dwelling microfilariae and their development can be supported by hard ticks. El Bihari et al. (1977) reported that the microfilariae of A. nilotica may also be ingested by ixodid ticks, although it has not yet been demonstrated whether the parasites develop further.

The genus Ackertia bears little similarity to that of Onchocerca, and A. globulosa was not chosen for this study on the basis of a close phylogenetic relationship between these genera. Instead, the interest in this parasite as a possible model for onchocerciasis accrues mainly from the habitat of the microfilariae in skin. The fact that A. globulosa occurs naturally in rodents has encouraged the present effort to establish this parasite in the laboratory.

Aims of the present work on *A. globulosa* reported in Part I

- 1) To collect rodents naturally infected with *A. globulosa* and study aspects of the life-cycle under natural conditions.
- 2) To identify vectors of *A. globulosa* in the field and colonise these in the laboratory.
- 3) To establish the parasite in laboratory rodents and develop methods for its routine maintenance.
- 4) To study the development and course of infection of *A. globulosa* in the intermediate and definitive hosts.
- 5) To study the host-parasite relationships of this filaria in rodents with special reference to interactions between the microfilariae, the host, and vectors feeding on the host.
- 6) To test a compound of known microfilaricidal action (diethylcarbamazine citrate) against the microfilariae in vivo and observe any host reactions (macro- and microscopically) during treatment.
- 7) To evaluate *A. globulosa* in rodents as a possible laboratory model (and primary chemotherapy screen) for onchocerciasis and streptocerciasis; based on the ease of its maintenance in the laboratory, similarities in the behaviour and pathology of the microfilariae in skin (with *O. volvulus* and *D. streptocerca* skin microfilariae), and the effect of diethylcarbamazine treatment on the microfilariae in skin.

CHAPTER 2FIELD STUDIES ON ACKERTIA GLOBULOSAINTRODUCTION

In order to establish A. globulosa in the laboratory it was necessary to obtain wild rodents harbouring natural infections of the parasite and susceptible arthropods to serve as intermediate hosts. This made it important to confirm that the hard tick Haemaphysalis leachii is a natural vector of A. globulosa, as suggested by Muller and Nelson (1975).

To meet these requirements, a field trip was made to the Machakos district of Kenya in December 1974, where it had been previously shown the infection exists (Muller, pers. comm., 1974). This chapter describes the work performed to collect and identify infected rodents for shipment to England and a search made for the natural vectors of this filaria.

The trapping programme

Trapping was conducted from the Division of Vector-Borne Diseases laboratory at Machakos town, 50 kilometres south-east of Nairobi. During most of the year Machakos has few bodies of permanent water, so rodent activity concentrates around the small streams that flow temporarily with the April and November rains (Kenworthy and Glover,

1958). The collection of rodents was therefore made from 2 sites close to water in and around the town.

The first of these lay within the town at Kytia river (Plate 1) and was bounded on either side by cultivated land and houses. At the time of trapping, Kytia was reduced to a diffuse stream filtering through tall reedbeds and in many respects resembled "swamp" as defined by Pratt et al. (1966). The second site lay 4 kilometres out of town at Manza river (Plate 2) which, in contrast to Kytia, formed a discrete stream at the time of trapping. This was bounded by fallow grassland with trees and low shrubs, having the characteristic appearance of "wooded and bushed grassland" (Pratt et al., 1966).

The collection of rodents was made at both sites concurrently, using live-catching traps with a maize porridge bait. To secure the greatest number and variety of rodents, traps were laid in the rodent runways that traversed the grassy banks and reedbeds, while others were placed at the foot of trees and bushes. Sited traps were covered with vegetation to conceal them and shield captured animals from the sun and rain.

Shortly after dawn (7.00 hours) and just before dusk (17.30 hours) the traps were examined and those containing rodents emptied into a large cloth bag. All traps, whether occupied or not, were rebaited at each visit. The supply of rodents from a given runway was rapidly exhausted so traps that failed to become occupied on 3 successive occasions were moved to new locations. The continual re-positioning, baiting and emptying of traps on a twice daily basis meant that only around 23 could be operated at any one time.





Plate 1. The trapping site at Kytia river



Plate 2. The trapping site at Manza river

Laboratory examination of captured rodents

After each "trap round", the collected rodents were transported directly to the laboratory. Here they were identified, sexed and housed individually in metal holding cages. Animals held in the laboratory were fed maize cobs and apples.

a) Examination for filarial infection

To identify rodents infected with A. globulosa, 3 milligram (mg.) biopsies of ear skin were taken from each animal using a chicken toe-punch (Holborn Surgical Instruments, Broadstairs) and searched for microfilariae. This punch cut a standard disc of tissue and had been previously calibrated for the weight of skin it removed (0.75 mg.), making it unnecessary to weigh individual biopsies.

Skin snips were placed in 3 drops of phosphate buffered saline (P.B.S.) on a microscope slide and teased apart with fine forceps to aid the escape of microfilariae. Slides were placed in a humid chamber for 2 hours and the pieces of skin rinsed in fresh P.B.S. on a second microscope slide. Searches were then made of both slides under a compound microscope (at x 100 magnification) and the number of microfilariae recorded. The microfilariae of A. globulosa could readily be identified by their small size, slow movements and the presence of refractile globules in the sheath.

In addition to skin snips, the blood of rodents was also examined for microfilariae. Animals were bled from the tail or by cardiac puncture and 20 cu.mm. samples of blood were lysed with water for immediate examination under a compound microscope.

A number of rodents, including 6 infected striped mice (Lemniscomys

striatus), were recovered dead from the traps or died shortly after capture. These were autopsied and the details of this work are given in Chapter 4, dealing with the biology of A. globulosa in the definitive host.

b) Examination of rodents for ectoparasitic arthropods

A number of rodents were examined for ectoparasitic arthropods in an attempt to identify possible vectors of A. globulosa. Animals were anaesthetised with ether and the fur thoroughly combed over a sheet of white paper. All rodents were examined within 4 hours of capture to minimise the loss of ectoparasites that constantly occurred from these hosts. The arthropods recovered were identified and counted and small samples of each species were fixed in 70% alcohol so that their identification could be verified at a later date.

The remaining arthropods were dissected in P.B.S. on microscope slides and examined for filarial larvae; mites were dissected in batches of 10. Larvae were allowed 30 minutes to emerge from the tissues and the slides then examined under a compound microscope. Microfilariae were fixed with methanol or acetone (for subsequent staining) and infective larvae placed in 70% alcohol containing 10% glycerine for fixation and clearing.

c) Collection of adult H. leachii

As it was suspected that H. leachii is a natural vector of A. globulosa (Muller and Nelson, 1975) a search was made for the adults of this tick on 3 dogs at Kytia river. Fully engorged female ticks were retained for egg-laying, while unengorged females and males were examined for filarial larvae. To determine whether the dogs harboured

filarial infections that might have been transmitted to the ticks, 2 of these animals were bled and skin-snipped from the ears at the sites of tick attachment.

## RESULTS

### Filarial infection in rodents

A total of 265 rodents of 7 species were caught during 27 days of trapping and a breakdown of these into their site of origin and rates of infection with A. globulosa is given in Table 2.

Four species of rodent harboured A. globulosa: these were Lemniscomys striatus, Tatera robusta, Aethomys kaiseri and Otomys anqoniensis (Plates 3 - 6). Of these, it is apparent that L. striatus (the striped mouse) was the principal host as 28 of the 33 infected rodents recorded in this survey were striped mice. Moreover, there was a high rate of infection in these animals and 82% of the 34 examined had patent infections. Significantly smaller numbers of the remaining host species harboured the parasite. Only 3 of 50 (6%) T. robusta and 1 of 22 (5%) A. kaiseri were infected and the prevalence of A. globulosa in O. anqoniensis could not be assessed as only a single animal was trapped. No filarial infections were found in any of the Mastomys natalensis, Rattus norvegicus or Thamnomys sp. examined.

Despite the differing ecologies of Kytia and Manza, there was no evidence that this affected the distribution of A. globulosa infection. Infected rodents were common at both sites, which it seems was largely because striped mice occurred in both of the habitats in equal number (see Table 2).

TABLE 2

Proportion of rodents infected with Ackertia globulosa  
at Kytia and Manza rivers, Machakos.

Rodent species	Kytia		Manza	
	No examined	No positive	No examined	No positive
<u>Aethomys kaiseri</u>	--	--	22	1
<u>Lemniscomys striatus</u>	17	16	17	12
<u>Mastomys natalensis</u>	58	0	42	0
<u>Otomys angoniensis</u>	1	1	--	--
<u>Rattus norvegicus</u>	2	0	--	--
<u>Tatera robusta</u>	50	3	--	--
<u>Thamnomys sp</u>	4	0	--	--
TOTAL	132	20	81	13



Plate 3. Lemniscomyz striatus



Plate 4. Tatera robusta



Plate 5. Aethomya kaiseri



Plate 6. Otomys angoniensis

Details of the prevalence of A. globulosa in male and female striped mice are given in Table 3. At the time of collection, 21 of 24 (88%) males and 7 of 10 (70%) females harboured patent infections which suggests that there was little or no difference between the sexes in their susceptibility to this parasite. When examined 2 months later, a further 4 striped mice had developed patent infections, increasing the prevalence of A. globulosa in these animals to 96% for males and 90% for females.

In total, 32 of 34 (94%) striped mice harboured A. globulosa. This was the only species of filarial worm found in these rodents or in any of the animals examined during this survey.

#### The recovery of ectoparasitic arthropods from rodents

Arthropods were collected from 24 L. striatus, 37 T. robusta, 19 A. kaiseri and 31 M. natalensis. All rodents, of either sex and from either trapping site, were heavily infested with ectoparasites.

Ornithonyssus bacoti (the tropical rat mite) was the commonest arthropod recovered and was found predominantly around the base of the tail. L. striatus, T. robusta and M. natalensis harboured comparable numbers of mites (with means of 25, 22 and 27 per host respectively), but A. kaiseri was relatively free of these arthropods (with a mean of 5 per host).

Fleas were also found on all 4 species of rodent but in much lower densities than mites. Mean infestations of 1.2, 1.0, 0.8 and 0.6 fleas per host were recorded on A. kaiseri, T. robusta, L. striatus and M. natalensis respectively. Most fleas occurred on the dorsal surface of the host in a mixed population of Xenopsylla cheopis and Echidnophaga gallinacea. However, only a sample of those collected was retained



TABLE 3

Prevalence of A.globulosa infection in male and female L.striatus.

	No. examined	On collection No. positive (%)	2 months after collection No. positive (%)
MALE	24	21(88)	23(96)
FEMALE	10	7 (70)	9(90)
TOTAL	34	28(82)	32(94)

for identification so it is possible that other species of fleas were missed.

The only ticks to be recovered from rodents were the nymphal stages of H. leachii. These occurred on all 4 species of rodent but were present in even lower densities than fleas. M. natalensis and L. striatus harboured mean infestations of 0.8 (0-5) and 0.6 (0-3) ticks per host, and A. kaiserii and T. robusta only 0.3 (0-2) and 0.2 (0-1) ticks per host. As with the fleas, ticks were restricted to the dorsal surface of the host, occurring predominantly on the ears, head and neck.

#### Recovery of A. globulosa from arthropods

No filarial larvae were found in a total of 259 mites and 61 fleas examined from infected rodents. However, a number of ticks (H. leachii) collected from each species of host contained microfilariae, infective stage larvae, or both. These findings are summarised in Table 4.

Microfilariae occurred in almost half (5 of 13) the ticks from L. striatus and in 1 of 9 from T. robusta. Only the ticks collected from infected hosts contained microfilariae. When stained with a 1 in 10 dilution of Revector Giemsa, these microfilariae were found to be identical with those obtained from the skin of rodents, although many were exsheathed. No microfilariae were present in any of the ticks recovered from A. kaiserii or M. natalensis.

Infective stage larvae occurred in ticks from all 4 species of rodent examined (see Table 4). Over half the nymphs (8 of 13) from L. striatus, 2 of 9 from T. robusta and 1 of 5 from A. kaiserii contained infective larvae. These were also found in 2 of 23 nymphs from M. natalensis. Generally, ticks harboured few infective larvae although

TABLE 4

Occurrence of A.globulosa in H.leachii collected  
off various rodent hosts.

Rodent	No.ticks examined	No.ticks with		No.infective larvae per infected tick Mean(range)
		Microfilariae only	Infective larvae only	
<u>L.striatus</u>	13	5	8	3.1(1-10)
<u>T.robusta</u>	9	1	2	1.5(1-2)
<u>A.kaiseri</u>	5	0	1	1 --
<u>M.natalensis</u>	23	0	2	3.5(3-4)

a maximum of 10 was recorded in one nymph.

Adult stages of H. leachii were found on 2 of the 3 dogs examined at Kytia river. Eighteen ticks were collected from around the ears, head, neck and genitals of one dog and a further 11 from the ears and head of the second. Five adult rhipicephalids (probably Rhipicephalus simus) were also obtained from the first dog. Nineteen ticks were dissected and infective larvae found in 2 male H. leachii.

The larvae collected from dog and rodent ticks were subsequently compared with the infective stage larvae of A. globulosa raised in the laboratory. Identifications were based on the criteria drawn up by Nelson (1960). Great care was taken to distinguish the infective larvae that had been recovered from dog ticks as it is known that at least 6 filarial species occur in dogs in Kenya (Nelson *et al.*, 1962). However, no microfilariae were found in the skin or blood of the dogs used for tick collection, and all of the infective larvae from dog and rodent ticks proved to be those of A. globulosa.

At the conclusion of the trapping, 36 rodents were shipped to London. These included L. striatus, T. robusta and A. kaiseri, of which 22 harboured patent infections of A. globulosa.

#### DISCUSSION

Results of the present study confirm the findings of Muller and Nelson (1975) that at least 4 species of rodent in the Machakos district of Kenya serve as hosts for A. globulosa. These authors reported natural infections in L. striatus, T. robusta, A. kaiseri and O. angoniensis which correspond with the 4 host species identified in the present work. As only 7 species of rodent were collected in

this study it may well be that other hosts were missed. However, there can be little doubt that striped mice (L. striatus) play a significant role in the transmission of this parasite as over 80% of those examined were infected. In comparison with the prevalence of A. globulosa in T. robusta (6%) and A. kaiseri (5%), infections in L. striatus must constitute an important reservoir of the parasite.

It seems somewhat surprising that no other species of filarial worm was detected in these animals. At least one filaria, belonging to the genus Onchocercella, has been discovered in rodents at Machakos (Nelson, pers. comm., 1975). This interesting parasite has been found in O. angoniensis (the swamp rat), where the adult worms form subcutaneous nodules and microfilariae occur in the blood. Only A. globulosa was found in the single swamp rat captured in this study. In the neighbouring Sudan, another rodent filaria has recently been discovered which has striking similarities with A. globulosa and has been designated Ackertia nilotica (El Bihari et al., 1977). This species occurs in Arvicanthus niloticus (the Nile rat) in which the adult worms live in the heart and microfilariae in the skin. As with A. globulosa, A. nilotica infects a high proportion of the host population and may also be transmitted by ticks, as microfilariae have been found in a number of Rhipicephalus simus collected from Nile rats (El Bihari et al., 1977).

In the limited time available for field work, it was not possible to make a detailed study of the biology of A. globulosa in nature. However, a number of conclusions may be drawn concerning the ecology of this parasite from the information obtained over the 4 weeks of trapping.

Rodents infected with A. globulosa occurred in both "swamp" and

"wooded and bushed grassland" at Machakos. From this it can be inferred that the distribution of the infection was not restricted to environments of only one specific ecological type, and that the transmission of this parasite may occur in a variety of habitats that provide suitable niches for the rodent hosts and hard tick vector (H. leachii).

Values for the prevalence of A. globulosa infection proved to be an underestimate, when based on skin-snips taken at the time of trapping as 12% of the striped mice collected in the field only developed patent infections after 2 months in captivity. Such an effect has also been reported by Williams (1948) who found that 20% of cotton rats naturally infected with Litomosoides carinii only became microfilaraemic some time after collection. Presumably, this phenomenon is common to most surveys of filarial infections and may be attributed to the relatively long prepatent period of the parasites involved.

Much attention has been paid to the question of host sex difference in filarial infections so it is interesting to note that no difference was found in the prevalence of A. globulosa in male and female striped mice: In this respect, the behaviour of the parasite in its mammalian host differs from that found with a number of filarial species.

Several surveys on human filariasis have shown that men have a higher susceptibility than women to infection with Wuchereria bancrofti, Dipetalonema perstans and Onchocerca volvulus (Murray, 1948; Jordan, 1955; Nelson, 1958; Heisch et al., 1959; Nelson et al., 1962). It has been argued by some authors that a differential exposure of men and women to the infective bites of the vectors may account for the higher

infection rates in the male population. However, Murray (1948) working on the sub-periodic form of W. bancrofti in the South Pacific, and Nelson et al. (1962) with the periodic form in East Africa, pointed out that such an exposure factor was inapplicable in the situations studied and could not explain the differences observed. In this study, no difference was found in the number of male and female striped mice infested with H. leachii.

In the realm of animal filariases it has also been reported that there are differences in the infection rates for male and female hosts. Wallenstein and Tibola (1960) found that 4 times as many male than female dogs were infected with Dirofilaria immitis although Thrasher et al. (1963) showed no such disparity. Nevertheless, both teams of workers found that Dipetalonema reconditum occurred more frequently in dogs than in bitches. Amongst the rodent filariae it has similarly been shown that male jirds (Meriones unguiculatus) are significantly more susceptible to infection with Brugia pahangi and Brugia malayi than are females (Ash and Riley, 1970a and b; Ash, 1971; El Bihari and Ewert, 1973). In contrast with many of the earlier studies, these observations were based on experimental infections so the course of filarial development could be examined after the controlled inoculation of infective larvae. The manipulation of hosts prior to infection also allowed underlying factors in the differential susceptibility of males and females to be investigated.

Nelson et al. (1962) and Nelson (1966) suggested that sex hormones might be a factor increasing the susceptibility of male hosts to infection with filarial parasites. This was confirmed by Wesley (1973, cited by Ash, 1974 - mimeo. doc.) who found that host androgens were responsible for the higher infection rates of B. pahangi in male jirds.

In a review on the role of sex hormones in parasitic infections, Solomon (1969) noted that males were less resistant to infection than females in many host-parasite relationships. He also pointed out that the susceptibility of males may be greatly enhanced in an abnormal host species. Thus, Haley (1958) found that the rat parasite, Nippostrongylus muris, infected both sexes of rat equally, while in hamsters (an unnatural host) it gave rise to far higher infections in males than in females. In this connection it is noteworthy that Denham (1974) found no difference in the infection rates of male and female cats with B. pahangi: Unlike birds, cats are natural hosts of this filaria. Similarly, Bertram (1966) found no difference in the susceptibility of male and female cotton rats to infection with their natural parasite, L. carinii. To these can be added the findings of the present study.

In the light of these observations it is tempting to speculate that in the most highly evolved host-parasite relationships, differences in the susceptibility of male and female hosts have been resolved. In this way parasites could adapt to exploit the maximum number of hosts available, increasing their chances of survival and transmission. On this premise, the equal susceptibility of male and female striped mice to infection with A. globulosa might be viewed as indicative of a highly evolved host-parasite relationship between this filaria and its principal host.

In this study, many of the ticks (H. leachii) recovered from rodents contained both microfilariae and infective larvae. Muller and Nelson (1975) also discovered infective larvae in H. leachii and suggested that this tick may be an intermediate host of A. globulosa in nature. The present findings support this view as they strongly



suggest that H. leachii is an important natural vector of A. globulosa at Machakos.

H. leachii is a three-host tick (Nuttall et al., 1908) and only the immature stages (larvae and nymphae) feed on rodents (Lewis, 1939; Hoogstraal, 1956, 1958; Walker, 1974). These are often found in the nests of their hosts (Roberts, 1935; Lewis, 1939; Hoogstraal, 1956, 1958) and have previously been reported from the region around Nairobi, infesting Otomys, Lemniscomys and other genera of rodents (Roberts, 1935; Lewis, 1939; Walker, 1974; Hoogstraal, 1975, pers. comm.). In contrast with the larvae and nymphae, the adults of H. leachii do not attach to rodents but occur chiefly on dogs and a number of wild carnivores (Hoogstraal, 1956; Walker, 1974). At Machakos, Lewis (1939) found many dogs were infested with this tick, in keeping with the experience of the present investigation. It therefore appears that while the reservoir of filarial infection is maintained in striped mice, that of the vector includes larger mammals such as dogs.

The precise identification of a vector species is of paramount importance to a study of this kind. In this respect, H. leachii presents great difficulties as both Hoogstraal (1956) and Walker (1974) have pointed out that this is not a uniform species but a broad and ill-defined group with at least 6 different forms in Kenya alone. Despite attempts to differentiate members of the H. leachii complex on morphological grounds, only 2 as yet can be identified with confidence (Hoogstraal, 1976, pers. comm.); these are H. leachii leachii and H. leachii muhsami. Interestingly, Hoogstraal (1956) found that all stages of H. leachii collected from rodent nests in Kenya and elsewhere were subsequently identified as the sub-species leachii. This suggests that H. leachii leachii is the most probable vector of A. globulosa at Machakos, especially as it was possible to exclude the

sub-species muhsami on the morphological features given by Hoogstraal (1956). In the laboratory a comparison was made of the susceptibility to infection of H. leachii originating from 4 different regions of East Africa. The results of this work are presented in Chapter 5.

For H. leachii to serve as a vector of A. globulosa, only the larval and nymphal stages can be involved as only they feed on rodents. Thus, ticks must acquire the infection as larvae in order to transmit it at the next, nymphal, feed. The probable sequence of events in the transmission of this parasite might therefore be as follows.

Larvae hatching from eggs deposited in the grass attach to passing hosts in the densely populated rodent runways. Those that attach to infected animals ingest microfilariae that the ticks carry as they detach from the host. The microfilariae then develop into infective stage larvae in the interval before the nymphal ticks feed. Hooker (cited by Nuttall et al., 1908) found that the immature stages of Haemaphysalis leporis-palustris detach from their lagomorph hosts mainly by day, when these nocturnal creatures are present in their forms. Arthur (1962) similarly reported that Ixodes tranguliceps drops off its hedgehog hosts during daylight hours while these animals are resting in their nests. In this way, moulting ticks emerge in a situation favourable for attachment to new hosts. It would be interesting to know if the same is true for H. leachii (but with detachment occurring at night as striped mice are diurnal) since Hoogstraal (1958) has observed "Larvae and nymphs, both engorged and unengorged, as well as recently moulted, unengorged adults can be found in grass-rat nests". Whether this is so or not, a proportion of infected nymphs must attach to new hosts, and possibly rodent pups, in the confines of the nest: others may find new hosts in the rodent runways. In either case,

infective larvae are then transmitted.

Despite the absence of A. globulosa infection in M. natalensis, a proportion of the nymphs recovered from these hosts harboured infective larvae. The presence of infected ticks on unsusceptible animals indicates that these can be transferred from host to host and the nichés of the rodents may overlap. Arthur (1973a) reported that Ixodes tranguliceps can be found on a number of rodent species as a direct result of the overlap in their home ranges. He suggested that by this mechanism, pathogens might be transmitted either trans-tadially or transovarially from one host species to another. It may therefore be assumed that the absence of A. globulosa infection in M. natalensis is not due to the exclusion of this rodent from the transmission cycle of the parasite. Instead, it seems likely that these animals exhibit either a natural or acquired resistance to A. globulosa infection.

The pattern of transmission of A. globulosa as outlined above is similar to that reported for other acarine transmitted rodent filariae. Species such as Ackertia marmotae transmitted by hard ticks, Dipetalonema viteae transmitted by hard and soft ticks, and Litomosoides carinii transmitted by mites all have vectors that live in the resting places of their hosts (Anteson, 1968 - Ph.D. thesis, Univ. of Connecticut; Ko, 1972a and b; Chabaud, 1954; Williams and Brown, 1945, 1946). This will ensure that transmission occurs to both adults and offspring of the rodents alike.

Nelson (1964) observed that the humidity and warmth afforded ectoparasitic arthropods by their intimate association with the host is an important factor in sheltering them from external conditions. This has allowed ectoparasite-transmitted filariae to exploit environments that might otherwise be inhospitable to other filariids. Humidity is

not only important for the survival of the arthropod but may markedly affect the development of filariae in their vectors. Thus, Nelson (1964) reported from Kenya that Dirofilaria immitis, Dirofilaria repens and Brugia pateri of dogs, which are transmitted by mosquitoes, are restricted to the humid coastal strip while Dipetalonema reconditum, which is flea transmitted, enjoys a wider distribution.

For much of the year the Machakos district of Kenya is dry. However, Hoogstraal (1956) noted that the microhabitat of rodent nests is significantly more humid than the surrounding environment. In Egypt, all stages of H. leachii may be found throughout the year in rodent nests in an area that would otherwise be far too arid for the survival of these ticks (Hoogstraal, 1958). Therefore, at Machakos it may well be that H. leachii is able to maintain the transmission of A. globulosa without a break during the seasons of drought. Unlike Canada, where the cold winter period interrupts the transmission of A. marmotae for up to 6 months in the year (Ko, 1972b), the transmission of A. globulosa is far less likely to be affected by the changes in temperature of equatorial Africa (seasonal range, 57-78°F at Nairobi - Kenya Atlas, 1962).

In this study it was found that 16 of 17 (94%) striped mice trapped at Kytia river in December 1974 harboured patent infections of A. globulosa. In contrast, Muller and Nelson (1975) found that only 4 of 17 (24%) striped mice collected from this site in March 1973 had patent infections. Although these results were obtained in separate years, they do raise the possibility that A. globulosa may have a seasonal cycle of transmission in spite of the considerations discussed above.

The most probable factor determining the prevalence of A. globulosa infections at various times of the year seems likely to be the breeding cycle of the rodent hosts. Striped mice, for example, which are principal hosts, exhibit 2 distinct breeding seasons coinciding with the April and November rains (Kingdon, 1974). In some years the "November" rains last from October to December and 2 striped mice trapped in the present study produced litters late in the month of December. As the vast majority of striped mice survive for only a single breeding season (Kingdon, 1974), those trapped in March may be expected to consist mainly of animals 4-5 months old. On the other hand, those trapped in December will be composed of offspring from the previous "April" (March - May) breeding season and will therefore be much older, at around 9 months of age. If it is assumed that host populations of increasing age sustain increasing rates of filarial infection, then the higher prevalence of A. globulosa in rodents trapped in December might be explained.

In terms of transmission, this would mean that as each breeding season is approached, the host population will be composed predominantly of mature animals with a high prevalence of filarial infection. Larval ticks that become infected on these hosts would then disseminate infective larvae to the influx of uninfected offspring that emerge at the close of breeding.

In nature, striped mice rarely survive for more than 6-12 months (Kingdon, 1974) so the transmission of sufficient numbers of infective larvae to hosts in the first weeks of life is of paramount importance if patent infections are to develop. In studies on the life-cycle of Dipetalonema johnstoni, a filarial parasite of Rattus fuscipes in Australia, Spratt and Varughese (1975) noted that the largest numbers of microfilariae appeared in the skin of hosts in October and November,

when the rodents start to breed. This, they suggested, makes optimal numbers of microfilariae available for the infection of vectors so that the transmission of D. johnstoni is ensured to offspring in the first months of life.

In this study it was found that densities of the tick vector were extremely low on rodents. Roberts (1935) also found that rodents around Nairobi harboured low numbers of H. leachii. This deficiency in the density of feeding vectors is compensated for by the very high rate of filarial infection in these ticks. Sixty-two percent of the nymphs recovered from striped mice harboured 1-10 infective larvae. Moreover, this figure is almost certainly an underestimate of the true infection rate in H. leachii as an unknown number of filarial larvae could already have been transmitted before the attached ticks were removed.

The dynamics of transmission of A. globulosa, as suggested by these findings, are in marked contrast with those described for a number of filarial species transmitted by free-flying insects. In the cases of Onchocerca gibsoni and Onchocerca cervicalis, Buckley (1938) and Mellor (1975) suggested that low infection rates in the Culicoides vectors are compensated for by the massive number of bites received by hosts each day. A similar situation has also been described for the transmission of human filariasis. Hairston and De Meillon (1968) and Wilson and Ramachandran (1971) calculated that man must be bitten by many thousands of infected mosquitoes to develop patent infections of Wuchereria bancrofti or Brugia malayi. The bovine, equine and human hosts of the above examples are all long-lived so inefficiencies in transmission can be tolerated. However, it is obvious that the transmission of A. globulosa could not be maintained in this way as comparatively few ticks feed on rodents and the hosts

are too short-lived.

In this study, 2 of the adult H. leachii recovered from dogs contained A. globulosa infective larvae. This is not altogether surprising as while the infective larvae in nymphal ticks may be transmitted to rodents, microfilariae ingested during the nymphal feed will develop to the infective stage in the adult tick. These will be unable to reach rodent hosts as the adults attach to dogs.

This aspect of the life-cycle of A. globulosa is somewhat perplexing as it represents a gross inefficiency in the transmission of the parasite. One possibility might be that A. globulosa has adapted to H. leachii from a tick species in which all stages of the arthropod feed on rodents. The propensity of filarial worms to adapt to new vectors in differing ecological environments is well known (Wharton, 1963; Laurence and Pester, 1967).

A. globulosa has been reported from only a restricted area around Nairobi. Muller and Nelson (1975) found infected animals at Kahawa and Machakos, and Gardener (1976, pers. comm.) has recently discovered a high prevalence of the infection at the University campus in Chiromo. However, both L. striatus and H. leachii occur over a wide belt of equatorial Africa (Kingdon, 1974; Hoogstraal, 1956), so until skin-snip biopsies are routinely examined during parasitological surveys, it is unlikely that the true range of this parasite will be fully revealed.

This field study provided a brief insight into the biology of A. globulosa in nature. The capture of live, infected rodents now enabled laboratory investigations to be performed in which the host-parasite relationships and experimental uses of this parasite could be further explored.



### CHAPTER 3

#### ESTABLISHMENT AND MAINTENANCE OF A. GLOBULOSA IN THE LABORATORY

Over the past 30 years a number of filarial species with mosquito, mite or soft tick vectors have been successfully established in the laboratory (see Schacher, 1973). However, hitherto, there have been no reports of the successful laboratory maintenance of a filarial worm transmitted by hard ticks.

This chapter describes the techniques used to establish and maintain A. globulosa in the laboratory. The life-cycle of this parasite, as elucidated by Muller and Nelson (1975) and confirmed in Chapter 2, is depicted in Figure 1. An attempt to find the most suitable method of transmitting the infection to laboratory rodents is also reported below.

#### GENERAL MATERIALS AND METHODS

##### Maintenance of the vector

Four colonies of Haemaphysalis leachii were established in the laboratory using ticks collected from dogs at Machakos, Muguga and Nairobi in Kenya, and Zomba in Malawi.

All stages of H. leachii (larvae, nymphae and adults) were stored in small perspex tubes, sealed with a plug of muslin and cotton wool, and held in large desiccators in which a relative humidity of 80% was maintained with potassium hydroxide solution (Solomon, 1951). Engorged





females, eggs and emerging larvae were kept at 20°C. At this temperature the larval ticks were ready to feed 7-10 days after hatching.

All 3 stages of the tick were fed on New Zealand White rabbits. Guinea-pigs were also used during the initial feeding trials but were later abandoned as alternative hosts since the adult ticks rarely engorged on them fully. To prepare a rabbit for infestation, the ears were shaved with electric hairclippers and enclosed in conical cloth bags affixed to the host with glue and adhesive tape (Plate 7). Before placing ticks in the bags to feed, twenty-four hours were allowed for fumes of the glue solvent to evaporate off. The rabbit was then infested, transferred to a cage over a water surround and a plastic "Elizabethan" collar secured around the neck to restrict host grooming (Watts et al., 1972).

Up to 3000 larvae were fed on a rabbit, completing engorgement in 3-5 days. When the majority of ticks were replete, the rabbit was anaesthetised with an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories Ltd.) at 25.4 mg/kg body weight, and the larvae collected. Fed ticks were divided into batches of 60 and stored at 26°C. However, larvae that failed to engorge were not normally recovered as most had died of desiccation.

Larval ticks moulted into nymphs 9-13 days after a blood meal. If required for immediate refeeding, the nymphs were transferred to 20°C and starved for 7 days before being placed on a rabbit. However, in this study it was necessary to have large numbers of nymphs available at all times for infection with A. globulosa so many were stored for far longer periods. Long-term storage of nymphs was achieved by transferring them from 20°C, through room temperature, to 4°C over a 3 week period. At 4°C, unfed nymphs remained viable for several months. For feeding, these were returned to room temperature 7 days prior to infestation.



Plate 7. Rabbit prepared for tick infestation

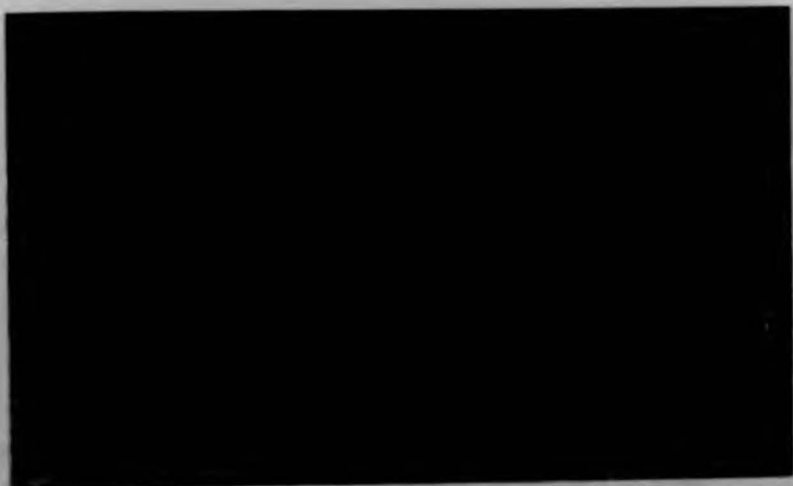


Plate 8. Bird prepared for tick infestation

400-600 nymphs were fed on a rabbit and took 5 days to complete engorgement. When replete, these were collected in batches of 25 and stored at 26°C. Nymphal ticks moulted into adults 13-16 days after feeding and were then transferred back to room temperature.

Adult ticks were starved for at least 7 days before the final blood meal. However, generally these were starved for several months as many more adults could be produced at one time than were immediately required for the purpose of breeding. 100 adult ticks were placed on a rabbit comprised of an equal number of males and females. Female ticks required 10-16 days to complete engorgement and during this period were collected each day to reduce the losses due to crushing and desiccation. As H. leachii copulates on the host (Hoogstraal, 1956), the males were discarded after feeding while the females that had engorged were placed individually in storage tubes.

At 20°C, female ticks commenced egg-laying 4-9 days after detachment and continued to oviposit for 18-23 days. Each fully engorged female laid between 1200 and 2100 eggs, as estimated by weight. Larvae began to emerge from the egg masses 25-33 days after the start of oviposition, by which time the majority of females were dead.

#### Maintenance of the natural definitive hosts

Lemniscomys striatus, Tatera robusta and Aethomys kaiseri that had been collected at Machakos during the trapping programme (see Chapter 2) were maintained in the laboratory for 2 years. During this period all 3 species of rodent survived well in captivity and few died of natural causes.

L. striatus and A. kaiseri were housed individually in 13 x 6 inch

mouse cages and the larger T. robusta in 15 x 9 inch rat cages. Animals were provided with hay for bedding and fed proprietary rodent pellet (Diet 86, Dixon Ltd., Ware) supplemented with sunflower seed and carrot; water was supplied by licking bottles.

All the wild rodents were aggressive when handled but striped mice (L. striatus) were particularly nervous and active. None of these animals became tamer with time despite regular handling over a long period. However, little use was made of anaesthetics as it was important to avoid accidental deaths with these valuable hosts. When required, Methoxyflurane B.P. (Penthrane, Abbott Laboratories Ltd.) was the anaesthetic of choice as it produced a light and controllable depth of anaesthesia not accompanied by a marked drop in body temperature.

A major threat to the health of striped mice in the laboratory was a periodic, exponential rise in the number of mites infesting these animals. To reduce mite densities, heavily infested rodents were treated with a 10% solution of tetraethylthiuram monosulphide (Tetmosol, I.C.I.) and the bedding changed twice weekly. However, the use of Tetmosol had to be kept to a minimum as striped mice were required for the infection of ticks.

Two striped mice trapped at Machakos in December, 1974 produced litters of 4 pups each shortly after capture. A litter of 2 was also obtained from A. kaiseri during the collection of rodents. As these provided a source of uninfected natural hosts for experimental infection, an attempt was made to obtain further litters by breeding the rodents in the laboratory. Single pairs of male and female L. striatus, T. robusta and A. kaiseri were housed in 22 x 15 inch stock cages and kept in an undisturbed room. To avoid fighting, rodent pairs were introduced into each cage simultaneously. Ample bedding was provided to afford the rodents privacy and was used by them in nest building. The diet of

these animals was supplemented with oestrogenic foods including germinating sunflower seed, wheat grains and grass seed. As L. striatus does not breed at a constant rate throughout the year (Kingdon, 1974), Happold (personal communication, 1975) suggested that variation in the day-night length ratio might stimulate the rodents to breed. This ratio was varied between 14-10 and 10-14 hours in half hour increments over 6 months by controlling room lighting with a time-switch.

It is not known which, if any, of the above factors were important in stimulating the rodents to breed as it proved extremely difficult to obtain litters from any of these animals. Nevertheless, 2 litters were produced by each host species although only 4 striped mice and 3 African gerbils (T. robusta) survived weaning.

#### Infection of the vector

Striped mice naturally infected with A. globulosa and with the highest densities of microfilariae in skin were used to infect ticks. Later in this study, experimentally infected hosts were also employed. Both larval and nymphal ticks were infected. However, for the routine production of infective larvae mainly nymphs were used as their greater size made them easier to handle and they also ingested more microfilariae.

Muller and Nelson (1975) reported that the microfilariae of A. globulosa concentrate in the ear skin of naturally infected hosts. Similar observations have been made in the present study and are described in Chapter 4. In order to take advantage of this phenomenon to infect ticks, a device was constructed to confine the feeding vectors

to the ears of the host. In appearance this device resembled a pair of stereophonic earphones (Plate 8) and consisted of two small perspex tubes with fitted lids lined around the lower rim with foam rubber. These were mounted in a light wire frame and were angled so that when in place on the head they fitted snugly over the ears with the foam rubber forming a tick-proof seal at the base. An elasticated chin-strap, tied across the frame, ran around the neck to hold the feeding capsules in place. As a precaution against rodents forcing the capsules off the head, a short length of "velcro" was attached to the centre of the wire frame and to a band of the same material secured around the chest immediately behind the fore-legs.

To fit the device it was necessary to lightly anaesthetise rodents with Penthrane. Before the animals were fully recovered, 30 nymphae or approximately 100 larvae of H. leachii were then placed in each capsule. When feeding was complete, 2-4 days later, the rodents were re-anaesthetised (with Penthrane) and the ticks collected. Despite vigorous attempts by the host to remove the feeding capsules when first fitted, they were later well tolerated and remained in place for the duration of tick feeding. The great advantage of this device was that it was light and required no glue or adhesive tape to hold it in place. This allowed rodents to be re-infested with ticks on a regular basis without the risk of injury.

After an infective blood meal, ticks were stored at 28°C and 80% relative humidity in the same manner as those of the stock colonies.

#### Recovery of infective larvae

Infective larvae for use in the routine infection of rodents were

collected from ticks 30-60 days post infection. The development of A. globulosa in H. leachii is described in Chapter 5. Live ticks were dissected with fine forceps in 15-20 mls of tissue culture medium 199 (B.D.H. Chemicals Ltd.) in a small petri dish. The infective larvae that emerged from the tissues were rapidly counted under a dissecting microscope and transferred by Pasteur pipette to watch glasses containing a minimal amount of medium 199. When they had settled to the bottom of these vessels, the larvae were loaded into 1 ml disposable syringes for immediate inoculation. Sluggish or damaged larvae were not included in the counted batches of worms.

#### Estimation of microfilarial densities in the skin of infected rodents

The technique used to estimate microfilarial densities in the skin of infected rodents was described in Chapter 2. In the laboratory, a Walser corneoscleral punch (L. Klein, Heidelberg) was used in addition to the chicken toe punch to take skin-snips from the ears of living rodents. This instrument proved easier to use and took a larger biopsy (1.5-3 mg) than the toe punch, but individual skin-snips had to be weighed. This was done on an Oertling electronic balance with a sensitivity of 0.1 mg. Mean numbers of microfilariae per milligram of skin were calculated for each rodent based on biopsies taken from both of the ears. In the case of dead animals, large skin-snips (5-15 mg) were taken from various regions of the body to estimate the distribution of microfilariae over the body (see autopsy procedure).

To standardize procedure, teased skin-snips were held for 6 hours in a humid chamber maintained at 28°C before counts were made of the emerged microfilariae.



Method of autopsy of experimentally infected rodents

Adult and neonatal rodents inoculated with A. globulosa infective larvae were autopsied at various intervals after infection.

Adult rodents were anaesthetised with an intraperitoneal injection of Nembutal and the fur removed with electric hairclippers. The thoracic cavity was opened, the animal exsanguinated by cardiac puncture, and the blood lysed with water and examined for worms under a dissecting microscope.

With rodents known to have patent infections from the previous examination of "ear-snips", skin biopsies were taken from the ears, head, neck, back, hind-legs, fore-legs, belly, nose and tail. These were weighed and examined for microfilariae as previously described. Further biopsies were taken from the ears and head and fixed in 10% formol saline for histological study.

The rodent was then skinned and all the internal organs removed. These were placed in separate petri dishes containing phosphate buffered saline (P.B.S.). Once the animal had been eviscerated, the peritoneal and thoracic cavities were rinsed with P.B.S. and the collected fluid searched for worms. The pelt and exposed musculature of the carcass were also examined for living or calcified parasites.

A thorough search was then made for worms in each of the isolated organs. To do this, each organ was crushed between glass plates and examined under a dissecting microscope. The crushed tissues were rinsed back into the petri dishes and thoroughly teased with fine forceps. The pelt and muscles were also torn by blunt dissection with forceps and left to soak in P.B.S. Fluid from the soakings of the carcass and organs was examined for parasites 6 hours later, and again the following day.

The procedure for autopsy of neonatal animals examined at 5, 10 and

18 days after infection differed only from adult autopsies in that the rodents were killed with chloroform, shaving was unnecessary and exsanguination was not performed.

Worms found in each region of the host were counted and living parasites transferred to 70% alcohol containing 10% glycerine for fixation and clearing. These were used for morphological study.

#### Experimental infection of laboratory hosts with *A. globulosa*

##### Experiment 1. Susceptibility to infection of laboratory-reared rodents

Six species of rodent were given subcutaneous inoculations of *A. globulosa* infective larvae. These included white rats, 4 strains of mice (TO, Sha Sha, CFI and C57 black), hamsters, Mongolian jirds (*Meriones unguiculatus*), multimammate rats (*Mastomys natalensis*) and laboratory-reared striped mice (*L. striatus*). The numbers of each species tested are given in Table 5.

Larvae were injected with 0.3-0.5 ml of medium 199 using a 1 ml disposable syringe coupled to a 21G x 1.5 inch needle. Usually 30 or more infective larvae were administered to each animal (see Table 5) but this number varied as a small number of rodents were given repeated inoculations of infective larvae as part of another experiment.

Skin snips were taken from the ears of rodents from 50 days after infection. The animals were considered susceptible to infection if microfilariae appeared in the skin within 200 days. Rodents which failed to develop patent infections within this time were then autopsied and a search made for living or calcified parasites.

TABLE 5

Experimental infections of laboratory reared rodents  
with A.globulosa.

HOST SPECIES	No.ANIMALS	No.OF INFECTIVE LARVAE PER ANIMAL MEAN(range)	No.OF HOSTS DEVELOPING PATENT INFECTIONS (%)
<u>Lemniscomys striatus</u>	14	63(12-143)	9(64%)
<u>Mastomys natalensis</u>	3	33(29-35)	0
<u>Meriones unquiculatus</u>	27	51(20-170)	17(63%)
<u>Mesocricetus auratus</u>	3	30(30)	0
<u>Mus musculus</u>			
TO	3	30(29-30)	0
Sha Sha	2	35(30-40)	0
CF1	2	29(27-30)	0
CF7 black	2	33(30-36)	0
<u>Rattus norvegicus</u>	3	31(30-33)	0

Only 2 species of rodent developed patent infections of A. globulosa in this experiment: These were the natural host, L. striatus, and laboratory rodent, M. unguiculatus (Table 5). Microfilariae appeared in the skin within 200 days in 9 of 14 striped mice and 17 of 27 jirds. However, no living worms were recovered from any of the remaining rodent species, although 2 calcified larvae were found in the subcutaneous tissues of a single hamster that died 90 days after infection.

Experiment 2. Administration of infective larvae by various routes

Since jirds were susceptible to A. globulosa, and were more readily available than striped mice, these were used to compare the efficiency of administering infective larvae by various routes. Three groups of 3 jirds aged between 6-8 weeks were injected subcutaneously, intraperitoneally, or intravenously (femoral vein) with 36-50 infective larvae. To obtain an accurate count of the number of larvae inoculated, those left in the syringe after injection were flushed out with medium 199 and their number deducted from that of the original inoculum. Two other jirds were anaesthetised with an intraperitoneal injection of Nembutal and 28 and 34 infective larvae were pipetted into the buccal cavity to see whether larvae could establish themselves in the host by penetrating the peribuccal mucosa.

Animals were autopsied 10 days after infection: The larvae recovered were counted and examined for evidence of growth and development. The morphological changes that occur in the development of A. globulosa in the definitive host are described in Chapter 4.

The results of this experiment, given in Table 6, suggest that only

TABLE 6

Worm recoveries from jirds 10 days after administration of A.globulosa infective larvae (L3) by different routes.

Route of L3 administration	No. Animals	Mean no. L3 per animal (range)	Total worm recovery 10 days post inf (%)	Larval stage
Sub-cutaneous	3	50(50)	5/150(3.3)	4th
Intraperitoneal	3	36(36-41)	2/107(1.9)	3rd
Intravenous	3	48(46-50)	0/145(0)	-
Oral*	2	31(28-34)	0/62(0)	-

\*larvae pipetted into buccal cavity of anaesthetised host.

the larvae inoculated by either subcutaneous or intraperitoneal routes survived in jirds. However, worm recoveries were extremely low and only 5 of 150 (3.3%) larvae inoculated subcutaneously and 2 of 170 (1.9%) inoculated intraperitoneally were recovered on autopsy. No worms were found in the jirds infected orally, and only a single dead larva was found in the liver of a jird inoculated intravenously.

An examination of the worms injected subcutaneously revealed that these had developed into fourth stage larvae. However, worms that had been inoculated intraperitoneally were identical with infective stage larvae. In view of this result and the evidence of higher worm recoveries from jirds inoculated subcutaneously, this route of administration of infective larvae was used in all subsequent experiments.

#### Experiment 3. Single and repeat infections with *A. globulosa*

In order to evaluate the parasitological success of infections in the definitive host resulting from single and repeated inoculations of *A. globulosa* infective larvae, rodents were skin-snipped from the ears at intervals from 50 days post infection. The prepatent period, duration of patency and peak microfilarial density attained in the host were then estimated.

The main problem associated with measuring these parameters was that only a limited number of biopsies could be taken from the ears. This imposed a severe restriction on the frequency of sampling the skin, especially as in many cases it was necessary to study the course of microfilarial densities in the host for over a year. Unfortunately it was not possible to use skin from other regions of the body as microfilarial densities were too low except in the ears.

#### The prepatent period

To obtain a value for the prepatent period, biopsies were taken at frequent intervals immediately prior to the expected date for the onset of patency. This was known to occur around 74-89 days post infection from a preliminary study of 3 experimentally infected rodents (J5, J13 and N1) that were biopsied at 10 day intervals from 40 days post infection. The prepatent period was then calculated as:-

$$\frac{\text{No. days to first "positive" biopsy} + \text{No. days to previous "negative" biopsy}}{2}$$

#### Duration of patency

This was a crude measure of the period over which microfilariae remained present in the skin of rodents and was calculated as:-

$$\text{No. days to last "positive" biopsy} - \text{No. days to first "positive" biopsy}$$

However, the measurement of this parameter was relatively inaccurate as skin-snips had to be taken at infrequent intervals (for reasons discussed earlier).

#### Peak microfilarial density

This was simply the highest microfilarial density recorded in ear skin during the period of the patent infection and was expressed as the number of microfilariae per milligram of skin (mf/mg).

While all of these measurements were imprecise, it was assumed the

inaccuracies in them would be constant, and they were therefore considered satisfactory to compare the course of infections between groups of animals. The course of microfilarial densities in the skin of jirds and striped mice experimentally infected with A. globulosa are shown in Figures 2 and 3.

Single infections with A. globulosa (Tables 7 and 8)

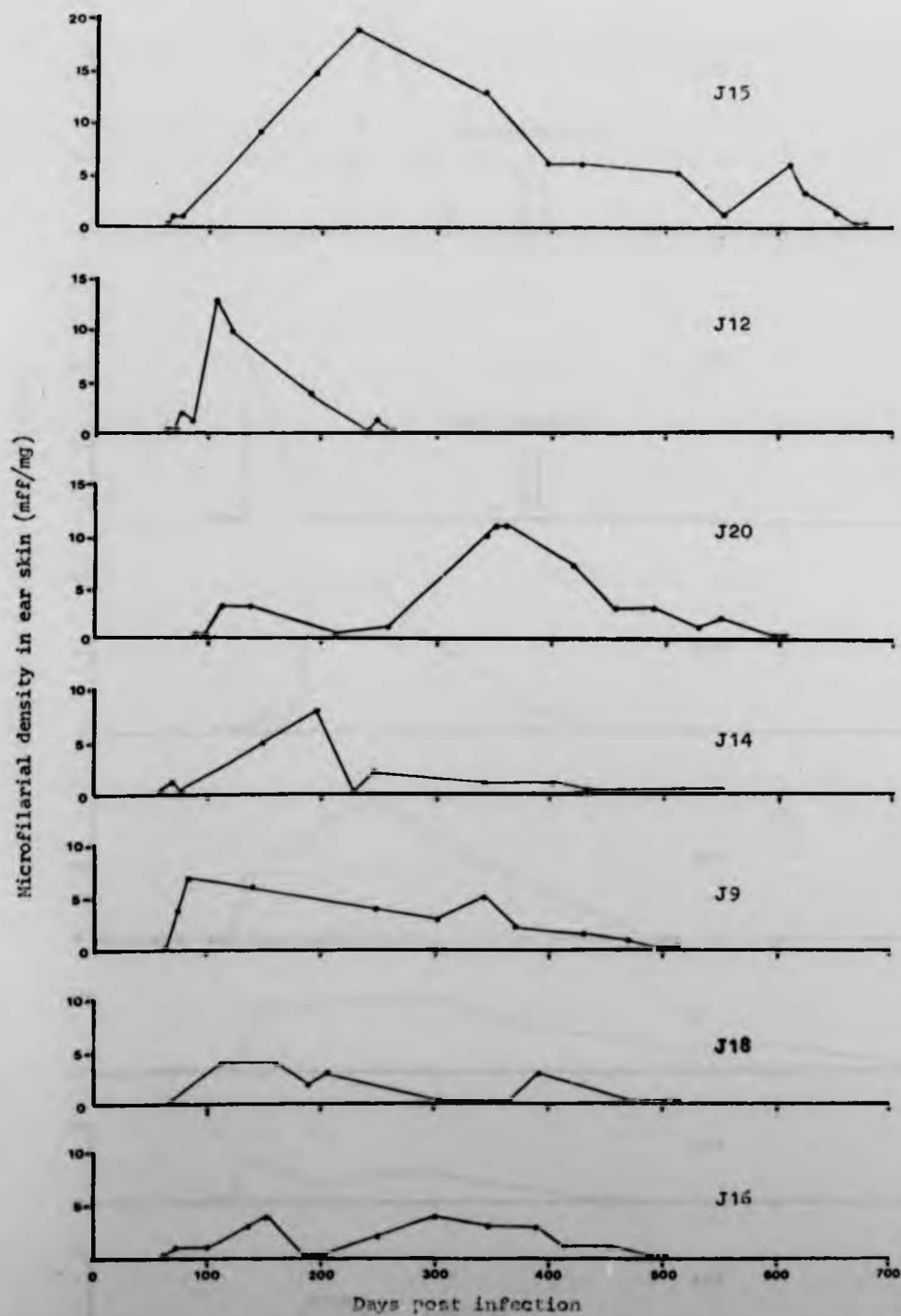
Twenty-five jirds and 8 striped mice were given single, subcutaneous inoculations of A. globulosa infective larvae. The number of larvae injected varied from 20-60 in jirds and 12-100 in striped mice. A further 3 striped mice were also infected on a single occasion by exposing them to the bites of experimentally infected ticks.

The results of this experiment, given in Tables 7 and 8, were that 15 of 25 jirds and 4 of 8 striped mice injected with known numbers of infective larvae developed patent infections. Microfilariae also appeared in the skin of all 3 striped mice infected by the bites of ticks. The mean length of the prepatent period was 74 (S.E. + 1.9) days in jirds and 88 (S.E. + 1.9) days in striped mice. There was no correlation between the development of patent infections or length of the prepatent period, and the dose of larvae received by the host within the range of inocula sizes used here.

In 5 jirds and 5 striped mice the course of microfilarial densities in the skin were studied until they subsided. With jirds, despite the small sample size, a positive correlation was found between the number of larvae administered and the duration of the patent infection ( $r = +0.95, 0.05 > P > 0.01$ ). The mean duration of patency was 370 (S.E. + 66) days in jirds but only 174 (S.E. + 54) days in striped mice.



**FIGURE 2** Course of microfilarial densities in ear skin of *M. unguiculatus* experimentally infected with *A. globulosa*.



**FIGURE 3** Course of microfilarial densities in ear skin of *L.striatus* experimentally infected with *A.globulosa*.

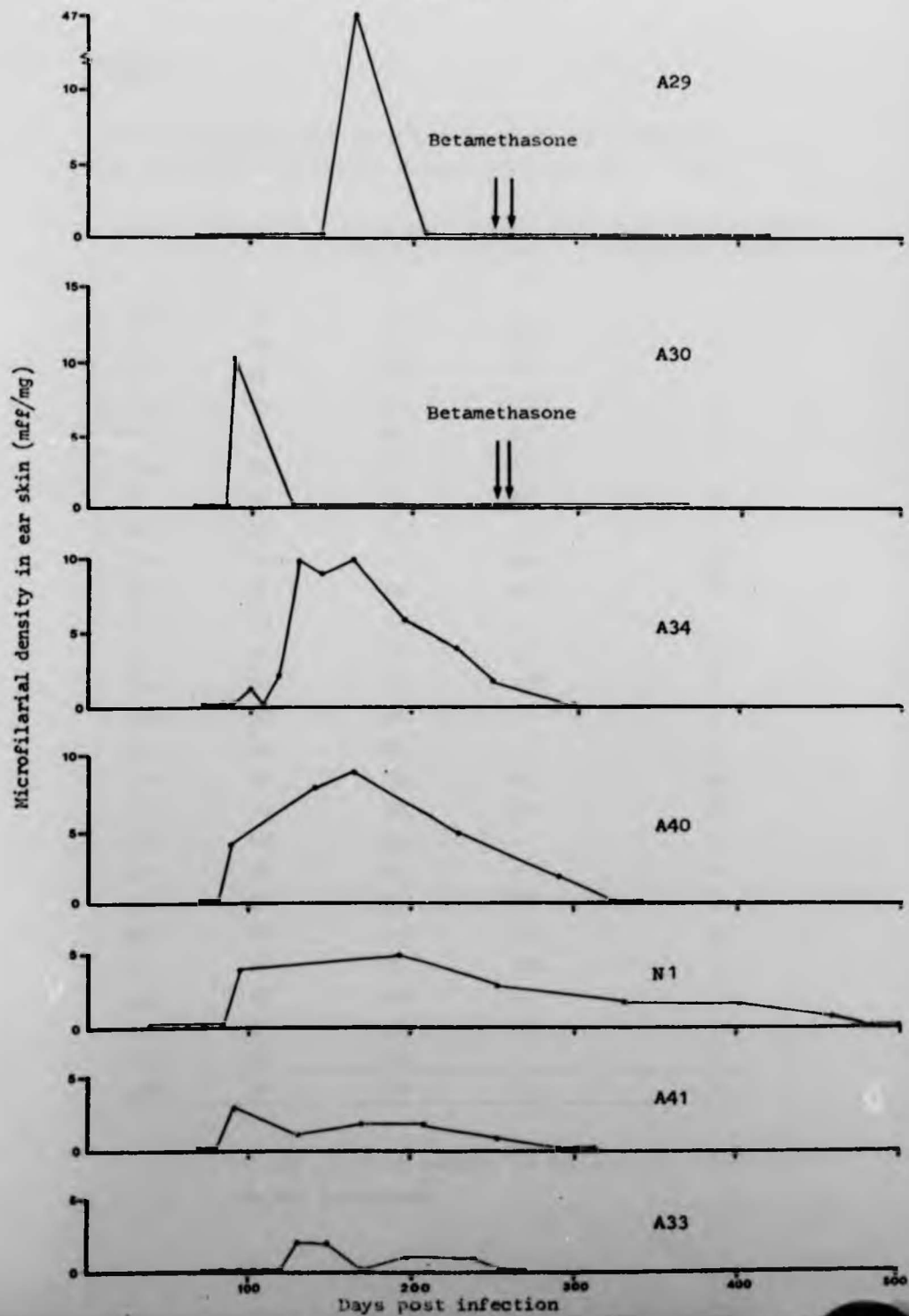


TABLE 7

Course of A.globulosa infection in jirds after single inoculations with various numbers of infective larvae.

Animal	Inoculum	Prepatent period	Duration of patency	Peak microfilarial density (mff/mg)
J19	20	NA	-	-
J5	30	74	ND	ND
J11	30	NA	-	-
J12	30	75	170	13
J13	32	81	ND	ND
J14	43	66	330	8
J7	47	90	ND	ND
AJ1	48	NA	-	-
AJ2	49	70	ND	ND
AJ3	49	80	ND	ND
J8	50	NA	-	-
J21	50	70	ND	ND
J16	50	69	376	4
J27	50	NA	-	-
J31	50	NA	-	-
CJ1	50	83	ND	ND
CJ2	50	73	ND	ND
CJ3	50	NA	-	-
CJ4	50	NA	-	-
CJ5	50	63	ND	ND
CJ6	50	73	ND	ND
J9	51	70	395	7
J10	55	NA	-	-
J15	57	66	581	19
J17	60	NA	-	-
MEAN	46	74	370	10.2

NA Not attained patency by 200 days post infection

ND Not determined

TABLE 8

Course of A.globulosa infection in striped mice after single inoculations with various numbers of infective larvae.

Animal	Inoculum	Prepatent period	Duration of patency	Highest mff density
N3	12	NA	--	--
N2	30	NA	--	--
N1	36	89	362	5
A30	50	88	39	11
A40	50	85	200	9
A41	50	85	124	3
A42	50	NA	--	--
A32	100	NA	--	--
A34	Infected tick bite	96	149	10
A35	Infected tick bite	80	ND	ND
A36	Infected tick bite	90	ND	ND
MEAN	47	88	174	7.6

NA Not attained patency by 200 days post infection

ND Not determined

In both host species, only low microfilarial densities were attained in the ear after experimental infection, with mean peaks of 10.2 (S.E. + 2.6) mff/mg in jirds and 7.6 (S.E. + 1.5) mff/mg in striped mice. There was no correlation between the number of infective larvae administered and the peak microfilarial densities attained in these animals.

Repeat infections with *A. globulosa* (Tables 9 and 10)

Repeated inoculations of infective larvae were given at 10 day intervals to 2 jirds and 3 striped mice. Jirds received a total of 59 and 170 larvae in 4 and 3 inoculations respectively (Table 9). Striped mice were given a total of 77, 100 and 143 larvae in 4, 3 and 4 inoculations respectively (Table 10). The prepatent period in repeatedly infected rodents was based on the interval between the first inoculation of larvae and the initial appearance of microfilariae.

Both jirds, and 2 of 3 striped mice developed patent infections in this experiment. Details of the infections arising in these hosts are given in Tables 9 and 10. Despite the small number of rodents used in this trial, it is striking that the duration of patency in jirds (281 and 440 days) was much greater than in striped mice (104 and 39 days). Unfortunately, it was not possible to perform more multiple infections because of a shortage of infective larvae. Therefore, a detailed comparison of infections arising in single and repeatedly infected hosts could not be made. Nevertheless, from the limited evidence available it appears that the additional work and large numbers of larvae required to infect rodents on a repeat schedule confer no advantage in producing useful infections of *A. globulosa* for the laboratory maintenance of

TABLE 9

Course of A.globulosa infection in jirds after repeated inoculations with infective larvae.

Animal	Inoculum	Prepatent period	Duration of patency	Peak microfilarial density (mfF/mg)
J18	32	90	281	4
	8			
	6			
	13			
Total	<u>59</u>			
J20	120	104	440	11
	20			
	30			
Total	<u>170</u>			
MEAN	115	97	361	7.5

TABLE 9

Course of A.globulosa infection in jirds after repeated inoculations with infective larvae.

Animal	Inoculum	Prepatent period	Duration of patency	Peak microfilarial density (mf/mg)
J18	32	90	281	4
	8			
	6			
	13			
Total	<u>59</u>			
J20	120	104	440	11
	20			
	30			
Total	<u>170</u>			
MEAN	115	97	361	7.5

**TABLE 10**

Course of A.globulosa infection in striped mice after repeated inoculations with infective larvae.

Animal	Inoculum	Prepatent period	Duration of patency	Highest mff density
A33	20			
	2			
	5			
	50			
Total	<u>77</u>	126	104	2
A31	50			
	25			
	25			
Total	<u>100</u>	NA	—	—
A29	20			
	40			
	25			
	58			
Total	<u>143</u>	158	39	47
MEAN	107	142	72	24.5

NA Not attained patency by 200 days post infection



this filaria.

Experiment 4. Comparison of the susceptibilities of adult and neonatal hosts to infection with *A. globulosa*

As the proportion of infective larvae that developed in jirds was extremely low (Experiment 2), an experiment was performed to see whether neonatally infected animals would prove more susceptible to infection than adults.

Breeding pairs of jirds were housed in separate cages and the animals were left in an undisturbed room. On production of a litter, both parents and 2 of the pups were infected with *A. globulosa*. The pups were each given 30 infective larvae by subcutaneous inoculation over the head within 24 hours of birth. The adults each received 50 infective larvae by the same route of administration. A total of 18 adult and 18 neonatal jirds were infected but despite care not to disturb the parents, 3 litters were killed (i.e. 6 neonatal jirds) and 1 adult died. These animals could not be necropsied as their cage-mates had eaten the bodies. Two neonatal jirds and their parents were autopsied at 5, 10, 18, 25, 80 and 105 days after infection. One of the surplus adult jirds was autopsied at day 10 and the 4 remaining animals at day 105 post infection. The number of living worms recovered was recorded and expressed as a percentage of the original number of infective larvae inoculated.

The results of this experiment are summarized in Table 11.

The recoveries of worms from neonatally infected jirds were far higher than from jirds infected as adults. However, the difference between these groups declined as the period of infection increased:

TABLE 11

Recovery rates of *A.globulosa* at various times after subcutaneous injection of infective larvae into adult and neonatal jirds.

	Length of infection in days.											
	5		10		18		25		80		105	
Age status of host at infection	<u>Neonatal</u>	<u>Adult</u>	<u>Neonatal</u>	<u>Adult</u>	<u>Neonatal</u>	<u>Adult</u>	<u>Neonatal</u>	<u>Adult</u>	<u>Neonatal</u>	<u>Adult</u>	<u>Neonatal</u>	<u>Adult</u>
Number of animals infected	2	2	2	3	2	2	2	2	2	2	2	6
Mean number of infective larvae per animal	28	50	30	50	29	50	30	50	29	50	24	49
Mean number of living worms recovered	10.5	2.5	8.5	1.7	3.5	0	2.5	1.0	2.0	1.0	0	1.5
(range)	(7-14)	(1-4)	(8-9)	(0-3)	(0-7)	-	(1-4)	(1-1)	(1-3)	(0-2)	-	(0-3)
% recovery of original inoculum	38	5	28	3	12	0	8	2	7	2	0	3

This was due to a progressive decrease in the number of worms recovered from animals infected as pups, while recoveries from adults remained consistently low (see Table 11). Thus, worm recoveries dropped from 38% to 7% between days 5 and 80 post infection in neonatally infected jirds while they varied only between 0 and 5% in adult animals during the equivalent period of infection.

The morphological changes that occur in the development of A. globulosa larvae in the definitive host are described in Chapter 4 based on worms collected in this study.

Experiment 5. The effect of corticosteroid treatment in rodents on the course of A. globulosa infections

In an effort to produce heavier and more persistent infections of A. globulosa, a number of animals were treated with the corticosteroid, betamethasone (Betsolan, Glaxo Laboratories Ltd.) to reduce host resistance to infection. To do this, the rodents were injected intramuscularly with 1 milligram of betamethasone daily for 7 days.

a) Treatment before infection. One week prior to infection, 3 jirds (J14, J19 and J10) were treated with betamethasone. The jirds were infected with 43, 51 and 55 infective larvae.

The results were that 2 of 3 jirds developed patent infections with prepatent periods of 66 and 70 days. In the infected animals, duration of patency was 330 days and 395 days with peak microfilarial densities of 8 and 7 mff/mg. The course of infection in these jirds closely resembled that seen in 22 untreated jirds infected with A. globulosa. In the untreated animals, 59% developed patent infections

after receiving a mean of 46 (S.E. + 2.2) infective larvae. The mean prepatent period was 74 (S.E. + 2.1) days, the duration of patency 376 (S.E. + 119) days and the peak microfilarial density 12 (S.E. + 4.4) mff/mg.

b) Treatment after infection. Two jirds (J15 and J20) with patent infections of A. globulosa were treated with betamethasone. Microfilarial densities in ear skin were measured 1 day prior to, and 3 days after, treatment. In addition, 2 striped mice (A29 and A30) that had exhibited microfilarial densities of 47 and 11 mff/mg on a single occasion were treated with betamethasone 54 and 134 days later: Skin-snips were then examined for the reappearance of microfilariae (see Figure 3). This was done to see whether the short duration of patency was due to the development of a latent infection produced in the host by an immunologically-mediated suppression of microfilariae in the skin.

In jirds, no difference was found in the level of microfilariae in skin-snips taken before (6 and 10 mff/mg) and after (3 and 11 mff/mg) treatment with betamethasone. No microfilariae reappeared in the skin of striped mice following betamethasone treatment.

#### Experiment 6. Passage of A. globulosa infection from jird to jird

This experiment was performed to see if A. globulosa could be "adapted" to jirds so that heavier infections would be produced after serial passage of the parasite from jird to jird.

Nineteen jirds were infected with 30-55 (mean 46) infective larvae originating from ticks fed on naturally infected striped mice. Four jirds were infected with 43-60 (mean 53) infective larvae originating

from ticks fed on experimentally infected jirds. Two jirds were infected with 20 and 50 infective larvae originating from ticks fed on jirds after 2 passages of the parasite through this host species.

The results were that 12 of 19 jirds infected with larvae from striped mice developed patent infections. The mean prepatent period was 75 (S.E. + 2.1) days and the mean peak microfilarial density, 10 (S.E. + 3) mff/mg. Three of 4 jirds infected with larvae after a single passage of the parasite through jirds developed patent infections. The mean prepatent period was 67 (S.E. + 1) days and the mean peak microfilarial density, 10 (S.E. + 4.5) mff/mg. Neither jird infected with larvae from 2 passages of the parasite through jirds developed patent infections. Therefore, further serial passage of A. globulosa through jirds was not possible.

#### DISCUSSION

Maintenance of A. globulosa in the laboratory proved difficult mainly because experimental infections in the definitive host were of low intensity. Microfilarial densities in the skin of experimentally infected rodents never approached those found in naturally infected hosts (see Chapter 4) and made the cyclical transmission of the parasite possible for only 3 serial passages.

In other respects, however, the establishment of A. globulosa in the laboratory was relatively simple. Ear capsules that confined feeding ticks to the area of highest microfilarial density on rodents were of great value in enabling the vector to be infected with the poor microfilarial levels of experimentally infected hosts. Because of the high reproductive potential of H. leachi, large numbers of ticks could

be easily reared. The longevity of these arthropods at cool temperatures also allowed them to be stock-piled in a manner unlike that possible with the vectors of many insect and mite transmitted filariae.

The natural definitive hosts of A. globulosa survived well in captivity but were unsuitable for laboratory maintenance because they failed to breed in large numbers. However, the susceptibility of Mongolian jirds to infection overcame this difficulty as these rodents breed readily in the laboratory and have been widely used in the routine maintenance of a number of filarial models (Weinstein and Highman, 1965; Thompson et al., 1968; Ash and Riley, 1970a and b; Ash, 1973; Denham and McGreevy, 1977).

In order to establish A. globulosa in the laboratory, a stock of experimentally infected hosts had to be built up before microfilarial levels in the naturally infected rodents declined. In addition to their use for transmission, some of these animals were required for a chemotherapy trial (reported in Chapter 6). This made it necessary to restrict the autopsy of experimentally infected rodents which meant that only small groups of animals could be used in most experiments.

Despite careful autopsies of the sacrificed rodents it is apparent that some worms must have been missed. 63% of experimentally infected jirds developed patent infection yet few adult worms (0-3 per host) were recovered. Only male worms were found when 2 jirds, which had positive skin-snips, were autopsied 105 days after infection. However, it is unlikely that many adults were overlooked as they are relatively large (1-1.5 cm long) and were easily recovered in greater numbers from naturally infected hosts (see Chapter 4). Instead, it seems probable that low worm recoveries from experimentally infected jirds reflected the low numbers of larvae that matured successfully. This would account for the low microfilarial densities attained in the skin of most

experimentally infected animals.

Nevertheless, jirds proved equally susceptible to infection (63%) as experimentally infected striped mice (64%). Jirds also attained microfilarial densities in ear skin (10 mff/mg) similar to experimentally infected striped mice (8 mff/mg) and the duration of patency was longer (370 days and 174 days respectively).

As striped mice are highly susceptible to A. globulosa in nature, developing heavy and persistent infections (see Chapters 2 and 4), it appears that the development of the parasite was in some way inhibited in experimentally infected animals. However, it was not possible to determine the reasons for this phenomenon with the limited number of striped mice available for experimental infection. Repeated inoculations of infective larvae, which will undoubtedly be the mode of exposure in nature, produced infections equivalent to those resulting from a single injection. Bertram (1966) noted that in Litomosoides carinii infections of cotton rats, repeat infections gave rise to higher microfilaraemias than did single infections provided that each challenge of infective larvae came after the prepatent period of the previous infection. Conversely, if challenge infections fell within the prepatent period, microfilaraemias diminished. If these observations on L. carinii (Bertram, 1966) are applicable to A. globulosa, then the experimental production of high microfilarial densities in rodents would be impractical to achieve as animals would have to be repeatedly inoculated with infective larvae at intervals of 74 days.

It seems unlikely that host immunity was primarily responsible for regulating A. globulosa infections in experimentally infected rodents. The treatment of animals with corticosteroids appeared to have no effect on the course of infection. Similar observations have been made by other workers who used cortisone therapy (Olson, 1959; Moon *et al.*, 1961;



Suswillo et al., 1977), cyclophosphamide treatment (Suswillo et al., 1977), or sub-lethal whole body irradiation (Siddiqui and Kershaw, 1975) in attempts to reduce host resistance to filarial infections.

It also appears unlikely that the low infections in jirds resulted from a physiological incompatibility between the parasite and host, as experimentally infected striped mice developed light infections too.

However, there was some evidence from Experiment 4 that jirds may develop an age-resistance to infection. The number of worms recovered from jirds infected as pups steadily declined between days 5 and 105 post infection while worm recoveries from adult jirds remained consistently low throughout this period. Unfortunately, it was not possible to infect jirds of various ages for autopsy after a standard interval of time as insufficient larvae were available for this experiment. Interestingly, Olson (1959) found that mature white rats developed an age-resistance to L. carinii infections. This was expressed by a decreased percentage of larvae completing migration to the pleural cavity and by an increased encapsulation of those worms that reached the pleural cavity. In contrast with white rats (which are abnormal hosts for L. carinii), cotton rats (the natural hosts) showed no resistance that could be correlated with age.

It may well be that the infection of rodents with A. globulosa by the bites of infected ticks could prove the most reliable method of transmission: All 3 striped mice infested with infected ticks developed patent infections. Unfortunately, it was not possible to study the full course of microfilarial densities in 2 of these animals as they were subsequently used in a chemotherapy trial.



## CHAPTER 4

BIOLOGY OF A. GLOBULOSA IN THE DEFINITIVE HOSTINTRODUCTION

The life-cycle of A. globulosa in nature was described in Chapter 2 and attempts to reproduce this in the laboratory were reported in Chapter 3. In this chapter, the biology of A. globulosa in the definitive host is considered in greater detail. This is a broad subject that encompasses many aspects of the host-parasite relationship from the introduction of infective larvae into the vertebrate tissues to the uptake of microfilariae from the body by the vector. However, little is known of this phase in the life-cycle of Ackertia species, involving the behaviour and development of the parasites in mammals.

Scott et al. (1951) gave the first detailed account of a complete filarial life-cycle when they described that of Litomosoides carinii in cotton rats. They discovered that filarial worms conform to the characteristic pattern of 5 larval stages seen in other nematodes and undergo 2 moults in both their intermediate and definitive hosts. Studies have since been made on the development of Dipetalonema viteae in jirds (Chabaud, 1954; Worms, 1970 - Ph.D. Thesis, University of London), Dirofilaria immitis in dogs (Kume and Itagaki, 1955; Orhiel, 1961), sub-periodic Brugia malayi in cats and jirds (Edeson and Buckley, 1959; Ash and Riley, 1970b), Brugia pahangi in cats and jirds (Edeson et al., 1960; Schacher, 1962; Ash and Riley, 1970a), Dipetalonema setariosum in jirds (Worms, 1970 - Ph.D. Thesis, University of London),

Brugia patei in cats and jirds (Buckley, unpublished - cited in Denham and McGreevy, 1977; Ash, 1973) and Breinlia booliata in rats (Singh et al., 1976). These investigations have shown that there is much uniformity amongst filarial worms in the sequence of morphological changes that occur during development, although their sites of maturation and rates of growth may vary considerably between the species.

The biology of Ackertia species in the definitive host has received little attention beyond descriptions of the morphology and location of adult worms and microfilariae (reviewed in Chapter 1). Anteson (1968 - Ph.D. thesis, University of Connecticut) described gametogenesis and the development of microfilariae in utero of Ackertia marmotae from groundhogs and Ko (1972b) expanded this work to examine the distribution of microfilariae in skin and their uptake by the vector (Ixodes cookei). Microfilariae were found to have an irregular distribution in the skin of groundhogs and to concentrate in the ears. Similar observations have recently been made for A. globulosa and Ackertia nilotica, where the skin microfilariae were also found to occur predominantly in the ears of their hosts (Muller and Nelson, 1975; El Bihari et al., 1977).

Ko (1972b) experimentally infected 5 groundhogs with the infective larvae of A. marmotae but recovered only 2 fifth-stage larvae from their final site of development in the lymphatics, 307 and 317 days after infection. In naturally infected groundhogs there was no evidence of histopathological changes due to either the microfilariae or adult worms (Ko, 1972b).

In this study, experimentally infected jirds were used to examine the development of A. globulosa in the definitive host and the eosinophil response to infection. Naturally and experimentally infected hosts were used to study the development of microfilariae in utero and their release into the blood, the distribution of microfilariae in the skin, the course

of microfilarial densities with time and the histopathology of infection. The behaviour of microfilariae in relation to the feeding habits of the tick vector was also examined.

#### Development of *A. globulosa* in the definitive host

The development of *A. globulosa* from the infective stage larva to the adult was studied in jirds autopsied at 5, 10, 18, 25, 80 and 105 days post infection. Observations were made on the location of worms in the host and of their stage of development at each time interval: The results are summarized in Table 12. These findings are based on autopsies made in Experiment 4 of Chapter 3, where the experimental techniques and worm recoveries are given (see Table 11). The morphology of larvae was studied using a Nikon phase microscope. Worms were measured and representative stages drawn with the aid of a camera lucida attachment. The major measurements of worms are given in Table 13 and illustrations in Figures 4-8.

#### 5 days post infection (Figure 4)

It is apparent that larvae had migrated widely from their subcutaneous site of inoculation within 5 days of infection. Of the 26 worms recovered, 15 were in the liver, 6 in the kidneys and 5 in the skin. These were all third stage larvae and showed little change in appearance from the infective stage. A morphological description of infective larvae collected from ticks is given in Chapter 5 (Figures 26 and 27; Table 21). The larvae from jirds were of similar length

**TABLE 12**

The development of A.globulosa in jirds and location in the host at various times post infection.

Numbers refer to worm recoveries (% of total recovery)

		DAYS POST INFECTION					
		5	10	18	25	30	105
SITE	Heart	0	2(9)	0	0	0	0
	Kidney	6(23)	2(9)	0	0	0	0
	Liver	15(58)	2(9)	0	0	0	0
	Lungs	0	4(18)	0	0	6(100)	9(100)
	Peritoneal cavity	0	5(23)	7(100)	7(100)	0	0
	Skin	5(19)	7(32)	0	0	0	0
STAGE	3rd stage larvae	26(100)	5(23)	0	0	0	0
	4th stage larvae	0	17(77)	7(100)	3(43)	0	0
	4th moult	0	0	0	4(57)	0	0
	Adult	0	0	0	0	6(100)	9(100)

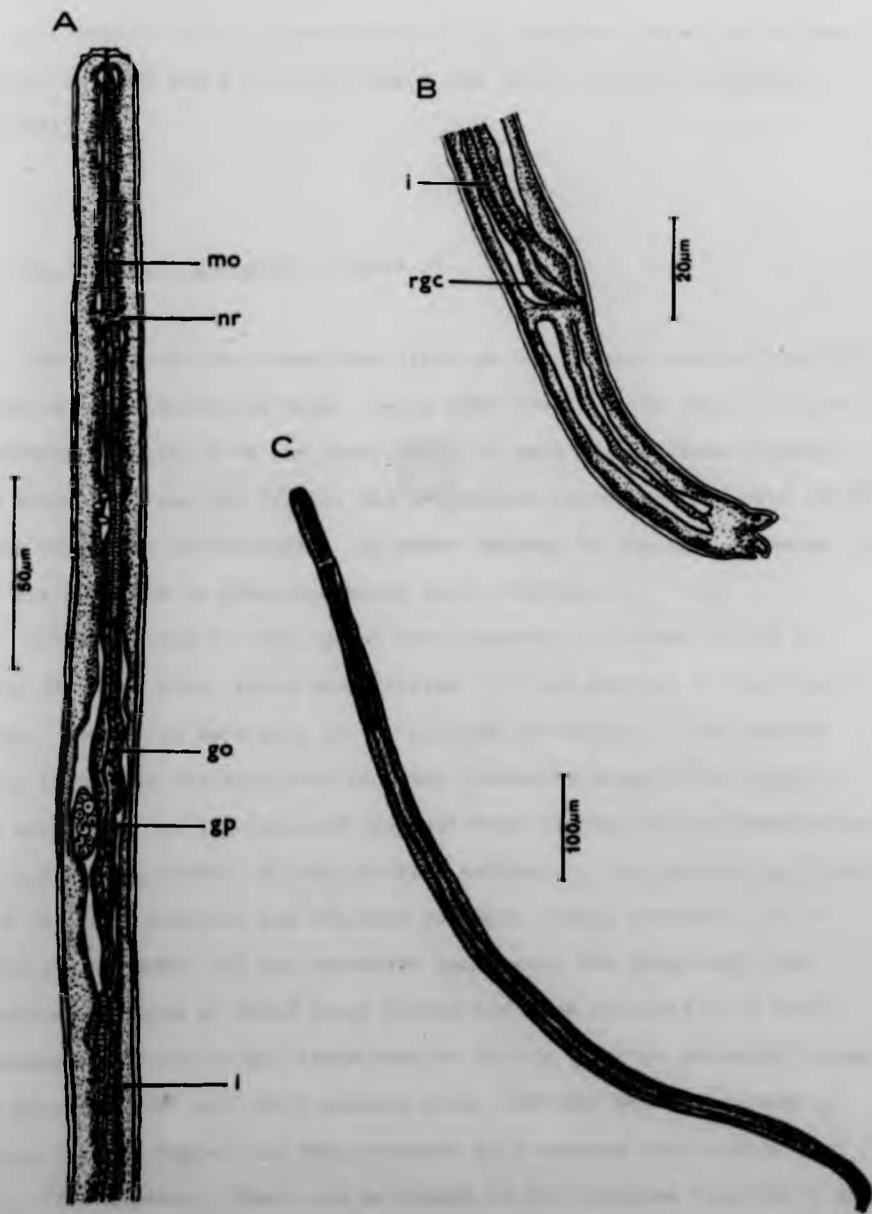
**TABLE 13**

Measurements of Ackertia globulosa larvae after various periods of development in jirds.  
Dimensions are given in microns as mean (range) or mean (% total length).

	DAYS POST INFECTION.				80	
	5	10	18	25*	Male	Female
<b>LENGTHS</b>						
Total	909	798(661-1123)	830(694-925)	5987	8333	10300
of oesophagus	248(27%)	240(30%)	240(29%)	461(8%)	248(3%)	311(3%)
of intestine	578(64%)	489(61%)	513(62%)	5445(90%)	7999(96%)	9890(96%)
of tail	83(9%)	69(9%)	72(9%)	82(1%)	86(1%)	99(1%)
Anterior end to:						
nerve ring	83(9%)	55(7%)	80(10%)	66(1%)	100(1%)	108(1%)
anus	826(91%)	729(91%)	758(91%)	5905(99%)	8247(99%)	10201(99%)
<b>WIDTHS</b>						
At nerve ring	11.6	21.6	24.0	32.9	14.8	34.1
Widest point	—	—	—	46.1	15.7	44.0
At anus	11.6	17.8	20.2	26.3	14.2	29.2

Measurements based on 1-10 specimens at each time interval

\* Female at 4th moult



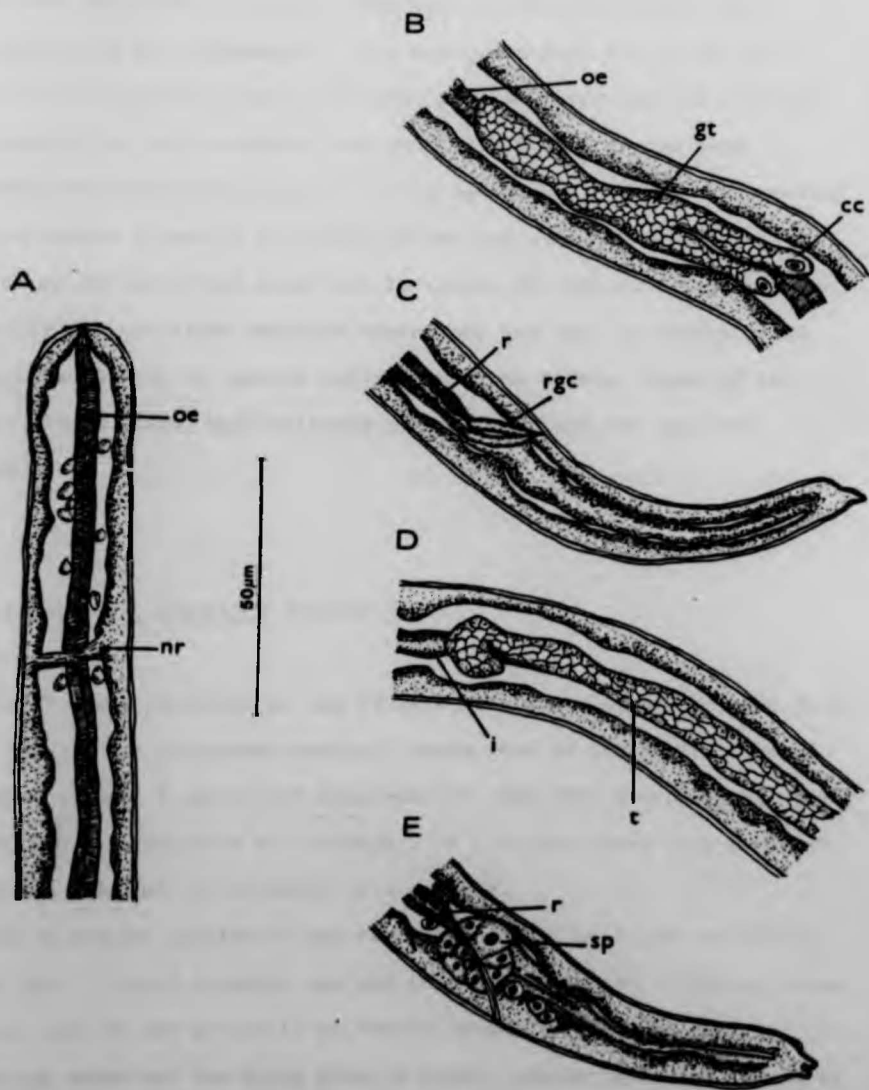
The third-stage larva, 5 days after infection : A Detail of the head ( lateral view ). B Detail of the tail ( lateral view ) C The whole larva.

(909 microns), width (11.6 microns) and internal morphology. However, the characteristically bulbous head of the infective larva had become bluntly rounded and a crescent shaped gap appeared at the cephalic extremity.

10 days post infection (Figure 5)

The 22 larvae recovered from jirds on day 10 were even more widely dispersed throughout the body. Seven were found in the skin, 5 in the peritoneal cavity, 4 in the lungs and 2 in each of the liver, kidneys and heart. Larvae lay free in the peritoneal cavity and occurred in the right ventricle of the heart. In other regions of the host, however, it was not possible to determine their exact location.

Obvious signs of development were apparent in larvae by day 10. While 23% were still third stage larvae, 77% had moulted to the fourth stage: No larvae were seen in the process of moulting. The fourth stage larva was distinguished from the preceding stage by changes in the morphology of the anterior and posterior extremities accompanied by an increase in width. At the anterior extremity, the cephalic papillae were far less distinct and the head was more evenly rounded than in third stage worms: At the posterior extremity, the large ear-like caudal appendages of third stage larvae had been replaced by 4 small processes. Fourth stage larvae were no longer than the preceding stage and measured 798 (661-1123) microns long, but they were considerably wider: At the nerve ring they measured 21.6 microns wide and at the anus, 17.8 microns. There was no change in the relative lengths of the oesophagus and intestine but the glandular region of the oesophagus was reduced.



The early fourth-stage larva, 10 days after infection : A Detail of head of male larva. B Detail of female larva showing development of genital tube. C Detail of female tail. D Detail of male larva showing development of testis. E Detail of male tail showing spicular primordium.

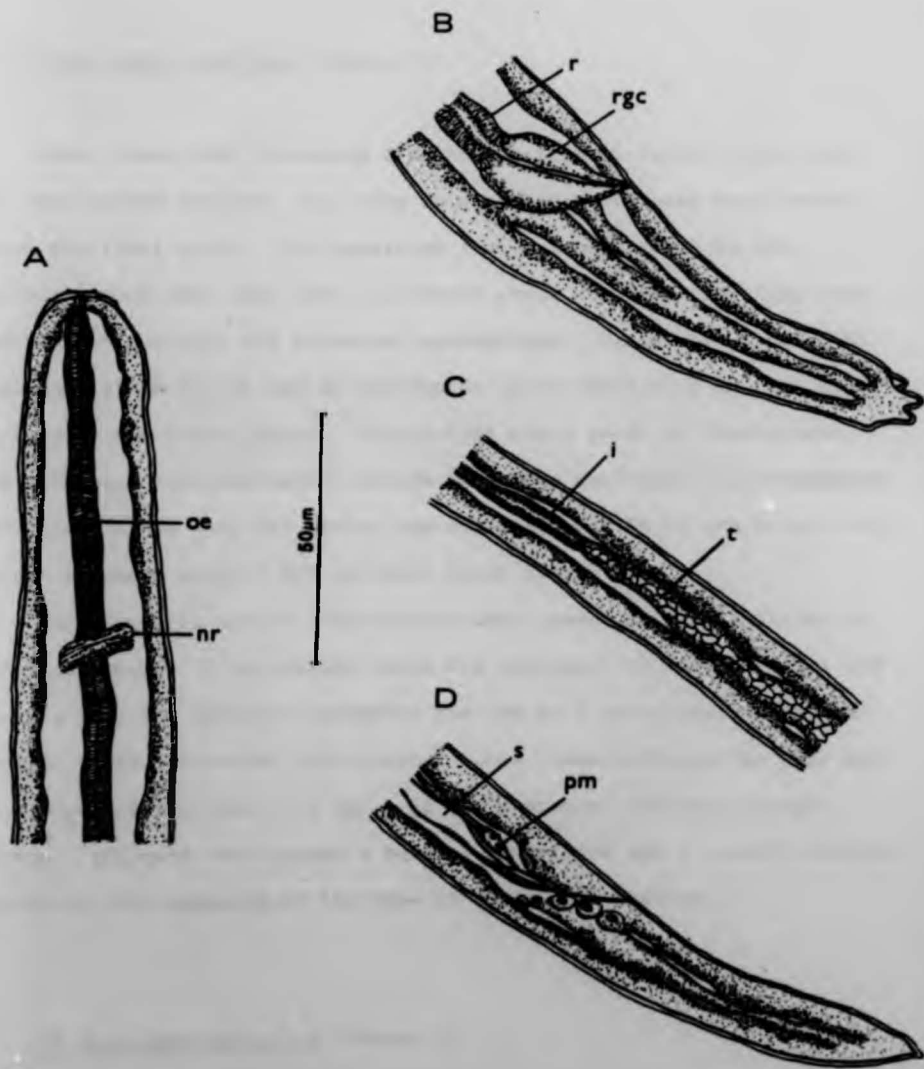


A prominent feature of fourth stage larvae was that the reproductive system had begun to develop. In the female, the genital primordium was situated 182 microns from the anterior extremity, about the midpoint of the oesophagus. This had grown into a tube of cells 116 microns long and was split distally into 2 ovario-uterine branches, each terminating in a prominent cap cell. In the male, the genital primordium was situated posterior to the oesophageal-intestinal junction. This had become flexed at its anterior end and extended distally as a long column of cells that could not be traced for its full length. The tail of the female larva remained unchanged, but that of the male had developed a cluster of hyaline cells around the lateral edges of the rectum. These large, multicellular groups comprised the spicular primordia.

18 days post infection (Figure 6)

The 7 worms recovered on day 18 were all fourth stage and were found lying free in the peritoneal cavity. These were no longer than larvae collected on days 5 and 10 and measured 830 (694-925) microns long, but their width had continued to increase: At the nerve ring they measured 24 microns wide and at the anus, 20.2 microns.

The glandular portion of the oesophagus could no longer be distinguished but in other respects the gut remained unchanged. Little change was also seen in the genitalia of female larvae, although a striking feature in males was the lying down of clear, tubular spicules served by protractor and retractor muscles. Development of the male reproductive system was rapid and the genital tube extended to within 47 microns of the spicular primordia. However, the male tail was not yet coiled and



The fourth-stage larva, 18 days after infection : A Detail of head of female larva. B Detail of female tail. C Detail of male larva showing development of testis. D Detail of male tail showing development of spicules.

the female tail remained simple.

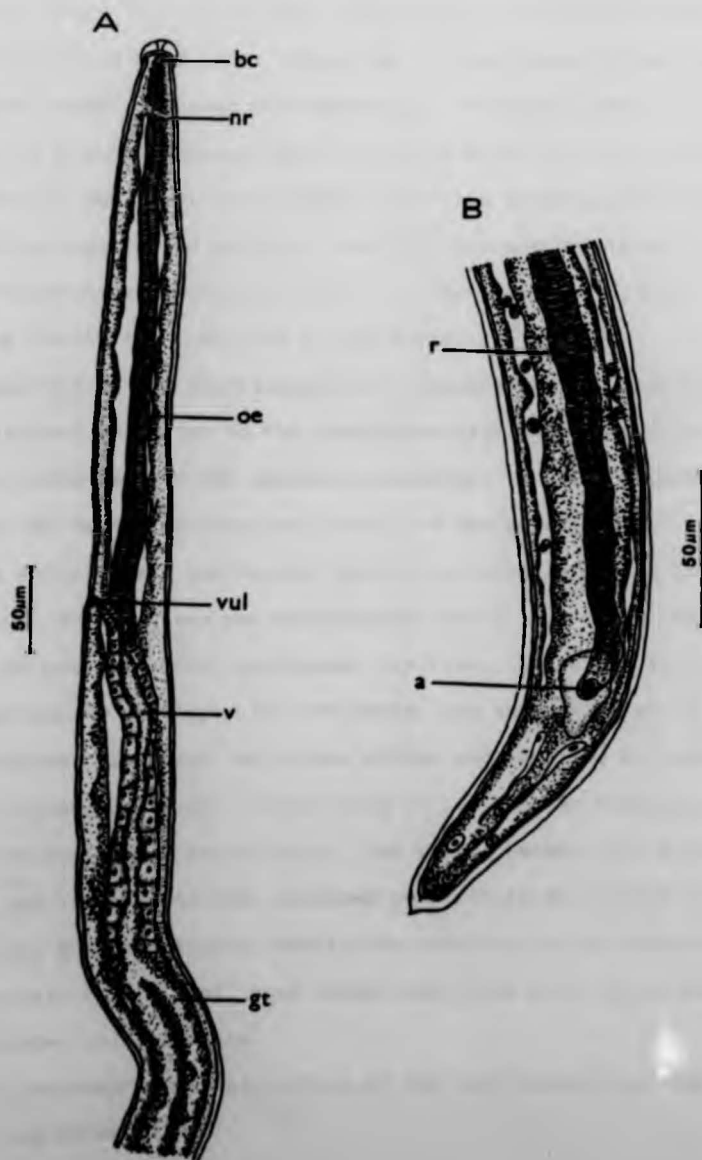
25 days post infection (Figure 7)

Seven worms were recovered from the peritoneal cavity where they lay free around the gut: All were females and 4 of these were undergoing the final moult. The remaining 3 were fourth stage larvae. Moulting worms were seen with the fourth stage cuticle separating from the body at anterior and posterior extremities. Larvae had grown considerably since day 18 and at the fourth moult measured 6 mm long by 15.7 microns at the widest point. During this rapid phase of development, the intestine had lengthened (5.4 mm long) far more than the oesophagus (461 microns) so that the latter now occupied only 8% of the total body length compared with 27-30% in third stage larvae.

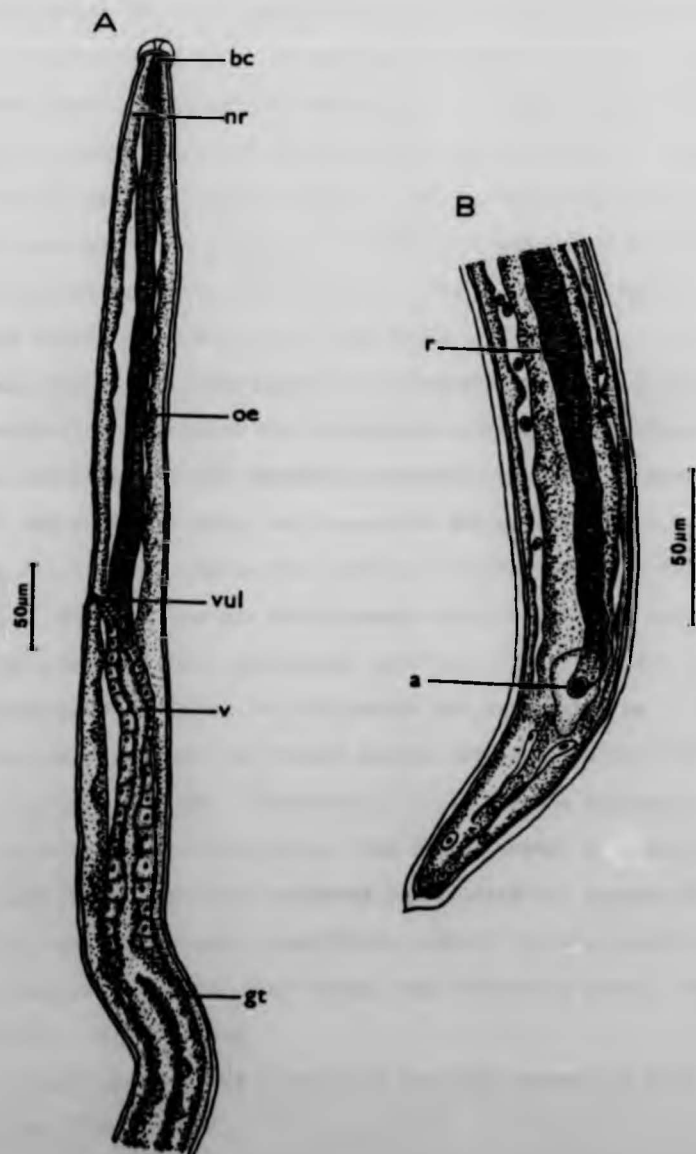
The genitalia showed considerable development in female larvae at the final moult. A non-patent vulva was implanted in the body wall 296 microns from the anterior extremity and led to a posteriorly directed vagina. This bifurcated into genital tubes which extended for over half the length of the body (3.5 mm). At the posterior end was a simple rectum. The head had assumed a bulbous appearance and a clearly defined cuticular ring appeared at the base of the buccal capsule.

80 days post infection (Figure 8)

Three male and 3 female adult worms were recovered, all from pulmonary blood vessels. Female worms had almost doubled in length since the final moult (10.3 mm long) and were nearly treble the width



Female larva at the fourth moult, 25 days after infection :  
 A Detail of anterior end showing development of the reproductive system. B Detail of the tail ( ventral view ).



Female larva at the fourth moult, 25 days after infection :  
 A Detail of anterior end showing development of the reproductive system. B Detail of the tail ( ventral view ).

(44 microns at widest point) but males were rather shorter (8.3 mm long) and far more slender (15.7 microns at the widest point).

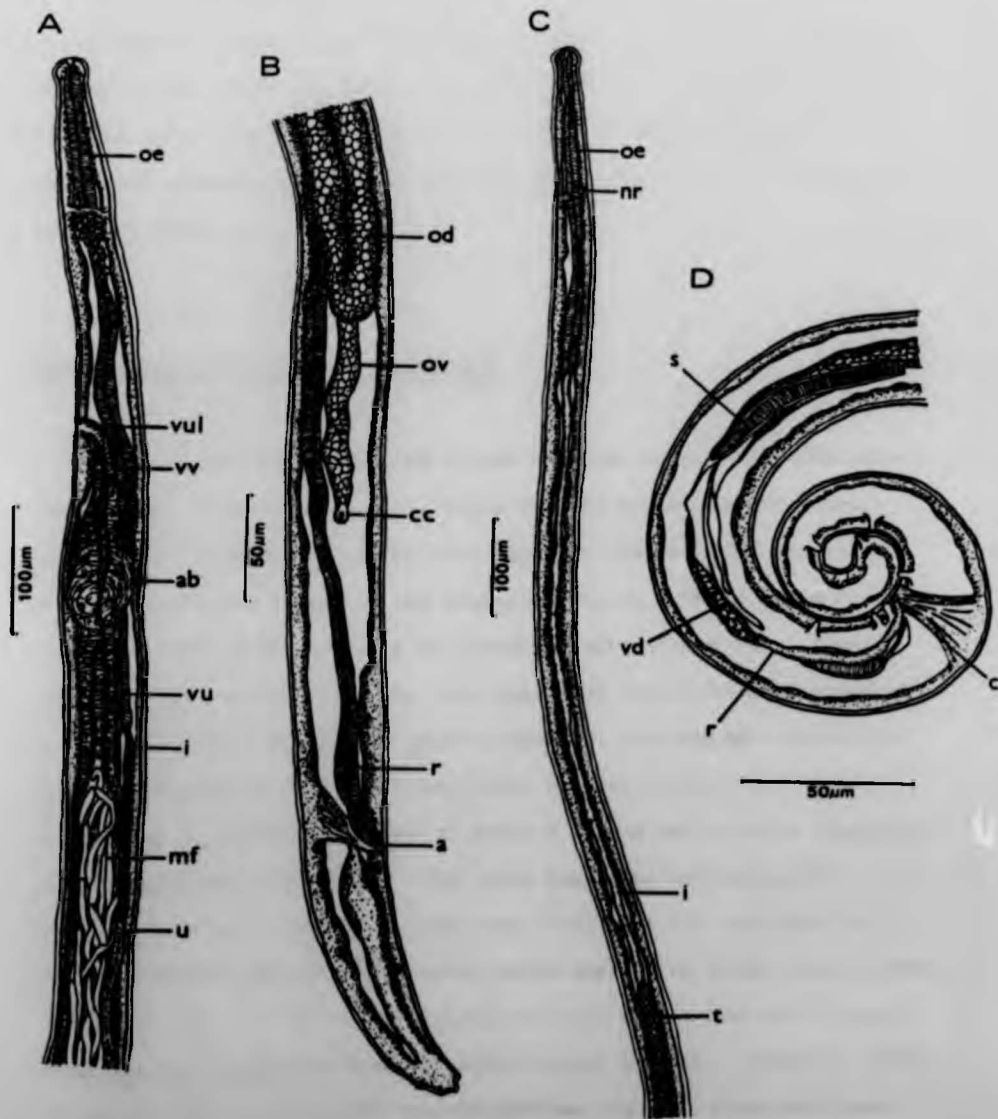
In both sexes the body tapered anteriorly and expanded terminally into a well-defined head bulb. There was a large funnel shaped buccal capsule with light cuticular strengthening. The rapid growth of the intestine had been maintained since the final moult so that it now occupied 96% of the total body length, while the oesophagus had grown little and was reduced to only 3%. The tail had not lengthened and during development had declined from 9% of the total body length in third stage larvae to 1% or less in the adult.

In male worms, the testis remained unattached to the body wall and was situated posterior to the oesophageal-intestinal junction at a variable distance from the anterior extremity. The vas deferens had fused with the rectum to form the cloaca and the spicules, which were dissimilar and unequal, had become lightly cuticularised with fibrillated walls. The tail was now corkscrewed with 4 or 5 turns and in lateral view bore pre- and postcloacal papillae. Depressor ani muscles were conspicuously developed in both sexes, but especially so in males.

In females, the vulva had become patent and remained situated in the mid-oesophageal region. Posteriorly it led via the vagina to a muscular ovejector with atrial bulb. The uteri, packed with microfilariae, led to oviducts that extended posteriorly for almost the full length of the body and looped immediately anterior to the simple rectum. The female tail bore 4 small appendages, but these were absent from the bluntly rounded tail of males.

A full morphological description of the adult worms has been given by Muller and Nelson (1975).

**FIGURE 8** DEVELOPMENT OF *ACKERTIA* *GLOBULOSA* IN JIRDS



The adult worms, 80 days after infection : A Anterior end of female worm ( lateral view ). B Posterior end of female ( lateral view ). C Anterior end of male worm ( dorsal view ). D Posterior end of male ( lateral view ).



105 days post infection

Nine adult worms were recovered on autopsy, also from pulmonary blood vessels. These were morphologically similar to adults collected 80 days after infection but were somewhat longer (males, 11.4 mm long; females, 13.9 mm long) than the latter worms. This implies that the growth of these parasites continued for some time after sexual maturity had been attained.

Development of microfilariae in utero

All female worms recovered 80 and 105 days after infection were fertilised. Uninucleated eggs (Plate 9) were present in the distal part of the oviducts and fertilised eggs, in the earliest stages of development, were packed in the posterior uterus. These had a distinct egg shell containing a morula of dividing cells (Plate 10). Towards the anterior end of the uterus were eggs that contained more advanced embryonic microfilariae. As these developed, the embryos became compact and assumed a C-shaped form within the egg (Plate 11), finally elongating and differentiating to produce a long coiled worm surrounded by the egg shell (Plate 12). The fully formed microfilaria then straightened out by stretching the egg shell with its head and tail. In the anterior part of the uterus, large numbers of fully formed microfilariae could be seen aligned longitudinally within the adult worm, with the egg shells retained as microfilarial sheaths. Sheathed microfilariae were released into the bloodstream via the vulva and those recovered from the uterus and blood were of equivalent lengths, measuring 135 (118-152) microns long.





Plate 9. Uninnucleated eggs

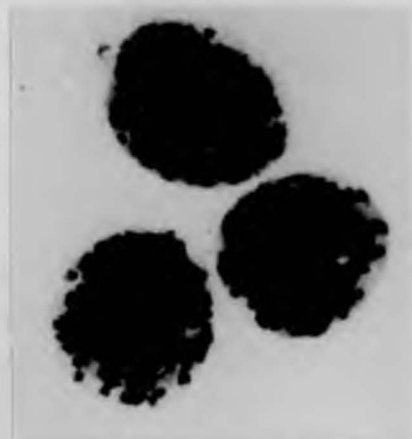


Plate 10. Morula of dividing cells



Plate 11. C-shaped embryos

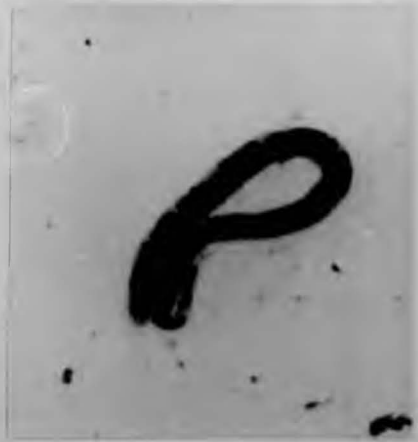


Plate 12. Coiled microfilaria

### Microfilariae in the blood

Blood was collected by cardiac puncture from 7 striped mice and 2 jirds known to have patent infections of A. globulosa by the presence of microfilariae in ear skin. Tail blood was collected from a further 11 striped mice with patent infections. 30 cu.mm. samples of blood were examined for microfilariae using the counting chamber technique described in Chapter 8.

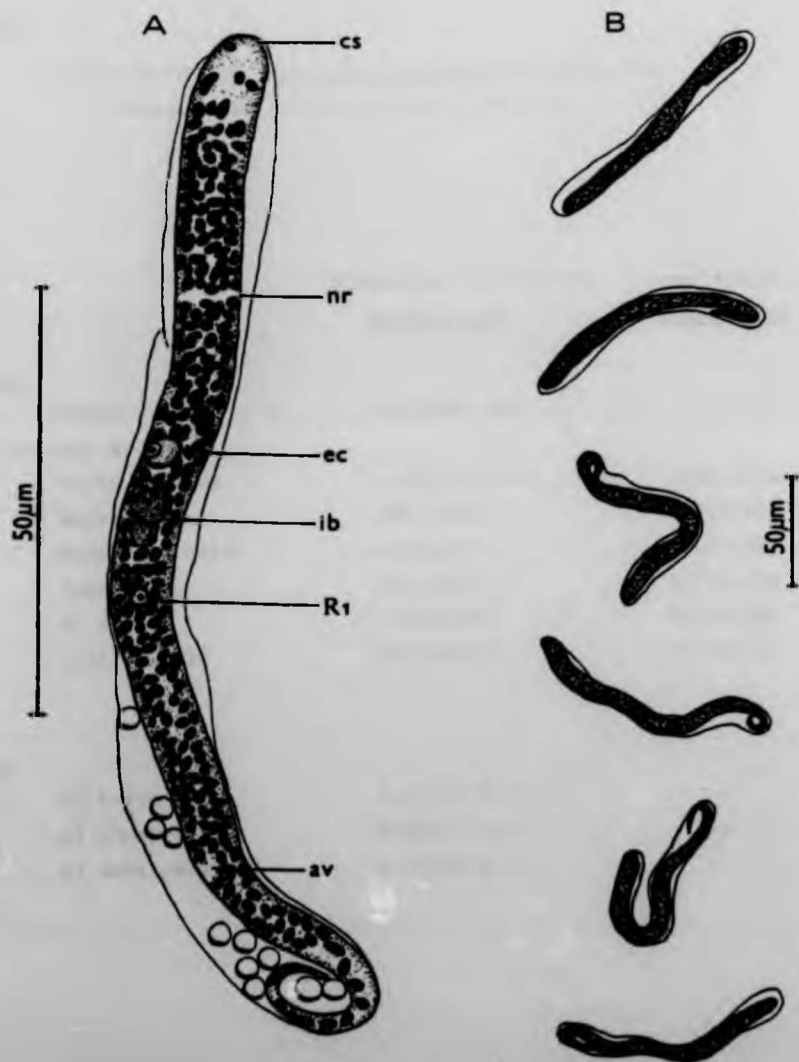
No microfilariae were found in the blood of jirds and the microfilaraemias in striped mice were extremely low. Four of 7 striped mice had between 4-15 microfilariae in 30 cu.mm. of heart blood, while 5 of 11 had from 1-4 microfilariae in an equivalent volume of tail blood. If the striped mice were anaesthetised with Penthrane, the microfilaraemias rose slightly so that 7 of 11 animals had between 2-12 microfilariae in 30 cu.mm. of tail blood.

### Morphology of skin microfilariae (Figure 9: Plate 13)

Microfilariae were extracted from host skin and fixed with methanol on microscope slides. These were stained with 10% Revector Giemsa stain, hot haematoxylin or alcian blue (Laurence and Simpson, 1968). Others were examined alive or fixed and cleared in 70% alcohol containing 10% glycerine.

The major measurements of microfilariae are given in Table 14. Skin microfilariae were of similar length to those from the blood and measured 139 (130-150) microns long. The microfilariae of A. globulosa are rather sluggish compared with those of most other filarial species and are invested in a loose fitting sheath in which the tail is usually doubled

**FIGURE 9**    THE MICROFILARIA OF *ACKERTIA GLOBULOSA*



**A** Details of the microfilaria seen in Giemsa stained specimens.  
**B** Attitudes assumed by microfilariae fixed with methanol on microscope slides.

**TABLE 14**

Measurements of Ackertia globulosa microfilariae  
based on 24 giemsa stained specimens.

	Dimension in microns	% of total length
	<u>Mean(range)</u>	<u>Mean(range)</u>
<u>LENGTHS</u>		
Total	139(130-150)	--
Anterior end to:-		
First nucleus	1.8(0.68-3.4)	1.3(0.5-2.4)
Nerve ring	28(21-34)	20(15-25)
Excretory cell	45(34-51)	32(25-37)
Inner body	53(45-62)	38(31-45)
R1 cell	75(58-82)	54(42-60)
Anal vesicle	98(72-123)	71(50-86)
<u>WIDTHS</u>		
At nerve ring	6.5(5.5-7.9)	--
At inner body	6.1(4.8-7.2)	--
At anal vesicle	4.8(3.4-5.5)	--

back. The sheath stains a bright pink in Giemsa, but may not be visible when haematoxylin is used. Within the sheath are 10-12 refractile globules which are best visualised in living microfilariae or those fixed in alcohol-glycerine. In these specimens the cephalic hook is prominent (Plate 14) but it may also be seen in microfilariae stained with alcian blue. Microfilariae fixed on slides usually assumed undulating bends or straightened out but some lay in a single, graceful curve (Figure 9 B). Those stained in Giemsa showed most features except the hook,  $R_2$ ,  $R_3$  and  $R_4$  cells and occasionally the globules. The excretory cell stained as a granular, ovoid body with a prominent nucleus and nucleolus and the inner body as a rectangular space with 1 or 2 large red globules. In many Giemsa stained microfilariae the  $R_1$  cell could be clearly seen as a large cell with filamentous cytoplasmic extensions. The nerve ring and anal vesicle appeared as clear spaces within the nuclear column. The tail was pointed and in its terminal section contained a column of 9-10 nuclei which rarely extended to the tip. Cuticular striations were evident in many specimens, especially on the tail.

Further details of the morphology of A. globulosa microfilariae are included in Chapter 5, where a full description has been given of the exsheathed microfilariae from the gut of the vector.

#### Spatial distribution of A. globulosa microfilariae in the skin of rodents

Two skin biopsies were taken from the ears, head, neck, back, fore-legs, hind-legs, belly, nose and tail of 7 naturally infected striped mice and 2 experimentally infected jirds and the mean microfilarial density at each site calculated. In skin-snips from the ears and legs

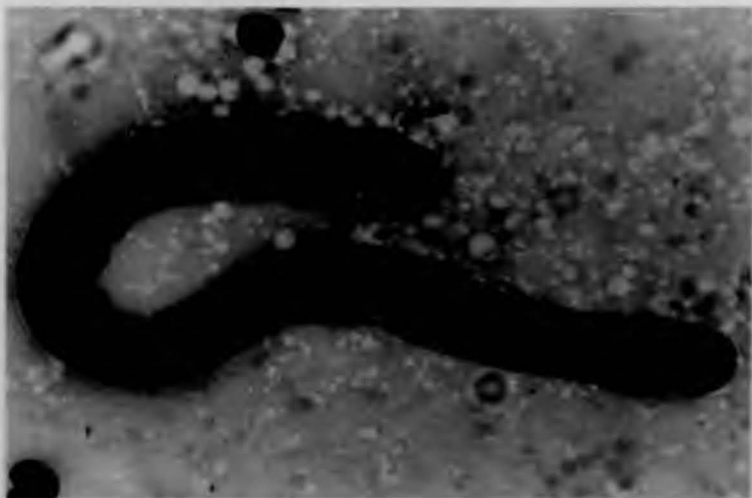
10  $\mu$ m

Plate 13. The microfilaria of A. globulosa extracted from host skin and stained with Giemsa.

10  $\mu$ m

Plate 14. Anterior end of a living microfilaria of A. globulosa showing the large cephalic hook.

it was found that the distribution of microfilariae was bilaterally symmetrical, so the results of biopsies taken from both sides of the body were pooled. The spatial distribution of microfilariae in striped mice is given in Table 15, and for jirds in Table 16.

In both species of rodent, microfilariae showed a highly distinctive distribution pattern in the skin with a marked concentration in the ears. The distribution of microfilariae in various regions of the body was constant, irrespective of the intensity of infection. Similar patterns of microfilarial distribution were seen in striped mice which had densities of microfilariae in ear skin ranging from 24-205 microfilariae per milligram (mff/mg). These animals had a mean of 93 mff/mg in the ear compared with only 14 mff/mg on the back, 12 mff/mg on the head and 10 mff/mg on the neck: In other regions of the body levels were even lower with an average of 2-4 mff/mg in the skin of the tail, belly, hind-legs, fore-legs and nose.

Densities of microfilariae in the skin of jirds were extremely low but a similar distribution pattern was found to that in striped mice. In J13, microfilarial levels were 14.2 mff/mg in the ears, 2.8 mff/mg on the head and 1.9 mff/mg on the back; levels in the rest of the body were below 1 mff/mg. In J5, ear skin contained 4.6 mff/mg and levels were below 1 mff/mg elsewhere in the body. However, the pattern of microfilarial densities was similar to that in J13 with head skin as the second most favoured site (0.7 mff/mg).

Relationship between the distribution of microfilariae and the attachment sites of the tick vector

In order to see whether the distribution of microfilariae in the skin of striped mice was related to enhancing their transmission to the

**TABLE 15**

Estimated numbers of microfilariae per milligram of skin from various regions of L.striatus naturally infected with A.globulosa.

ANIMAL	EARS	BACK	NECK	HEAD	NOSE	FORE-LEGS	HIND-LEGS	BELLY	TAIL
AA01	117	22	13	15	5	4	3	3	2
AA02	189	27	21	22	7	5	5	6	2
AA03	34	6	4	6	2	2	1	2	1
AA04	54	9	8	6	3	3	2	2	2
AA05	27	5	3	4	1	2	0	0	1
AA06	205	26	21	24	10	7	8	6	4
AA07	24	4	3	4	0	2	1	0	2
MEAN	93	14	10	12	4	3	3	3	2



**TABLE 16**

Estimated numbers of microfilariae per milligram of skin from various regions of M.unquiculatus experimentally infected with A.globulosa.

ANIMAL	EARS	BACK	NECK	HEAD	NOSE	FORE-LEGS	HIND-LEGS	BELLY	TAIL
J5	4.57 (100%)	0.22 (5%)	0.30 (7%)	0.73 (16%)	0 (0%)	0.22 (5%)	0.10 (2%)	0.05 (1%)	0 (0%)
J13	14.20 (100%)	1.94 (14%)	0.70 (5%)	2.82 (20%)	0.73 (5%)	0.69 (5%)	0.54 (4%)	0.13 (1%)	0 (0%)

Percentage of maximum in parenthesis

vector, the distribution of attachment sites for H. leachii nymphs on the body of these rodents was determined.

In nature, the juvenile stages of H. leachii attach to their murine hosts in the confines of the nest (Hoogstraal, 1956). To simulate this, approximately 80 nymphs were placed in a small glass jar that had been loosely filled with grass bedding and fitted with a nylon gauze top. Half an hour was allowed for ticks to disperse in the "nesting chamber" and a striped mouse, which had been lightly anaesthetised with Penthrane, was placed inside. Rodents were left in the nesting chamber for 6 hours. They were then removed to a wire-mesh cage over a water surround and held for 24 hours so that the natural process of grooming could occur.

To assay the distribution of ticks on rodents, each animal was anaesthetised with an intraperitoneal injection of Nembutal and a search made of the entire body surface under a dissecting microscope. The anatomical site of each attached tick was recorded. The results of 5 separate infestations with ticks were pooled and are given in Table 17.

TABLE 17

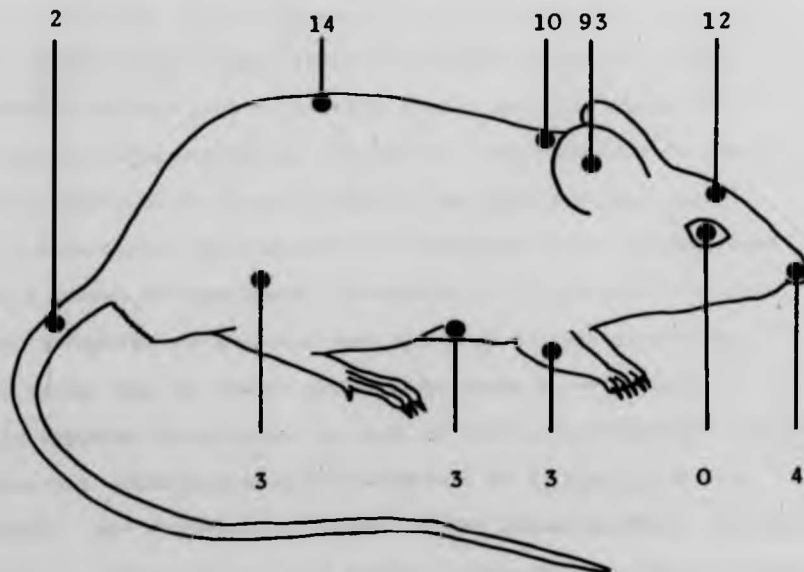
Number of ticks recovered from various regions  
of striped mice (% total recovery)

<u>Anatomical site</u>	<u>Dorsal surface</u>	<u>Ventral surface</u>
Ears	80 (23)	-
Head	214 (62)	0
Neck	48 (14)	0
Torso	4 (1)	0
Fore-legs	0 -	0
Hind-legs	0	0
Tail	0	0
Genitals	-	0
Totals	346 (100)	0
Grand Total		346

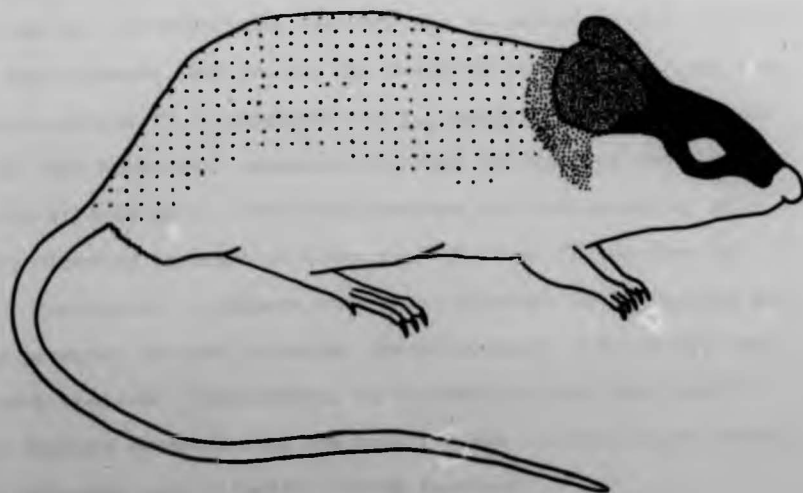
As can be seen from Table 17, ticks attached exclusively to the dorsal aspect of the host, concentrating on the head, ears, and neck. Despite the large surface area afforded by the torso, few ticks attached to this site.

The distribution of tick attachment sites and of microfilariae over the body of striped mice are compared in Figure 10. Attaching ticks concentrated solely on the dorsal surface of the host, corresponding with the regions of highest microfilarial density in infected rodents. However, the correlation between tick and microfilarial distributions was not perfect as microfilariae showed a strong predilection for the ears which were only the second most favoured site of attaching ticks.

**FIG. 10** MEAN NUMBERS OF MICROFILARIAE PER MILLIGRAM OF SKIN  
FROM DIFFERENT REGIONS OF L. STRIATUS NATURALLY  
INFECTED WITH ACKERTIA GLOBULOSA (SEVEN ANIMALS).



DISTRIBUTION OF ATTACHMENT SITES FOR H. LEACHII  
NYMPHS ON L. STRIATUS (FIVE ANIMALS).



Intensity of shading expresses percentage of ticks  
attached at each site.

Nevertheless, a comparison of the distribution patterns of microfilariae and feeding ticks by Spearman's rank correlation coefficient revealed a highly significant association between them ( $P = 0.001$ ).

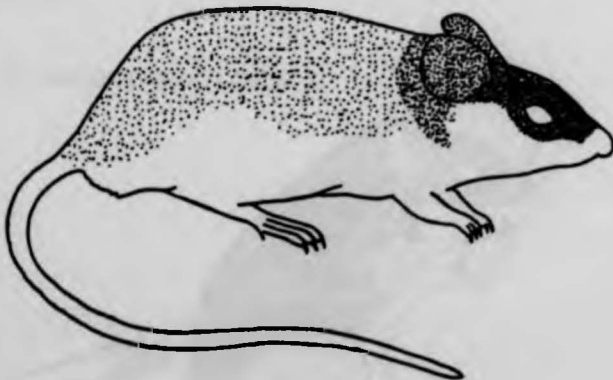
A brief study was made of possible mechanisms that might direct ticks to their favoured sites of attachment. The influence of light and shaded regions on the host, access to the skin surface, and gravity in determining the selection of attachment sites was assessed in a simple series of experiments illustrated in Figure 11. The procedure for infesting striped mice was identical to that previously described except for the single modification made in each trial.

It is apparent from Figure 11, that of the factors examined, only gravity has any significance in the behaviour of H. leachii on its murine hosts. The attachment of nymphs to the dorsal surface of rodents is therefore ascribed in part to a strong, negatively geotactic response in the unfed tick, a feature long recognised in questing ixodids (Arthur, 1962).

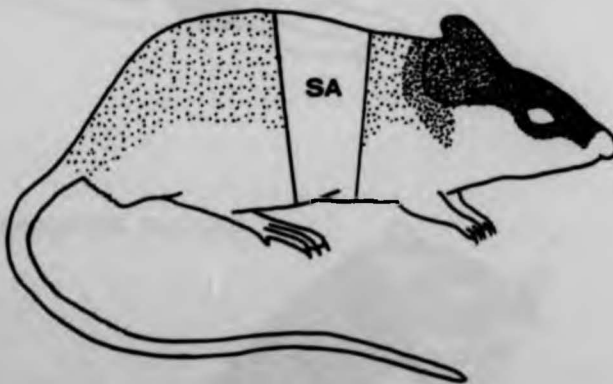
The selection of attachment sites by H. leachii was also assessed on an "abnormal" host species, Meriones unguiculatus. Three infestations were carried out in which jirds were allowed to groom and 3 in which no grooming was allowed. The results are depicted in Figure 12. On this second host species it is apparent that H. leachii showed a different pattern of site selection, concentrating less on the ears and head on jirds as on striped mice. Under the pressure of host grooming, an anteriorly directed movement of ticks from the back to the neck was observed. Therefore, it appears that site selection by H. leachii may partly be governed by host grooming, the efficiency of which may vary between host species. Furthermore, it is possible that more subtle extrinsic factors determined by the host species to which ticks attach may also influence site selection during feeding.

**FIG. 11** DISTRIBUTION OF ATTACHMENT SITES FOR H. LEACHII NYMPHS ON L. STRIATUS AFTER EXPOSURE TO INFESTATION UNDER VARIOUS CONDITIONS

IN TOTAL DARKNESS



WITH DIRECT ACCESS TO SKIN SURFACE VIA SHAVED AREA (SA)



WITH HOST SECURED IN SUPINE POSITION



Intensity of shading expresses % of ticks at each site

DISTRIBUTION OF ATTACHMENT SITES FOR H. LEACHII NYMPHS  
ON M. UNGUICULATUS

A

WITH NO GROOMING ALLOWED



B

WITH GROOMING ALLOWED



Intensity of shading expresses percentage of ticks  
attached at each site

Relationships between the location of microfilariae within the skin,  
the mode of tick feeding, and the ingestion of microfilariae

Histological sections of skin from the ears and head of 9 naturally infected striped mice and 2 experimentally infected jirds were examined. Skin was taken at autopsy, fixed in 10% formol saline, and routinely processed, sectioned and stained in haematoxylin and eosin.

In transverse sections of skin, microfilariae showed no affinity for particular structures such as hair follicles, nor were they restricted to any given depth. Microfilariae were common in both the dermis (Plate 15) and adipose tissue (Plate 16) of ear skin but occurred mainly in the dermis of head skin. Occasionally, sections of microfilariae also appeared within the cartilaginous tissue of the ears. Their distribution in transverse sections taken across the length and breadth of the pinna was uniform and no concentration or clumping of microfilariae was seen in any region of the ear.

To observe the mode of tick feeding on the host, striped mice were infested on the ears with the larvae and nymphae of H. leachii and killed 42 hours later. The pinnae were immediately excised and dropped in liquid nitrogen with the ticks still attached. When the nitrogen had boiled off, the ears were transferred to fixative and processed for histological study using the techniques described in Chapter 5 for tick histology. If liquid nitrogen was not used, ticks survived long enough to detach in the fixative. Serial sections were cut through the points of tick attachments to the skin and stained with haematoxylin and eosin.

An examination of attached and unattached ticks revealed that in H. leachii the hypostome is short and cement is laid down during feeding to aid attachment. The nymphal hypostome measured 148 microns long and



30  $\mu$ m

Plate 15. *Microfilaria* of *A. globulosa* in the dermis of the ear of a naturally infected striped mouse. Note the absence of local tissue reaction ( in contrast with Plate 34 ).

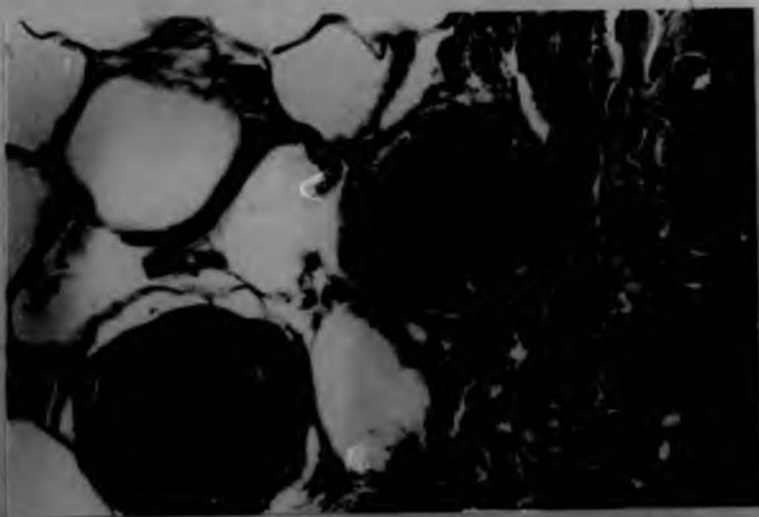
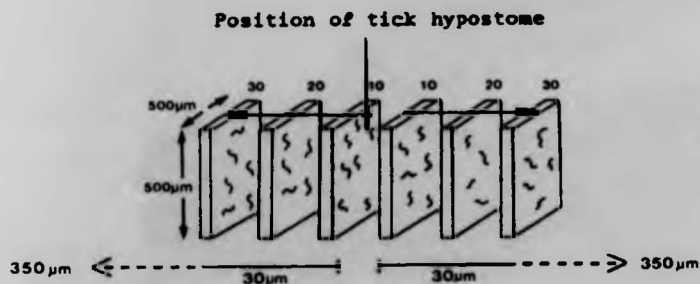
30  $\mu$ m

Plate 16. *Microfilaria* of *A. globulosa* in the adipose tissue of the ear of a naturally infected striped mouse.

that of the larva was 56 microns long. While the hypostome of nymphal ticks penetrated host skin deep into the reticular layer of the dermis, larval attachment was superficial and the mouthparts only reached the upper layers of the dermis. Nevertheless, there was an extensive zone of haemorrhage, oedema, necrosis and cellular infiltration in host skin (Plate 17) beneath the mouthparts of both the larvae and nymphae, extending through the dermis to underlying adipose tissue. Sections of microfilariae were observed in the dermis adjacent to, and within, these foci of inflammation and further microfilariae were seen in the gut caecae of feeding ticks.

In the course of this work an attempt was made to quantify the distribution of microfilariae in the region of tick attachment sites. Three striped mice, with microfilarial densities in ear skin of 6-7 mff/mg were infested with a single nymph on each ear. The excised pinnae were processed as described above and serial sections cut at 10  $\mu$ m thickness through the point of tick attachment. These were numbered with care so that the precise distance of each skin section from the site of the embedded hypostome could be calculated. The method used to estimate the distribution of microfilariae in the skin in the region of tick attachments is illustrated in Figure 13.

**FIGURE 13** Method used to estimate the distribution of microfilariae in the region of tick attachment sites : Number of portions of microfilariae in each skin section counted



The number of portions of microfilariae in each skin section was counted within an area of  $500 \times 500 \mu\text{m}$  (i.e.  $0.25 \text{ mm}^2$ ) for a total of 70 sections cut in a strip through the position of attachment (i.e. a tract of skin  $350 \mu\text{m}$  long on either side of the mouthparts). The hypostome itself occupied a depth of one,  $10 \mu\text{m}$  section. Animals with low microfilarial densities were used to make it easier to assess any concentration of microfilariae beneath the mouthparts of the vector and only a single nymph was allowed to attach to each ear so that the distribution of microfilariae in the skin would not be influenced by neighbouring ticks.

The results of examining the distribution of microfilariae around the attachment sites of 5 individual ticks are given in Figures 14 and 15. In 3 cases, a marked concentration of microfilariae was observed

200  $\mu$ m

Plate 17. Nymph of H. leechii attached to the ear of an infected striped mouse. Note the marked infiltrate of inflammatory cells beneath the mouthparts.

30  $\mu$ m

Plate 18. Accumulation of microfilariae of A. diambloana beneath the attachment site of H. leechii.

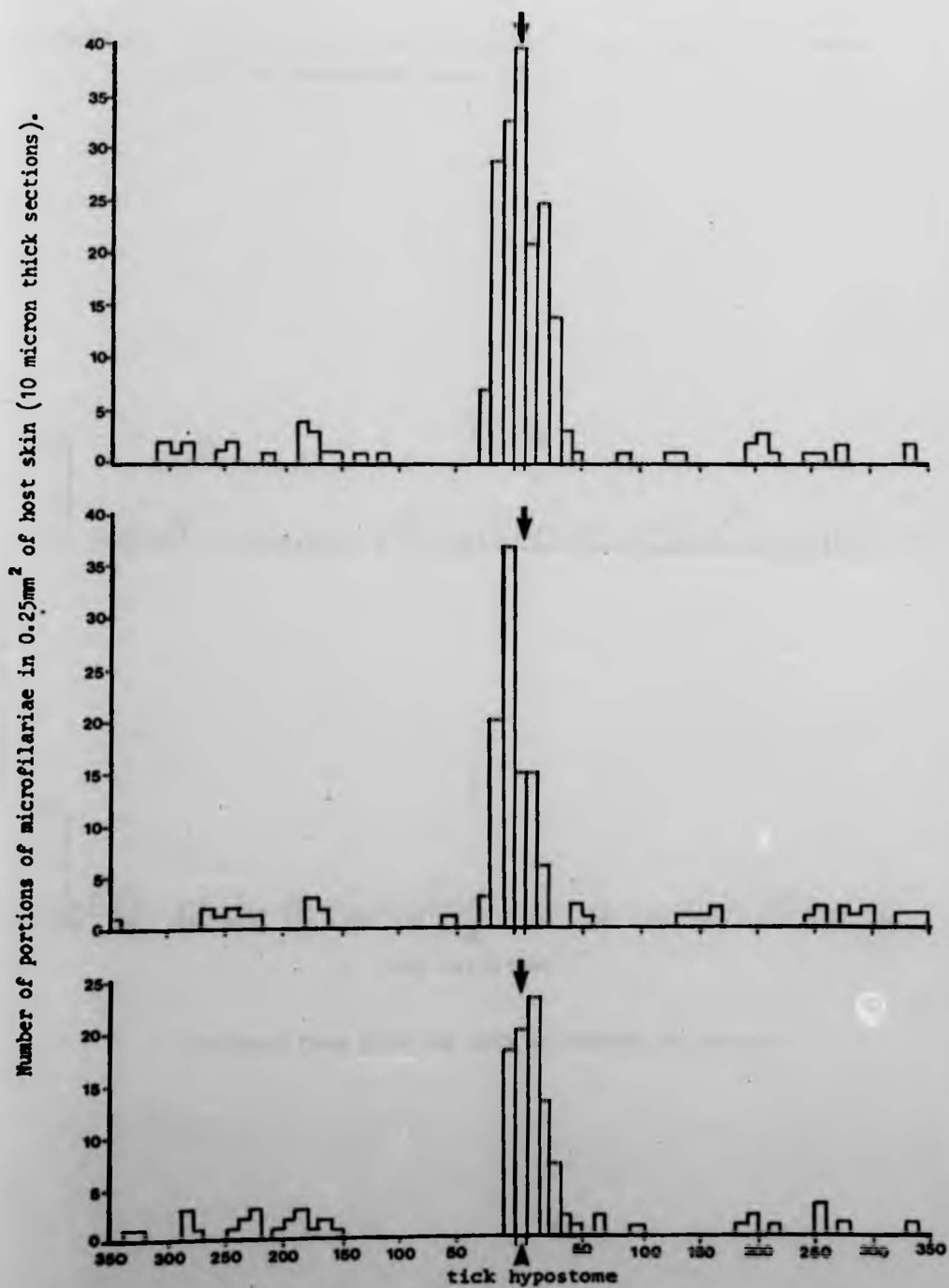
beneath the mouthparts (Figure 14; Plate 18). 75 (69-81)% of the total number of microfilarial portions recorded in a strip of skin 710 microns long were found within 30 microns on either side of the tick attachment site. In the 2 remaining cases, microfilariae were uniformly distributed in the region of attachments (Figure 15).

The technique used here to quantify the distribution of microfilariae around feeding ticks incurred 2 main sources of error. Despite the use of animals with low microfilarial densities it proved impossible to trace individual microfilariae in skin sections, so only portions were counted. This meant that many microfilariae would have been recorded more than once with the consequent exaggeration of their absolute number. However, if it is assumed that the orientation of individual microfilariae in the skin was random, then this error might be expected to apply equally to all of the skin sections and not distort the estimated distribution pattern of microfilariae around the mouthparts. In addition, no account was taken of the number of microfilariae ingested by ticks over the 42 hours prior to fixation since to do so would have meant relating absolute numbers of microfilariae in the vector with portions of microfilariae in the skin. This would undoubtedly have led to an underestimate of the power of feeding ticks to concentrate microfilariae in the skin.

Nevertheless, there was good evidence of a striking accumulation of microfilariae beneath the mouthparts of 3 feeding ticks (Figure 14). This was an exceptional observation, since no clumping of microfilariae was seen in hundreds of skin sections taken where no ticks had attached (see earlier in this chapter).

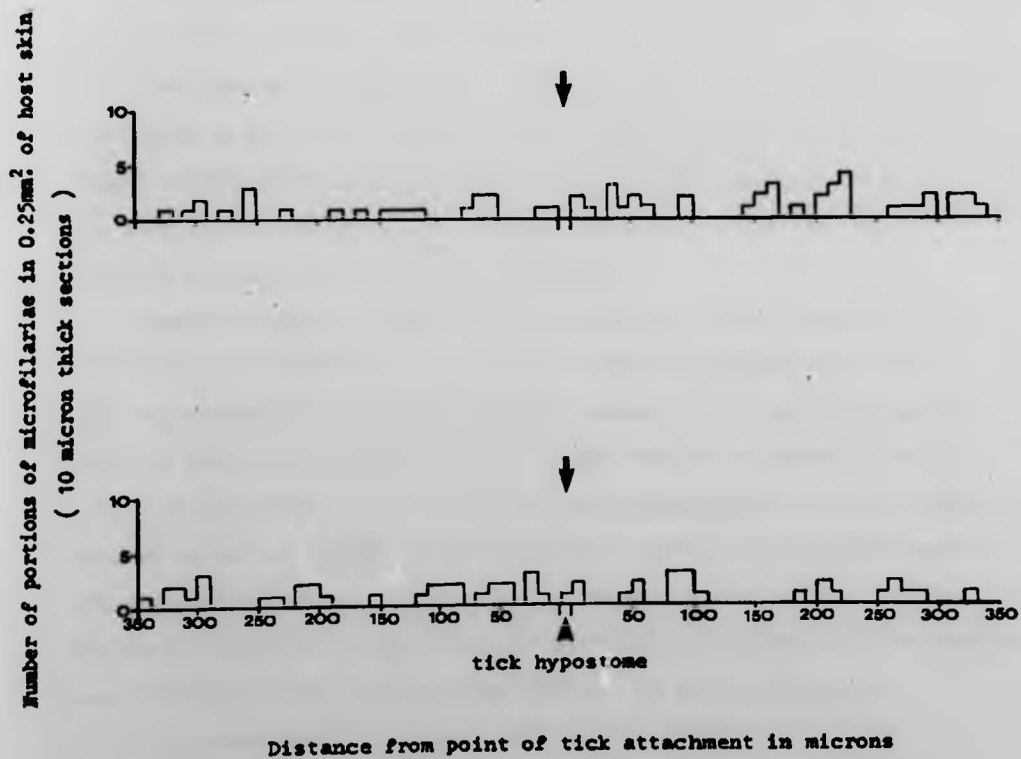
**FIGURE 14**

Distribution of microfilariae in host skin in the region 132 of tick attachment sites (↓).



Distance from point of tick attachment in microns.

**FIGURE 15** Distribution of microfilariae in host skin in the region of tick attachment sites (↓).



Recovery of adult worms and microfilariae from striped mice naturally infected with *A. globulosa*

Seven striped mice trapped at Machakos town were autopsied shortly after capture. The results of this work, in terms of adult worm recovery and microfilarial density in ear skin, are summarized in Table 18.

All 7 animals harboured adult worms which were recovered exclusively from the lungs. In most rodents these comprised a similar number of males and females. A mean of 6.9 (3-11) worms were collected from each host, made up of 3.7 (2-6) males and 3.1 (1-6) females. All of the female worms contained embryonated eggs and microfilariae and all of the rodents had patent infections, with a mean of 93 (24-205) microfilariae per milligram (mff/mg) of ear skin.

From the results in Table 18, a trend appears which suggested that the level of microfilariae in the skin of naturally infected striped mice was related to the number of adults present, and that high microfilarial densities (greater than 100 mff/mg) may be attained in the ear with 4 or more worm pairs. To confirm this observation, the relationship between adult worm burden and microfilarial density in respective animals was assessed by the coefficient of correlation. This was done by comparing the total worm burden with the microfilarial density and the female worm burden with the microfilarial density. In both instances, a directly proportional relationship was found to exist ( $r = +0.89$ ,  $0.001 < P < 0.01$ ). Therefore, it appears that under conditions of natural transmission, the number of adult worms or of fertile females in the host may play a significant role in determining the density of microfilariae in the skin.



**TABLE 18**

Relationship between worm load and microfilarial density  
in ear skin of L.striatus naturally infected with A.globulosa.

ANIMAL	MICROFILARIAL DENSITY (mf/mg)	ADULT WORM RECOVERY		
		Female	Male	Total
AA06	205	6	5	11
AA02	189	4	5	9
AA01	117	4	4	8
AA04	54	2	6	8
AA03	34	3	2	5
AA05	27	2	2	4
AA07	24	1	2	3
MEAN	93	3.1	3.7	6.9

The course of microfilarial densities with time

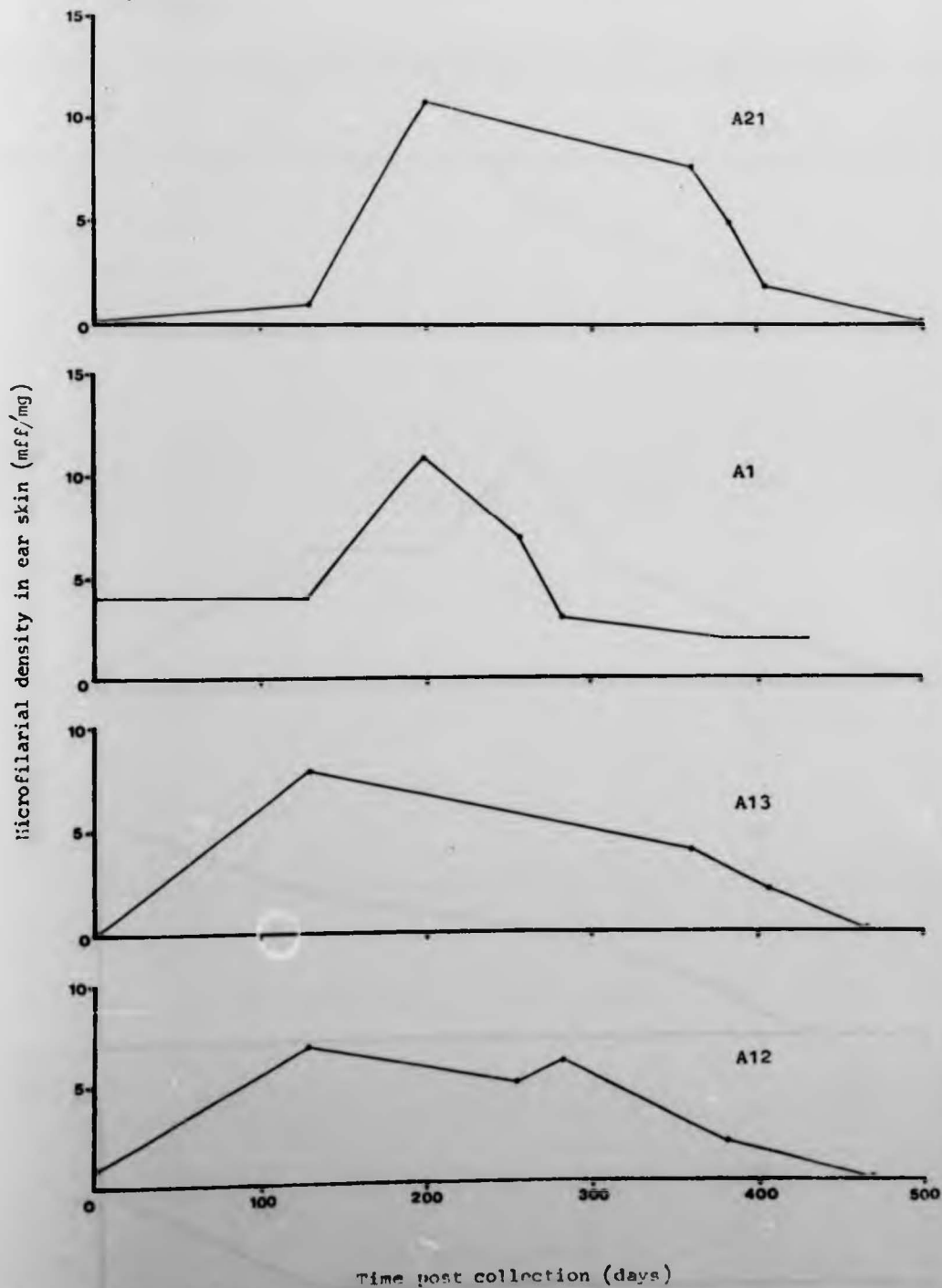
The course of microfilarial densities in ear skin were studied in 14 striped mice and 2 African gerbils (Tatera robusta) for over 400 days. These animals were naturally infected with A. globulosa and all but 2 had already developed patent infections at the time of collection. Rodents developed light, moderate or heavy infections and have been grouped accordingly in Figures 16, 17 and 18 for striped mice and Figure 19 for African gerbils.

The course of microfilarial densities in gerbils did not differ from those in striped mice but only low levels were attained (9 and 7 mff/mg) in the former host species. Fourteen of 16 striped mice and gerbils showed a rising level of microfilariae while in the laboratory, which in 12 of these rodents reached a peak after 100-200 days followed by a decline over the next 300 days. Peak microfilarial levels were between 7-417 mff/mg. Despite this wide range of values, 15 of 16 rodents maintained patent infections for over 400 days and microfilariae disappeared from the skin 400-500 days after their arrival in the laboratory. There appeared to be no seasonal influence on the numbers of microfilariae in the skin.

The 2 main courses of microfilarial density in rodents are typified by those of striped mice A11 and A14 (Figure 18). In A11, the density of microfilariae climbed to a peak by day 129 and was followed by a progressive decline over the next 370 days. In A14, the microfilarial density climbed to a peak by day 129 but was followed by a sharp decline over the ensuing 70 days: Microfilarial levels then stabilised for 103 days and gradually diminished over the final 139 days of observation. In both animals, low residual microfilarial densities persisted for around 70 days at the end of the patent period.

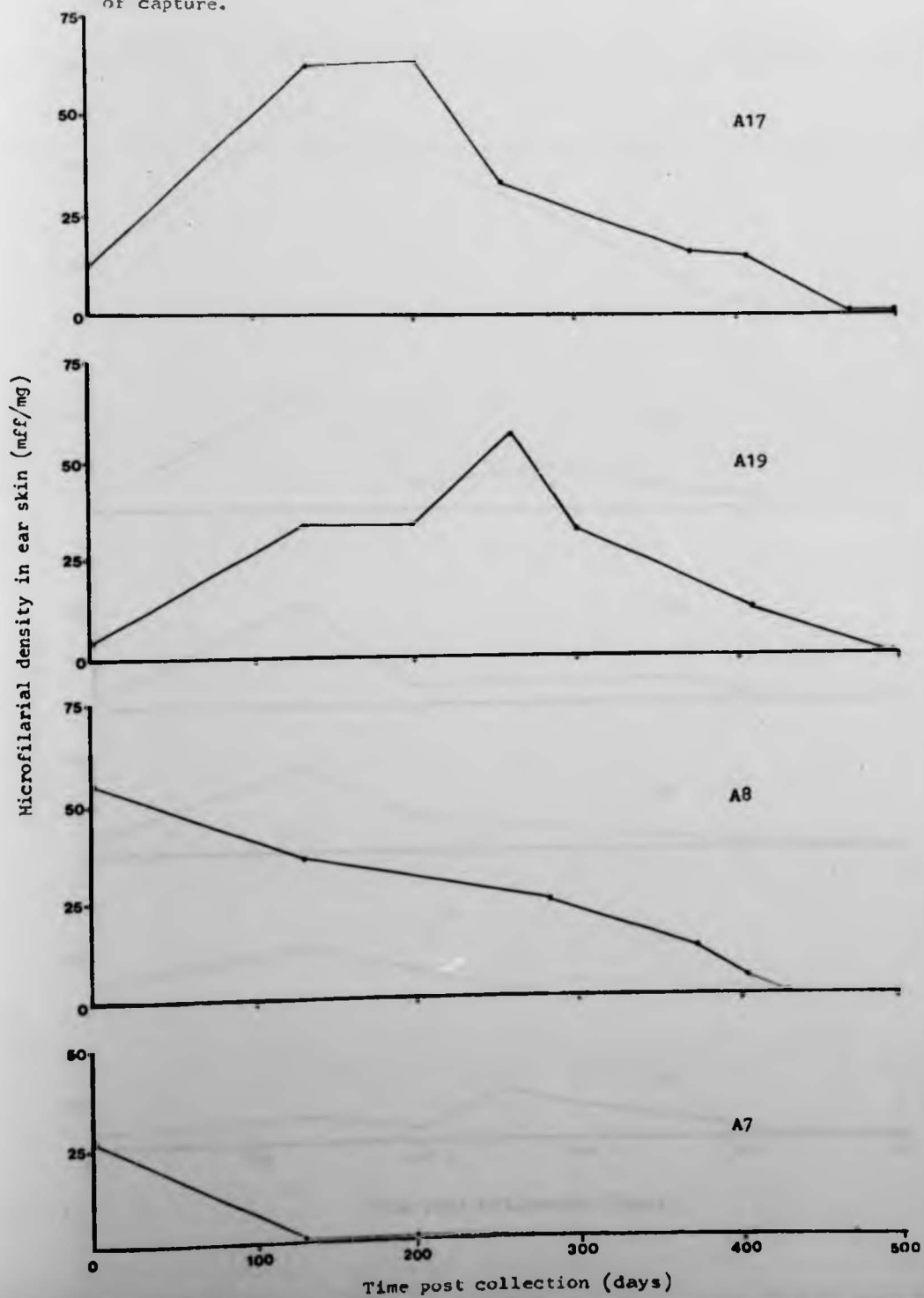
**FIGURE 16**

Course of microfilarial densities in the ear skin of *L. striatus* naturally infected with *A. globulosa*. Readings taken from time of capture.



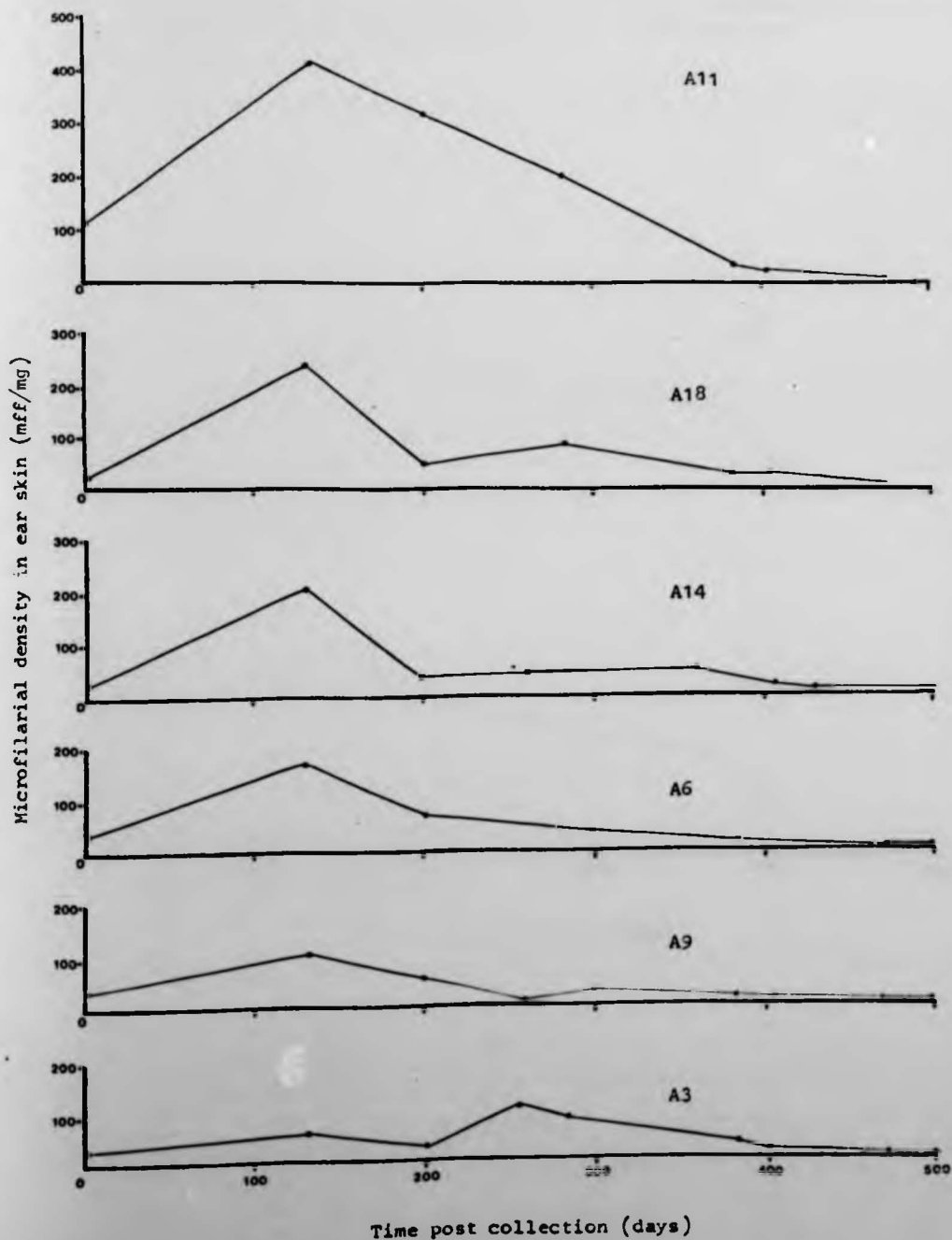
**FIGURE 17**

Course of microfilarial densitie in the ear skin of *L. striatus* naturally infected with *A. globulosa*. Readings taken from time of capture.



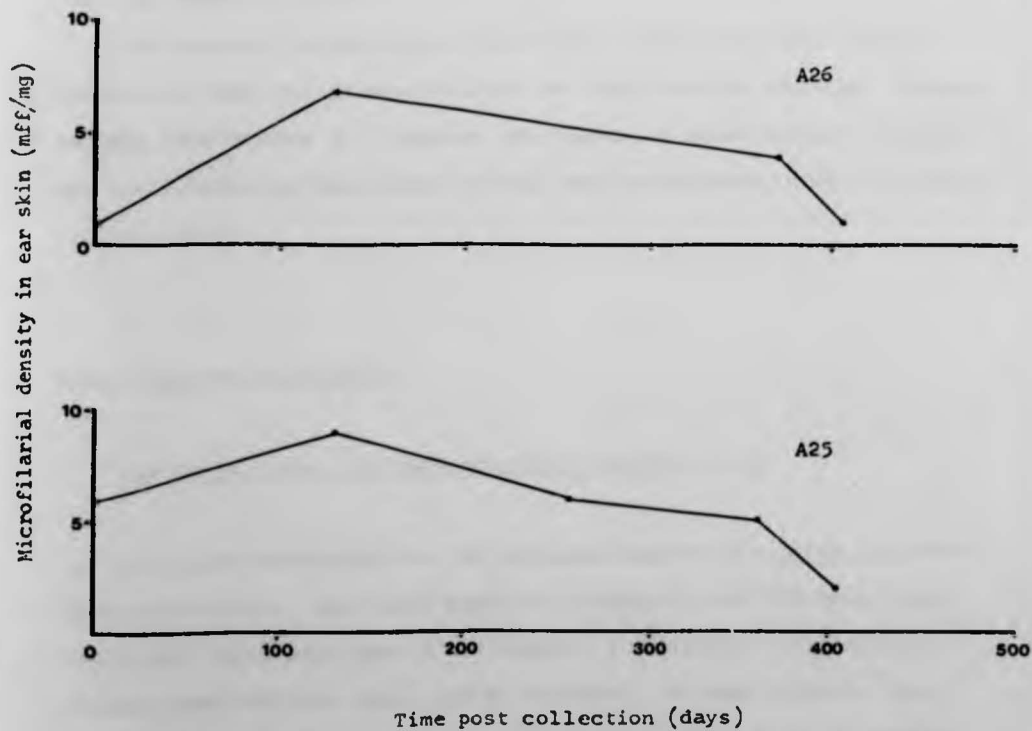
**FIGURE 18**

Course of microfilarial densities in the ear skin of L.striatus naturally infected with A.globulosa. Readings taken from time of capture.



**FIGURE 19**

Course of microfilarial densities in the ear skin of *T. robusta* naturally infected with *A. globulosa*. Readings taken from the time of capture.



Two rodents developed patent infections some weeks after capture (A13 and A21) and acquired only low microfilarial densities whilst in the laboratory (8 and 11 mff/mg respectively). In these cases, it seems likely that the animals were collected after a relatively short period of exposure to transmission. The 2 striped mice in which microfilarial levels steadily declined from the time of capture (A7 and A8) were probably older animals that had been exposed to infection earlier than the remaining stock.

The course of microfilarial densities in experimentally infected rodents was similar, if more erratic, to that found in naturally infected rodents (see Chapter 3). However, the levels of microfilariae attained, and the duration of the patent period, were far greater in most naturally infected hosts.

#### Host responses to infection

##### Eosinophil counts in experimentally infected jirds

Six jirds inoculated with 50 infective larvae of A. globulosa were bled at intervals from 1 week prior to infection until 105 days after infection, and counts made of the number of circulating eosinophils. Three uninfected jirds were used as controls. On each occasion, the animals were bled from the tail between 14.00 - 15.00 hours to reduce possible errors due to diurnal fluctuations in eosinophil levels (Archer, 1963; Basten et al., 1970). Blood samples were drawn up into a white cell counting pipette and diluted 1 in 20 with Discombe's diluting fluid, containing 5 volumes of 1% aqueous eosin Y, 5 volumes of acetone and 90 volumes of distilled water. Only freshly prepared diluent was used.

Cells were counted in a "Bright line" improved Neubauer haemocytometer under the X 100 magnification of a compound microscope. The total number of eosinophils in the 8 corner squares of the chamber were counted and multiplied by 25 to give the number of eosinophils in 1 cu.mm. of blood.

### Results

Only 4 of the 6 infected jirds developed patent infections so eosinophil counts recorded from the 2 remaining animals were excluded from the interpretation of results.

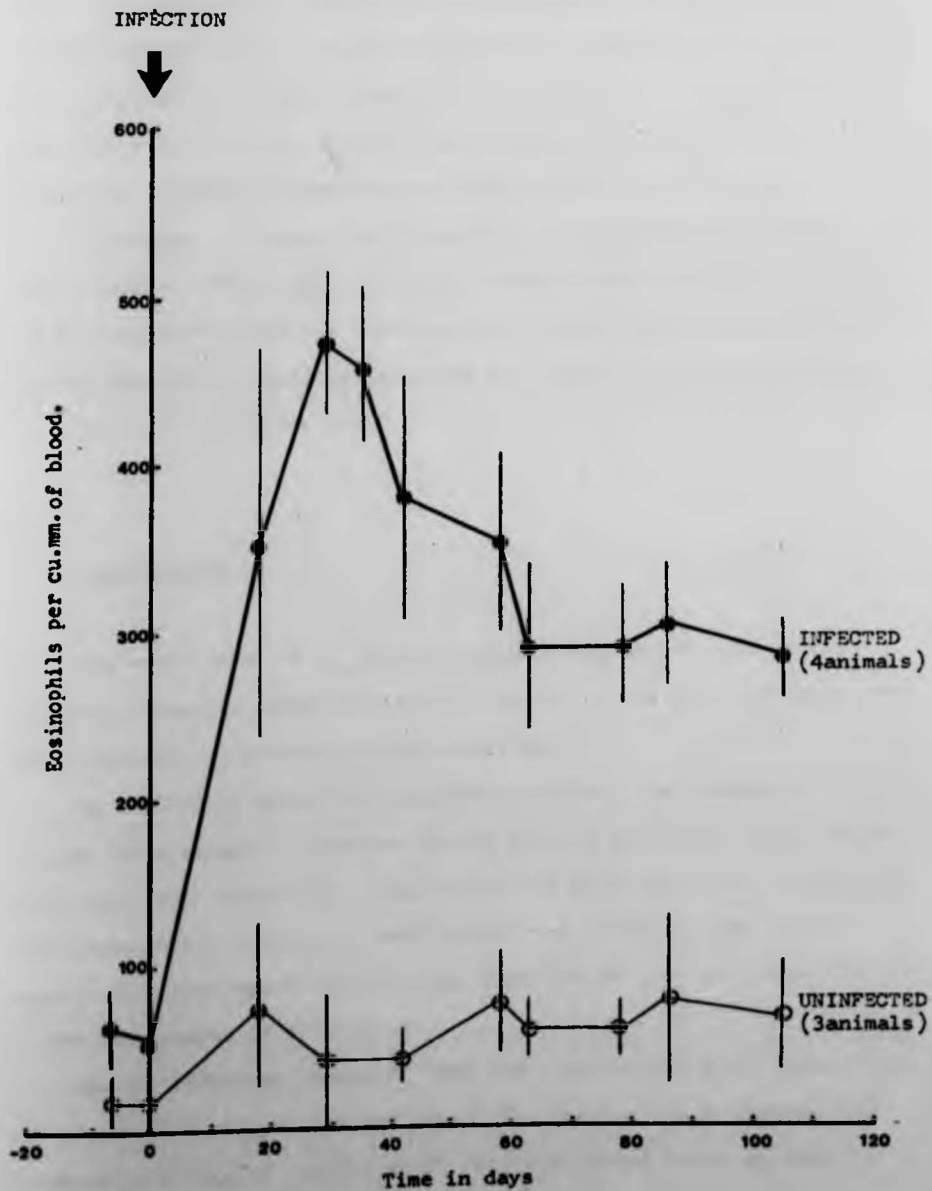
As shown in Figure 20, jirds infected with A. globulosa showed a significant rise in the number of circulating eosinophils, while the counts in the control group remained at a constant, low level. Eosinophilia developed within 18 days of infection and reached a peak cell count of 469 per cu.mm. of blood by 29 days post infection. The level of eosinophils then declined until day 63 when their number stabilised at between 281-300 per cu.mm. of blood for the remaining period of observation. The cell count in control animals varied between 17-75 per cu.mm. of blood.

Although the level of eosinophils attained in infected animals was not high, peak eosinophilia coincided with the timing of the final moult of A. globulosa (day 25). The onset of patency in these animals between 60-80 days post infection did not, however, give rise to an increased level of circulating eosinophils.



**FIGURE 20**Eosinophil counts in jirds infected with A.globulosa.

Mean values in peripheral blood shown with 95% confidence limits.



### Adult worms in the definitive host

As experimentally infected rodents developed only light infections (see Chapter 3), histological observations on adult worms in the definitive host were made on naturally infected animals which were autopsied at various times after capture. Four striped mice were autopsied after 50 days, 3 after 225 days and 4 after 500 days. An uninfected, laboratory bred striped mouse was used as a control.

On autopsy, the heart and lungs were removed and fixed in 10% formal saline. These were processed, vacuum embedded in paraffin wax, and sectioned at 5 microns thickness for routine staining with haematoxylin and eosin. Selected sections were stained with methyl green and pyronin Y for plasma cells.

### Observations

The adult worms of A. globulosa were found exclusively in the pulmonary arteries and arterioles (Plate 19) in the lumen of large and small vessels, unattached to the vessel wall.

In the "early stage" of infection (day 50), the presence of worms in the lungs caused no apparent damage and did not elicit any histological reaction (Plate 19). Lung structures were normal and no cellular infiltration was evident. Female worms were often seen in close association with males and had uteri packed with eggs and microfilariae at various stages of development.

As the infection "matured" (day 225), worms provoked 2 major forms of reaction in the parasitised lung. The first was a widespread and intense perivascular cuffing which occurred around large and small

100  $\mu$ m

Plate 19. An adult female of A. globulosa in the pulmonary artery of a naturally infected striped mouse.

50  $\mu$ m

Plate 20. Perivascular cuffing of a pulmonary arteriole containing a living adult of A. globulosa. The cellular infiltrate consists mainly of plasma cells together with lymphocytes and eosinophils.

vessels containing living worms (Plate 20). This consisted of a heavy cellular infiltration made up predominantly of plasma cells, with lymphocytes and some eosinophils. Cellular infiltrations in the lung were restricted to the arterial system and were not found around bronchioles. Where cuffing occurred around an artery in close proximity to a bronchiole, this was discontinuous in the region adjacent to the airway. Perivascular cuffing was widespread, but in histological sections was most intense around vessels containing sections of worms. There was also a mild, monocytic infiltration of the pericardium made up mainly of plasma cells.

The second form of reaction in parasitised lungs was a hypertrophy of the vessel walls. This was invariably associated with perivascular cuffing, being restricted to regions where worm sections were evident. The worms, like others recorded on day 225, appeared morphologically normal. In the larger arteries and arterioles only a slight thickening of endothelial cells was observed. However, reactions were most severe in the small arterioles at the periphery of the lung where the vessel walls showed a gross thickening due to fibrosis of the intima (Plate 21). Nevertheless, even the most intense wall reactions did not fully occlude the vessel lumen.

In longstanding infections that had terminated naturally (day 500), granulomatous reactions were seen around dead parasites that occluded the vessel lumen (Plate 22). The morphological integrity of worm structures had broken down so that parasites appeared only as amorphous bodies surrounded by a heavy infiltration of cells. The obliterative granuloma consisted of multinucleated foreign body giant cells, macrophages and neutrophils. Much of the surface of dead parasites was coated with multinucleated foreign body giant cells. The presence of thrombin within the affected vessels indicated a cessation of blood flow which had not

30  $\mu$ m

Plate 21. Fibrosis of the intima of a pulmonary arteriole containing a living adult of A. globulosa. Perivascular cuffing is also evident around this vessel.

50  $\mu$ m

Plate 22. Granulomatous reaction containing a pulmonary arteriole containing a dead and degenerating adult of A. globulosa. The reaction is composed of multinucleated foreign body giant cells, macrophages, neutrophils and fibroblasts.

been associated with earlier stages of infection. Surrounding the vessel remained a cuffing of plasma cells to which macrophages had been recruited.

In the most advanced condition, fibroblasts had entered the obliterated vessels and scar tissue formation had been initiated. Despite these gross pathological changes, only peripheral arterioles appeared to harbour dead parasites and were consequently the only vessels to be occluded. It therefore seems unlikely that the host response that provoked these changes caused any ill-effects in infected hosts. Certainly, infected striped mice remained active and healthy for the full period of their captivity.

#### Microfilariae in the definitive host

The distribution of microfilariae within the skin and over the body surface of striped mice and jirds has already been described. No pathological changes due to the presence of microfilariae in the skin were observed throughout the natural course of the patent period, either macro- or microscopically.

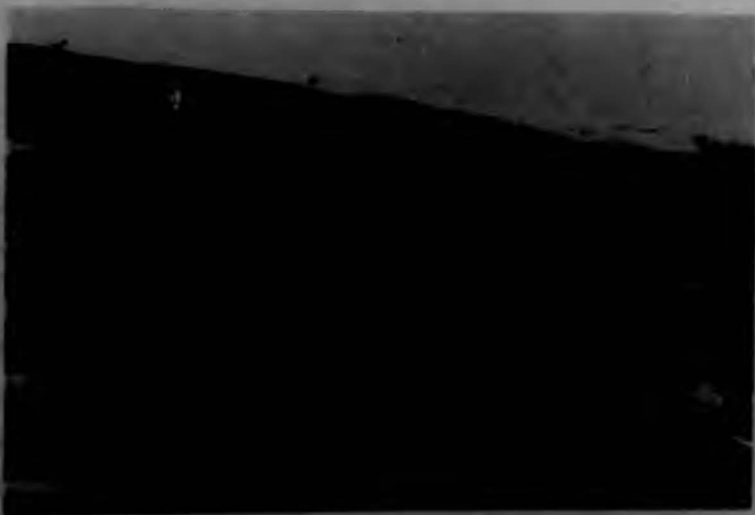
A histological examination of the eyes removed on autopsy from 6 naturally infected striped mice revealed the presence of microfilariae in the anterior segment in 4 of these animals. The eyes of 1 uninfected, laboratory bred striped mouse were examined as controls. The histological techniques used for the fixation and processing of eyes were identical to those used for ticks (described in Chapter 5).

In 3 striped mice, microfilariae were found in both eyes. Their number was extremely low and only 3.1 (1-5) microfilariae were detected in semi-serial sections taken of 7 "infected" eyeballs. Most microfilariae



30  $\mu$ m

Plate 23. *Microfilaria* of *A. globulosa* in the anterior sclera of the eye of a naturally infected striped mouse.



30  $\mu$ m

Plate 24. Degenerating *microfilaria* of *A. globulosa* in the cornea of a naturally infected striped mouse : Note the mild infiltration of plasma cells accompanying the parasite in the cornea.

were found in the anterior sclera (Plate 23), corneo-scleral envelope or sub-conjunctival epithelium. In these situations microfilariae provoked no host reaction and no damage could be ascribed to their presence in the tissues. However, 2 microfilariae were found in the cornea of one eye taken from a heavily infected striped mouse that had been autopsied 225 days "after collection". Here they had penetrated approximately 500 microns from the periphery of the cornea where their presence was accompanied by a localised monocytic infiltration of plasma cells (Plate 24). In 13 eyes where no microfilariae could be demonstrated in the cornea, no cellular infiltrations were observed. No microfilariae were found in other regions of the eye.

#### DISCUSSION

The early development of A. globulosa in jirds was marked by a rapid and widespread migration of larvae throughout the organs of the body, suggesting that infective stages may travel via a circulatory system in the host. Larvae were positively identified in the vascular system by day 10 when 2 worms were found in the right ventricle of the heart. At this stage some larvae had already reached the lungs. On 18 and 25 days post infection worms were found only in the peritoneal cavity. However, adults were recovered from the lungs at 80 and 105 days post infection where they occurred in pulmonary arteries, as observed by Muller and Nelson (1975).

The worm recoveries from experimentally infected jirds were too low for a detailed study of the route of invasion of A. globulosa. However, the distribution of larvae during development has features compatible with the migratory pathway described by Wenk (1967) for



Litomosoides carinii in cotton rats and laboratory mice. Wenk (1967) found that the infective larvae of L. carinii moved within 24 hours from their site of deposition in the skin to lymphatic spaces and whence to regional lymph nodes. From there, either directly or via other lymph nodes, larvae reached the vascular system and proceeded to the lungs via the right heart. In natural hosts, maximum invasion of the lungs occurred between 5-10 days post infection and larvae then migrated through the lungs to the pleural cavity. Wenk (1967) suggested that the few worms found in the peritoneal cavity of his animals had probably reached this site by leaving the abdominal lymph vessels. He also postulated that if the migratory pathway described for L. carinii larvae in the definitive host was paralleled by other filarial parasites, the site of adult worms would depend on how far along the route of invasion each species proceeded. If this contention is accepted, A. globulosa represents a species that follows the migratory pathway as far as the pulmonary arteries, and larvae found in the peritoneal cavity had presumably left this route of invasion prematurely.

The larvae of A. globulosa showed considerable development during the first 18 days in the definitive host but did not increase in length. The gross difference in size of worms between 18 and 25 days after infection suggests that growth was rapid after larvae had reached the peritoneal cavity, although it cannot be excluded that the worms may have been stunted during early development in jirds (an unnatural host). Nevertheless, the larvae of L. carinii also develop little while migrating through the host body and only start to mature on reaching the final site of development in the pleural or peritoneal cavities (Scott and Macdonald, 1953; Zein-Eldin, 1965). In contrast, the larvae of Conispiculum flavescens, Dirofilaria immitis and Breinvlia booliata mature in separate site from those occupied by the adult worms (Menon et al., 1944; Kume and Itagaki,

1955; Orhiel, 1961; Singh et al., 1976). Since the adults of A. globulosa were found only in the pulmonary arteries, it would be of interest to know whether larvae that enter the peritoneal cavity subsequently reach the lungs. An attempt to transplant fourth stage larvae (collected on day 25 post infection) into the peritoneal cavity of new hosts was unsuccessful as the worms were dead when recovered 1 week later. Unfortunately this experiment could not be repeated because of the dearth of developing larvae.

As with other filarial parasites whose life-cycles are known, A. globulosa has a third stage in the definitive host of relatively short duration, lasting around 10 days. Dipetalonema viteae moults to the fourth stage in about 7 days (Chabaud, 1954), B. booliata in 6-8 days (Singh et al., 1976), Brugia pahangi in 8-9 days (Schacher, 1962), Brugia malayi in 9-10 days (Edeson and Buckley, 1959), D. immitis in 9-12 days (Orhiel, 1961), L. carinii in 10-15 days (Scott et al., 1951) and Dipetalonema setariosum in 13-14 days (Worms, 1970 - unpublished thesis).

The fourth stage larvae of A. globulosa develop rapidly and enter the final moult at around 25 days post infection. A similar rate of maturation occurs in other rodent filariae: L. carinii undergoes the final moult in 23-24 days (Scott et al., 1951), D. viteae in 20-21 days (Chabaud, 1954) and B. booliata in 24-28 days (Singh et al., 1976). Nevertheless, the timing of the fourth moult is not so uniform amongst all filarial species. B. pahangi moults to the fifth stage in 23-33 days (Schacher, 1962), D. setariosum in 30-35 days (Worms, 1970), B. malayi in 35-40 days (Edeson and Buckley, 1959) and D. immitis in 60-70 days (Orhiel, 1961). Variations in the rate of maturation of fourth stage larvae do not seem to reflect the timing of the previous moult but may be an important factor governing the prepatent period of filarial species.

The prepatent period of A. globulosa in jirds and striped mice was

found to be around 74-88 days (see Chapter 3) which is of the same order as that reported by Singh *et al.* (1976) for B. booliata in rats (77-88 days). In other species of Ackertia the prepatent period is unknown, although Ko (1972b) suggested that this may be at least one year for Ackertia marmotae: Worms recovered from experimentally infected groundhogs 307 and 317 days post infection were still immature and wild hosts less than 1 year old never harboured patent infections (Ko, 1972b). The prepatent period of filarial parasites varies considerably and for species with short-lived hosts it will be important that this be brief. L. carinii and D. viteae of rodents produce microfilariae in about 50 days (Bertram, 1947; Hawking and Sewell, 1948; Chabaud, 1954; Worms *et al.*, 1961; Beaver *et al.*, 1974), and avian filarioids develop even faster: The prepatent period of Ornithofilaria fallisensis in ducks and of Cardiofilaria nilesi in chicks is about one month (Anderson, 1956; Gooneratne, 1969; Ponnudurai, 1971). In contrast, Dirofilaria species take far longer to mature. Microfilariae appear at around 6 months in D. repens (Webber and Hawking, 1955) and D. immitis (Orhriel, 1961) infections of dogs and not for 11 months in D. aethiops infections of monkeys (Hawking and Webber, 1955). The prepatent period of Onchocerca volvulus is estimated to be 10-20 months (Duke, 1962).

A. globulosa shares with other filarial worms the capacity to produce microfilariae before growth is complete. This feature has been noted for L. carinii (Scott, 1946), B. malayi (Edeson and Buckley, 1959), D. immitis (Orhriel, 1961), B. pahangi (Schacher, 1962; Denham *et al.*, 1972a) and D. viteae (personal observation). In such a way filarial worms may increase their reproductive potential, producing microfilariae soon after maturation and in increasing numbers as growth proceeds.

The development of intrauterine microfilariae in A. globulosa

appears to be similar to that reported for L. carinii (McFadzean and Smiles, 1956; Taylor, 1960a), A. marmotae (Anteson, 1968) and B. pahangi (Rodgers et al., 1976). In these species the egg shell is retained as the microfilarial sheath unlike D. viteae (Terry et al., 1961; Ellis et al., 1978) and Onchocerca gutturosa (Rodgers et al., 1976) where the microfilariae are unsheathed and are freed from their shells in the proximal part of the uterus.

The presence of 10-12 refractile globules in the sheath of A. globulosa microfilariae is an unusual feature although not restricted to this species. Dissanaik (1974) reported similar bodies in an unidentified microfilaria from Rattus muelleri in Malaysia and these also occur in the microfilarial sheath of Ackertia nilotica from Arvicanthus niloticus (El Bihari et al., 1977).

A low microfilaraemia was associated with natural infections of A. globulosa in striped mice. This is not uncommon for filarial parasites with skin microfilariae and occurs in hosts infected with A. marmotae (Ko, 1972b), Dipetalonema johnstoni (Spratt and Varughese, 1975), Onchocerca armillata (Patnaik, 1962) and Onchocerca volvulus (Fuglsang and Anderson, 1974; Duke et al., 1975). In fact the microfilariae of A. globulosa, A. marmotae and O. armillata were first discovered by their presence in the blood and only later was it discovered that the site of predilection is the skin (Muller and Nelson, 1975; Ko 1972b; El Bihari and Hussein, 1975). It seems probable that the blood-borne microfilariae of A. globulosa are the recent progeny of adult worms in the pulmonary arteries and are carried in the bloodstream to the skin. Microfilariae released in the pulmonary arteries would travel via the lungs and heart to the general circulation. As they pass through the capillary bed of the dermis they may leave the blood, assisted by the narrow lumen of capillaries, and enter the connective tissue of the skin.

Ko (1972b) suggested that the microfilariae of A. marmotae also reach the skin by circulating in the blood and the same may be true of O. armillata, since adult worms occur in the aorta (Patnaik, 1962). In contrast, the adults of many Onchocerca species live outside the circulatory system (see Table 1) and it has been suggested that the microfilariae of O. cervicalis and O. volvulus may migrate to their locations in skin via the subcutaneous fascial planes (Mellor, 1973a; Figuera-Marroquin, 1974).

In the present study, the microfilariae of A. globulosa were found to have an irregular distribution in the skin of rodents and to concentrate in the ears, corresponding with the distribution of microfilariae shown by Muller and Nelson (1975) in L. striatus and T. robusta. Interestingly a similar phenomenon occurs in groundhogs infected with A. marmotae (Ko, 1972b), rats infected with D. johnstoni (Spratt and Varughese, 1975) and Nile rats infected with A. nilotica (El Bihari et al., 1977). In addition to these rodent parasites, many species of skin microfilariae exhibit distinctive distribution patterns in their vertebrate hosts. These include O. volvulus (Kershaw et al., 1954a; Mazzotti, 1951a; Nelson, 1958; Lagraulet et al., 1964) and Dipetalonema streptocerca (Kershaw et al., 1954b; Duke, 1954) in man, Onchocerca gutturosa (Supperer, 1952; Eichler and Nelson, 1971; Hussein et al., 1975) and O. armillata (El Bihari and Hussein, 1975) in cattle, O. cervicalis in horses (Mellor, 1973a), numerous species of Onchocerca and other genera in deer (Hibler, 1965; Weinmann et al., 1973; Schulz-key, 1975c; Schulz-key et al., 1975) and Litomosa filaria in bats (Nelson, 1964).

The concentration of A. globulosa microfilaria in the head region of the host is a good adaptation to the feeding habits of H. leachii which preferentially attaches to the ears, head and neck on striped

mice. Most studied ixodids have preferential attachment areas on their hosts and the factors that control these distributions have been reviewed by Arthur (1962) and Balashov (1972). Irregular tick distributions on the host body may be associated with the microclimate next to the skin, the length of the capitulum in relation to vertebrate skin thickness, barriers formed by the host hair coat, the physiological condition of attaching ticks, and the grooming ability of the host (Arthur, 1962; Balashov, 1972).

In the present study, H. leachii was found to attach exclusively to the dorsal surface of the host and appeared to be directed to this site by a negative geotactic response. This behaviour is common to many ixodids when seeking hosts (Arthur, 1962; Balashov, 1972), but after attachment it may also protect ticks from being dislodged when feeding. Under the pressure of host grooming, ticks moved towards the head region on rodents. According to Arthur (1973a), the sites of predilection of feeding ticks on small mammals are the ears and head. The specificity of H. leachii for the ears and head was stricter on striped mice than on jirds and may reflect differences in the grooming ability of the two species. Kucheruk et al. (1955, 1956) found that 96% of Dermacentor marginatus larvae attached to the ears of field mice but were uniformly distributed on hedgehogs. They also noted that infestation rates with this tick varied greatly between 5 species of rodent and increased several fold if grooming was restricted by placing collars on the hosts.

The small nymphs of H. leachii could readily penetrate the pelage of rodents and did not attach by preference to hairless regions of the host. In addition, the distribution of H. leachii on rodents did not appear to be influenced by lighting conditions on the skin surface. This was perhaps to be expected since eyes are absent in the genus



Haemaphysalis (Hoogstraal, 1956), which suggests that photoreception is poorly developed. Some species of tick feed on different regions of the host according to the season. For example, in central Asia, Hyalomma detritum attaches to the dorsal surface of cattle during the cool winter months but during the warm summer avoids the direct sunlight by attaching to the ventral surface of the host (Galuzo, 1947 cited in Balashov, 1972). However, like most other species of ixodid ticks, H. leachii appears to exhibit a more stable distribution pattern on a given host species which must be an important factor that has enabled the microfilariae of A. globulosa to adapt to the feeding habits of this arthropod.

Many workers have noted that the distribution of microfilariae in the skin corresponds to the preferred biting site of their vectors and it is clear that this is a key adaptation of skin microfilariae to enhance transmission. In human infections, it has long been recognized that the microfilariae of O. volvulus occur in the upper or lower regions of the body according to whether the local simuliid vectors are high or low biters (reviewed by Nelson, 1970). Similarly in animals, Ko (1972b) observed that the accumulation of A. marmotae microfilariae in the ears and head of groundhogs makes them well adapted for transmission by Ixodes cooki, which preferentially attaches to these regions of the host. The distribution of microfilariae may be extremely localised or more evenly spread over an anatomical quarter of the host in response to the feeding behaviour of the vector. Ninety percent of O. gutturosa microfilariae are restricted to the umbilicus of cattle in Hertfordshire since Simulium ornatum has a strong predilection for this site (Eichler and Nelson, 1971), while the microfilariae of O. cervicalis occur more evenly over the belly of horses as Culicoides nubeculosus bites over the whole underside (Mellor, 1973a).

Mechanisms that might control the distribution of microfilariae in the skin have been much discussed in the literature but as yet remain obscure. The distribution of A. globulosa microfilariae appeared to be a highly stable character that did not vary between striped mice, which are natural hosts, and jirds, which are not. Irrespective of the intensity of infection, microfilariae occurred in each anatomical region of the host in constant proportions which suggests that a "spill-over" of parasites from the ears was not responsible for the lower microfilarial densities in the rest of the body. These observations imply that the microfilariae of A. globulosa possess an intrinsic, directional behaviour.

Several hypotheses have been advanced to explain the distribution of skin microfilariae in their hosts but most seem inapplicable to A. globulosa infections. The adults of A. globulosa are highly site specific to the pulmonary arteries and microfilariae released into the circulation should be able to reach any site in the body, so it is improbable that the location of adult worms has any influence on the distribution of microfilariae, as suggested for O. volvulus (Kershaw et al., 1954a) and Cutifilaria wenki (Schulz-key, 1975a). Ko (1972b) suggested that there are extensive arterio-venous anastomoses in the ears of groundhogs that trap the circulating microfilariae of A. marmotae and cause them to leave the capillary bed for the dermis in greater numbers in the ears than elsewhere in the body. This is an attractive theory that could play some part in the distribution of A. globulosa microfilariae but further work is necessary to show that this could explain the pattern of microfilarial densities observed. When microfilariae of the ungulate parasites, O. gutturosa, O. cervicalis and O. armillata are injected into rats and mice they concentrate in the ears and nose (Nelson et al., 1966; Eichler and Nelson, 1968; Mellor,



1973a; El Bihari and Hussein, 1975), while those of O. cervicalis accumulate in the inguinal region and tail of jirds (Rabalais, 1974). From these observations it has been suggested that in laboratory rodents, the microfilariae respond to a thermal gradient and congregate in the coolest regions of the body; however, there was no evidence of this in the natural hosts. It is unlikely that the microfilariae of A. globulosa are distributed in striped mice and jirds in response to a thermal gradient as they are very scarce in the nose and tail which are 2-3°C cooler than the rest of the body (see Rabalais, 1974 for chart of skin temperatures in jirds).

One possibility to explain the directional behaviour of A. globulosa microfilariae in the host is that this is governed by a negative geotactic response. This would account for the peak microfilarial density in the ears, the moderate concentration over the dorsal surface, and low numbers of microfilariae on the belly, legs and tail. Hawking (1959) observed that the large, blood-dwelling microfilariae of Dirofilaria magnilarvatum accumulate in the dependent tail of monkeys and a similar effect of gravity has been noted by Nelson (1964) with the skin microfilariae of L. filaria which concentrate in the face of leaf-nosed bats. In both of these examples, microfilariae respond to gravity by a positive geotaxis; but in much the same way as the microfilariae of Loa loa in man respond to a rise in host body temperature by entering the peripheral blood while those of Edesonfilaria malayensis in monkeys leave the peripheral circulation under the same stimulus (Hawking, 1967), so the microfilariae of A. globulosa may react differently from D. magnilarvatum and L. filaria to the effect of gravity. In this connection, it may be interesting to note that the free-living stages of several parasitic nematodes show an upward migration on grass-stalks and plants (Croll, 1975).

While a gravitational effect may explain the distribution of A. globulosa microfilariae in rodents, it is equally possible that they respond to a migratory stimulus which is far more complex and has developed between the parasite and host over a long period of association. However, this stimulus must be common to a variety of rodent species since microfilariae exhibit the same characteristic distribution pattern in L. striatus, T. robusta and M. unguiculatus. Whatever the mechanism that causes microfilariae to concentrate in the ears and head, it appears that this is primarily an adaptation to ensure transmission.

Skin microfilariae rely on the local tissue damage produced by their vectors when biting in order to escape from the tissues and be ingested with the blood meal, and many species concentrate in the upper layers of the dermis to accommodate the superficial mode of feeding their vectors employ. This has been seen with Eufilaria cypseli of swifts which is transmitted by mallophagan lice (Nelson, 1964), O. volvulus and O. gutturosa which are transmitted by simuliid flies (Nelson, 1970; Eichler and Nelson, 1971), and Onchocerca gibsoni and O. cervicalis which are transmitted by ceratopogonid midges (Buckley, 1938; Mellor, 1973a). Recently, Nelson and Davies (1976) have also shown that the microfilariae of Mansonella ozzardi concentrate in the superficial dermal capillaries of man in the West Indies where the vectors are Culicoides spp.

In contrast to these forms, the microfilariae of A. globulosa occur randomly throughout the entire depth of the skin. The skin of rodents is thin and the mode of tick feeding such that microfilariae may be ingested from all depths of the host integument. Unlike the simuliid and ceratopogonid vectors of Onchocerca species that use scarifying mouthparts to create a shallow wound in the skin, H. leachii

produces a relatively deep feeding lesion that extends well beyond the depth of the embedded hypostome. This type of feeding lesion was seen beneath the mouthparts of both the larvae and nymphae and is common to several other species of ixodid ticks (Arthur, 1965, 1973b; Moorhouse, 1967; Balashov, 1972; Ko, 1972a). When ixodids feed, saliva is inoculated into the host which spreads from the mouthparts into the surrounding tissues. Histolytic enzymes in the saliva may cause specific vascular damage (Moorhouse and Tatchell, 1966) and initiate lysis of dermal tissues (Arthur, 1965, 1973b), but further damage is caused by host inflammatory cells that respond to the presence of tick saliva and produce an extensive area of tissue necrosis (Tatchell and Moorhouse, 1968; Arthur, 1973b). In the present study, the feeding lesions seen beneath the mouthparts of H. leachii extended through the dermis to underlying adipose tissue, freeing microfilariae from all depths of the skin. Those enveloped in the feeding cavity, and others that may have entered from the surrounding area, could then be ingested during the imbibition of blood and tissue fluid.

There have been few studies on the uptake of microfilariae by ixodid ticks, so it is of much interest that an accumulation of A. globulosa microfilariae was seen beneath the attachment sites of H. leachii as similar observations have been made by Moorhouse (1969) and Ko (1972b). Moorhouse (1969) reported an accumulation of microfilariae (of an undescribed filarioid) in the dermis of a greater glider (Schoinobates volans) beneath the attachment site of Ixodes tasmani, and Ko (1972b) observed that the microfilariae of A. marmotae concentrated beneath the mouthparts of Ixodes cooki attached to groundhogs. Ko (1972b) further showed that oedematous fluid extracted from swellings at the site of tick attachments contained high numbers of microfilariae relative to their level in the surrounding tissues.

In the present study, the pronounced accumulation of A. globulosa

microfilariae was found at only 3 of 5 tick attachment sites. This result suggests that not all ticks were equally capable of concentrating microfilariae from the skin although it is also possible that in the 2 remaining cases, ticks had already ingested the bulk of accumulated microfilariae prior to fixation. Nevertheless it is clear that where microfilariae do congregate beneath the mouthparts, this will greatly enhance the chances of H. leachi ingesting a large number of parasites or concentrating them from skin with only a low microfilarial density.

Ko (1972b) suggested that the accumulation of A. marmotae microfilariae beneath the mouthparts of I. cookei might be due to the feeding habits of the tick vector. There is usually a copious flow of tissue fluid towards the feeding sites of ixodid ticks induced by pharmacologically active components of salivary secretions and the immune response of the host (Tatchell and Moorhouse, 1968; Tatchell, 1969). This flow is encouraged by the pharynx of the tick, which acts like a pump to suck tissue fluid and blood into the hypostome (Gregson, 1960). Ko (1972b) argued that by a combination of these events, microfilariae may be carried from the surrounding tissues into the feeding lesion and thus lead to their accumulation beneath the mouthparts. While this might influence the distribution directly beneath the attachment site of an ixodid vector, it seems improbable that the striking accumulation of A. globulosa microfilariae could have been brought about in this way alone. The microfilarial densities in animals used for this work were extremely low (6-7 mff/mg) and microfilariae must have been drawn from a considerable distance to reach the concentration seen beneath the mouthparts of some of the feeding ticks (see Figure 14).

An alternative hypothesis to explain the concentration of A. globulosa microfilariae might be that these possess a chemotactic

response to components of the salivary secretions of H. leachii. Moorhouse (1969) suggested that such a phenomenon may be responsible for the accumulation of skin microfilariae beneath the attachment sites of I. tasmani, and in the older literature several authors have proposed that vectors concentrate microfilariae from the skin or blood in this manner (Manson, 1883; Ashburn and Craig, 1907; Craig, 1932; Strong et al., 1934; Galliard, 1936; O'Connor and Beatty, 1937).

Studies such as these on the relationship between the number of microfilariae in the host and the number ingested by the vector have been reviewed by Lavoipierre (1958b), Hawking and Worms (1961) and Nelson (1964). These authors concluded that for blood microfilariae, the number ingested is roughly proportional to the number expected from the size of the blood meal, although large variations occur between individual arthropods. Support for this view came from the work of Gordon and Lumsden (1939) who made direct observations on the feeding mechanism of mosquitoes on frogs infected with Foleyella. They found that variations in the number of microfilariae ingested depended on whether the insect fed directly from a capillary with a high or low microfilaraemia or from a pool of blood emanating from torn capillaries in the skin. There was no evidence of microfilariae becoming entangled around the mouthparts or of a chemotactic response to mosquito saliva (Gordon and Lumsden, 1939), as suggested by some earlier workers (Manson, 1883; Ashburn and Craig, 1907; Craig, 1932; Galliard, 1936; O'Connor and Beatty, 1937).

In contrast to blood microfilariae, there is much evidence to suggest that some skin-dwelling forms are concentrated by their feeding vectors. Apart from the tick-transmitted species mentioned above, several reports have shown that simuliid flies appear to exert a powerful attraction on the microfilariae of O. volvulus in the skin. Strong et al.

(1934) first revealed this effect when they showed that a massive influx of microfilariae took place beneath the feeding sites of blackflies fed on patients infected with onchocerciasis. An ambitious series of experiments in support of this work was later performed by De Leon and Duke (1966). They found that when Simulium ochraceum, Simulium metallicum and Simulium callidum were fed on patients infected with the local Guatemalan strain of O. volvulus, 10-25 times as many microfilariae were ingested by the local flies as by Simulium damnosum of African origin. The highly specific nature of this attraction to a naturally evolved vector-parasite couple was further demonstrated by the fact that S. ochraceum ingested 20-25 times as many microfilariae of the Guatemalan strain of the parasite as of a West African strain. Similar studies by Duke et al. (1967), Duke (1970), Garms (1973) and Omar and Garms (1975) have shown that simuliids often take up more microfilariae with a blood meal than would be expected from their density in the skin. In East Africa, Wilkinson (1949) exploited this phenomenon for the xenodiagnosis of onchocerciasis as have De Leon and Duke (1966) in Guatemala. This raises the possibility that an isolate of blackfly saliva may provide a useful diagnostic tool to identify patients lightly infected with onchocerciasis. In this connection it would be interesting to study the distribution of A. globulosa microfilariae in the skin after an intradermal injection of saliva from H. leachii. Any accumulation of microfilariae might then be ascribed more to a chemotactic response than to a passive transport of microfilariae to the bite site from adjacent tissues, as suggested by Ko (1972b).

While the evidence of a chemical attraction of microfilariae remains circumstantial, it is easier to envisage how this might operate for skin-dwelling forms than for those in the blood. Saliva inoculated into the host by the feeding vector will spread slowly through dermal tissues,



creating a chemical gradient that skin microfilariae will encounter and may follow to its source. Providing the migration of microfilariae occurs before feeding is complete, many more might be ingested than would otherwise be possible. It has been observed that the vectors of O. volvulus, O. gutturosa and O. cervicalis ingest more microfilariae when feeding is prolonged, irrespective of the size of the blood meal (Wegesa, 1967; Eichler, 1971; Mellor, 1975). Since a characteristic of ixodid ticks is their long period of attachment to the host, these may be amongst the most suitable vectors of filarial parasites to which a skin microfilariae such as A. globulosa might have evolved a chemotactic response to enhance transmission.

For many species of filarial worms it has been shown that microfilarial levels in the definitive host are regulated by a dynamic relationship between the parasites and their hosts. Results obtained in the present study suggest that under conditions of natural transmission, striped mice infected with A. globulosa develop higher microfilarial densities in the skin as they acquire more adult worms. Probably it will be the number of gravid females in the host that will reflect the microfilarial density more closely than the total number of worms. However, this relationship cannot be verified until a comparable study is possible in experimentally infected rodents, in which the course and duration of infection is known, and the transmission or transplantation of parasites to the host may be controlled. Bertram (1958, 1966) found that in cotton rats lightly infected with L. carinii, there also appeared to be a directly proportional relationship between the number of adult worms in the host and the level of microfilariae. In heavy infections, however, the microfilaraemia ceased to increase proportionately and a crowding effect developed manifested by the retarded growth of maturing larvae and reduced fecundity of adult females (Bertram,

1958, 1966). Although the intensity of infections examined in the present study covered a broad spectrum of those encountered in the field, it is possible that worm burdens of A. globulosa might also reach an upper limit above which the relationship with microfilarial density is no longer maintained.

In many filarial infections it has been found that there is a lack of correlation between the adult worm burden and levels of microfilariae. For example, Beaver et al. (1974) showed that maximum levels of microfilaraemia in D. viteae infections of jirds were approximately the same with 1 pair of worms as with 5, although 10 worm pairs gave rise to an exponential rise in microfilaraemia that often overwhelmed the host. Denham et al. (1972a) similarly found that primary infections of B. pahangi in cats gave rise to a level of microfilaraemia that did not increase significantly with an increase in the number of adult worms. However, after repeated infection with B. pahangi, the control of microfilarial levels in cats was modified such that an increase in the number of adult worms was reflected by an increase in the number of circulating microfilariae (Denham et al., 1972b).

Most striped mice harbouring natural infections of A. globulosa showed a similar pattern of microfilarial density over the 16 months in which they were studied. This pattern was essentially the same as that seen in experimentally infected rodents (see Chapter 3) but differed in that microfilarial densities were far higher, and persisted for much longer, in most naturally infected hosts. The course of microfilarial densities was characterised by a rise in level during the first 4 months of observation followed by a gradual decline over the next 10-12 months, producing a peak type curve similar to that seen in cotton rats with moderate infections of L. carinii (Bertram, 1966). However, Bertram (1958, 1966) demonstrated that the course and level of microfilarial



densities in L. carinii infections may be radically modified by changes in the incident transmission of this parasite. Thus, the intermittent transmission of L. carinii to cotton rats led to high and prolonged microfilaraemias, while continuous transmission over several months produced a crowding effect amongst the adult worms that resulted in far lower microfilarial levels of relatively short duration.

As the striped mice used in this work had been removed from the natural transmission cycle for study in the laboratory, it may well be that the pattern of microfilarial densities shown by these animals differs from that in the wild. In nature, striped mice will acquire their worm burden through constant exposure to reinfection. Therefore, as the successive worm populations mature, microfilarial densities might continue to rise throughout the short life-span of the host. Alternatively, microfilarial levels may reach a peak and then be maintained at a plateau, as observed with D. viteae in jirds (Beaver et al., 1974), B. pahangi in cats (Denham et al., 1972a) and D. immitis in dogs (Worms, 1971).

Many animal filariae appear to live and reproduce for the natural life-span of their hosts. L. carinii of cotton rats lives for 15 months (Bertram, 1966), D. viteae of jirds for up to 2 years (Johnson et al., 1974), D. setariosum of mongooses for 4-6 years, and D. immitis and D. repens of dogs for up to 9 years (Worms, 1970 - unpublished thesis). The longevity of A. globulosa was difficult to ascertain as striped mice were autopsied at infrequent intervals. However, microfilariae were detected for up to 500 days and a live male worm was recovered from one striped mouse 506 days after collection from the field. According to Kingdon (1974), the natural life-span of L. striatus is around 6-12 months although a few individuals may survive for 2 years. As A. globulosa has a prepatent period of almost 3 months in striped mice and peak microfilarial densities are not attained for at least another

4, it would appear that this species will also live and reproduce for the natural life-span of its host.

A. globulosa appeared to be well tolerated in the definitive host and provoked few pathological changes. During the early stages of infection, a slight, but significant, eosinophilia developed in the peripheral circulation which reached a peak by the twenty-ninth day after infection. Eosinophil levels then subsided, although they remained significantly higher in infected animals than in uninfected controls for the remaining period of observation. Raised eosinophilia is a common feature of many helminth infections (Archer, 1963, 1970; Zvaifler, 1976) and has been reported in both human and experimental filariasis (Goodman et al., 1945; Hodge et al., 1945; Oothuman, 1976 - Ph.D. thesis, University of London; Zvaifler, 1976). The rather weak eosinophil response in jirds infected with A. globulosa may have been due to the low number of worms maturing in these hosts, as jirds infected with equivalent numbers of D. viteae infective larvae supported many worms and developed high eosinophilias (personal observation). Peak cell counts were 21 times higher (9,825 eosinophils per cu.mm. of tail blood) in D. viteae infections, although the response curves were basically the same in both infections.

Skin microfilariae can be severely pathogenic in the vertebrate host in contrast to forms in the blood, although Klei et al. (1974) have reviewed pathological changes in the lung, spleen, liver and kidneys attributed to blood-borne microfilariae. It has long been recognised in man that the microfilariae of O. volvulus may cause progressive pathological changes in the skin (reviewed by Rodger, 1962; Nelson, 1970) and recently Meyers et al. (1972) showed that the skin microfilariae of Dipatalonema streptocerca may also be pathogenic. It is generally believed that the pathogenesis of onchocerciasis is due to an

immunological reaction of the host to dying microfilariae (Nelson, 1970), although Rodger (1962) suggested that a direct toxic effect of the dead parasites is responsible.

In some cases of onchocerciasis, even high densities of microfilariae in the skin produce no apparent damage (Nelson, 1970; Duke, 1971; Anderson et al., 1974a). The reasons for this may be due to a complex interaction of factors governed by the parasite and host, but in such cases it is apparent that the parasites are well tolerated. With several animal filarioids, it appears that skin microfilariae are also well adapted. The microfilariae of L. filaria in bats, E. cypseli in swifts, and A. marmotae in groundhogs all exist in the skin without producing skin lesions (Nelson, 1966; Ko, 1972b) and it is apparent from the present study that the microfilariae of A. globulosa also produce no pathological changes in the skin of jirds and striped mice. 14

Inflammatory skin lesions in cattle and horses have sometimes been attributed to the microfilariae of O. gutturosa and O. cervicalis (reviewed by Nelson, 1966, 1970) but the role of these parasites in the aetiology of seasonal dermatitis and sweet itch requires further study. Eichler and Nelson (1971) and Mellor (1973b) suggested that generalised inflammatory reactions seen in the skin of cattle and horses in England may be due to an allergic reaction to biting insects rather than to Onchocerca infection. Support for this view has been given by the studies of Riek (1954) and Mellor and McCraig (1974).

The presence of A. globulosa microfilariae in the eyes of naturally infected striped mice is of particular interest. The microfilariae of O. volvulus are recognised to be an important cause of eye disorders in man (reviewed by Rodger, 1960; Nelson, 1966, 1970; Anderson et al., 1974a) but few other filarial parasites have microfilariae that are known to enter the eye. Lagraulet (1962) found good evidence to suggest that

horses heavily infected with O. cervicalis develop eye lesions similar to those seen in human cases of ocular onchocerciasis and Patnaik (1962) incriminated O. armillata microfilariae as a cause of eye lesions in cattle. The microfilariae of O. gutturosa may also enter the conjunctiva of cattle, but as yet no eye lesions have been observed (Nelson, 1966). It is believed that the present report is the first to describe the natural entry of microfilariae into the eyes of small mammals.

A. globulosa microfilariae probably enter the anterior segment of the eye from the surrounding skin as microfilarial densities are highest in the ears. Rodger (1959) and Anderson et al. (1974b) have suggested that the microfilariae of O. volvulus may also enter the cornea from the surrounding skin and the risk of developing eye lesions in the anterior segment has been related to the level of microfilarial densities at the outer canthus (Fuglsang et al., 1976).

Only in one eye were the microfilariae of A. globulosa found in the cornea but in this instance their presence was accompanied by a localised infiltration of plasma cells. This suggests that the parasites had provoked an immunological reaction in the sensitised host. Studies on the pathogenesis and pathology of rabbit eyes after the injection of O. volvulus microfilariae into the subconjunctiva and cornea have revealed much about the development of anterior segment eye lesions. In contrast to man, rabbits rapidly develop corneal lesions to the microfilariae of O. volvulus (Duke and Anderson, 1972b). In normal rabbits, these soon resolve and cause little impairment of vision, but Duke and Garner (1975) found that if rabbits were pre-immunised with living microfilariae, such reactions were more severe and persisted to cause a sclerosing keratitis similar to that in man. It is generally believed that dead microfilariae provoke the inflammatory changes in human eyes

(Nelson, 1966) which Rodger (1960) confirmed in the rabbit. However, Duke and Anderson (1972b) found that living microfilariae appeared to elicit more severe inflammation in the eyes of rabbits. In the present study, the microfilariae of A. globulosa were scarce in the eyes of striped mice and caused little pathology. As it is probable that the number of microfilariae entering the eye will be proportional to the intensity of infection, significant pathological changes might be found in more heavily infected hosts.

Throughout the course of naturally acquired infections, A. globulosa caused no signs of overt disease in striped mice. However, adult worms in the pulmonary arteries provoked a sequence of histopathological reactions that culminated in the occlusion of some minor arterioles in longstanding infections. Initially, adult worms appeared to elicit no host reaction, but as the infection matured and microfilarial densities reached their peak, there was a strong inflammatory response manifested by a widespread perivascular cuffing. There can be little doubt that this was associated with A. globulosa, but the widespread nature of the response suggests that the antigenic stimulus from adult parasites involved a worm product swept in the bloodstream throughout the entire arterial network of the lungs. The predominance of plasma cells in this reaction is highly suggestive of a humoral component in the host response to adult parasites. The presence of eosinophils further suggests that the host had produced specific antibody against parasite antigens and that these cells had been attracted to the lungs by the formation of immune complexes (Litt, 1964). However, it is not known whether the host response had a deleterious effect against any stage of A. globulosa.

Accompanying the perivascular cuffing were localised changes in the artery wall. Hypertrophy of the intima of the pulmonary arteries, as seen in striped mice, has also been reported in dogs infected with

D. immitis (Nelson, 1966; Worms, 1970 - Ph.D. Thesis, University of London) and cows infected with O. armillata (Chodnik, 1958). It is probable that this reaction to the adults of A. globulosa was due to the direct contact of living worms with the vessel lining and that mechanical, chemical or immunological factors played a role in its cause. Support for this view comes from the occurrence of vessel wall reactions only in the immediate vicinity of adult parasites and by the higher incidence of such reactions in small vessels where contact between the worm and vessel lining would be most intimate. Poynter (1966) showed that the larvae of Strongylus vulgaris in horses provoked an endarteritis by direct irritation of the nematode on the endothelium of pulmonary arteries. Mural deposits were formed and these were eventually incorporated into the intima which thereby became thickened by fibrinous deposits.

By the end of the patent infection, dead and necrotic worms were found surrounded by a granulomatous reaction that occluded the vessel lumen. Reactions of this kind have also been reported in the pulmonary arteries of seals infected with Dipetalonema spirocauda (Taylor et al., 1961) and dogs infected with D. immitis (Worms, 1970). In striped mice it seems noteworthy that such reactions were found only in the smaller vessels of the lung while living adults occurred throughout the pulmonary arteries. This suggests that dying worms may be unable to maintain their position in the larger arteries and are swept into smaller arterioles where they become lodged in the narrow lumen. This may well protect the host from the serious consequences of an obliterative granuloma involving the major pulmonary blood vessels.

From the present study, it is clear that A. globulosa is well adapted to its principal natural host, L. striatus. The delayed onset of a host response and absence of severe pathology are indicative of a highly evolved host-parasite relationship which enables A. globulosa to



live for a long period in striped mice without prejudice to host survival. The adaptations of A. globulosa microfilariae to ensure transmission also suggest that there must be a highly evolved relationship between the parasite and its tick vector, a topic that will be examined further in the following chapter.

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CHAPTER 5BIOLOGY OF A. GLOBULOSA IN ITS HARD TICK VECTOR  
AND THE TRANSMISSION OF INFECTIVE LARVAEINTRODUCTION

Since 1878, when Sir Patrick Manson showed mosquitoes to be intermediate hosts of Wuchereria bancrofti, a vast literature on the development of filarial parasites in their arthropod hosts has accumulated. The rapid growth in knowledge of filarial life-cycles in the vector is amply illustrated by a succession of excellent reviews on the subject: Chabaud (1954) listed 29 known vectors of human and animal filariae, which has risen in subsequent reviews to 35 (Lavoipierre, 1958b), 39 (Hawking and Worms, 1961), 50 (Nelson, 1964), 57 (Macdonald, 1971) and 93 (Schacher, 1973).

Hard tick-transmitted filarial worms

Ixodid ticks were first shown to be vectors of filarial parasites in 1890 when Grassi and Calandruccio reported the presence of Dipetalonema larvae in Rhipicephalus sicanus. These were believed to be D. reconditum although Nelson (1962) has suggested they were probably D. grassii.

Few filarial worms are known to have hard tick vectors and their development in these arthropods had rarely been described. Noé (1908)

reported the growth of D. grassii larvae in the muscles of Rhipicephalus sanguineus, and Ko (1972b) gave a detailed account of the development of Ackertia marmotae in the epidermis and fat body cells of Ixodes cookei. Chabaud (1954) stated that Dipetalonema viteae may be transmitted by both hard and soft ticks, but only described its development in the latter family of arthropods.

In addition to the examples given above several ixodid species have been found to contain microfilariae or developing larvae, although their role as vectors remains unclear. Filarial species recorded in hard ticks include Filaria martis in Ixodes ricinus (Baldasseroni, 1909 - cited in Chabaud, 1954), Filaria mitchelli in Dermacentor venustus (Smith, 1910), a microfilaria from Schoinobates volans in Ixodes tasmani (Moorhouse, 1969), Wehrdikmansia rugosicauda in Ixodes ricinus (Schulz-Key, 1975b) and Ackertia nilotica in Rhipicephalus simus (El Bihari et al., 1977).

In Chapter 2 it was confirmed that Haemaphysalis leachii is a natural vector of A. globulosa, as originally suggested by Muller and Nelson (1975). This chapter reports studies on the development of the parasite in its hard tick vector and the susceptibility of other blood-sucking arthropods to infection. Pathological changes in infected ticks and their response to the presence of developing larvae were examined. An attempt was also made to study the transmission of infective larvae from H. leachii nymphs to the natural definitive hosts.

Attempts to infect haematophagous arthropods

Various species of arthropod including hard and soft ticks, mites, fleas, and mosquitoes, were fed on striped mice naturally infected with A. globulosa. Six animals with microfilarial densities between 27-33 microfilariae per milligram (mff/mg) of ear skin were used. The 8 species of arthropod tested and numbers examined are listed in Table 19. Engorged arthropods were examined for filarial parasites immediately after the blood meal, and at 7 and 30 days post infection. This approach was not taken in the case of fleas and mites: Instead, rodents were infested for 30 days and the arthropods recovered were then dissected immediately.

Results of the feeding trials are given in Table 19. Only 2 ixodid ticks, Haemaphysalis leachii and Rhipicephalus sanguineus, supported the development of A. globulosa to the infective stage. A comparison of H. leachii originating from 4 sources in East Africa revealed a marked disparity in the susceptibility of ticks to infection. The Machakos (Kenya) and Zomba (Malawi) biotypes of H. leachii showed a greater uptake of microfilariae, and larger yields of infective larvae, than either the Nairobi (Kenya) or Muguga (Kenya) biotypes. Nevertheless, all 4 types of H. leachii tested proved better intermediate hosts for A. globulosa than R. sanguineus. This was because fewer microfilariae developed to infective stage larvae in the nymphs of R. sanguineus, even though these ticks ingested comparable numbers of microfilariae to H. leachii nymphs from Nairobi and Muguga.

Haemaphysalis punctata did not attach readily to striped mice but of 12 ticks that did, active microfilariae were seen in the blood meals of 4 immediately after detachment. Only a few disintegrating microfilariae were found in the gut on day 7 and no filarial larvae could

**TABLE 19**

Presence of filarial infection in various haemaphagatous arthropods after experimental feeding on L.striatus naturally infected with A.globulosa.

Arthropod species	No. examined	Presence of viable larvae		
		Day 0	Day 7	Day 30
<u>Haemaphysalis leachii</u>				
"Machakos form"	40	++++	++++	+++
"Nairobi form"	49	+++	+++	++
"Muguga form"	37	+++	+++	++
"Zomba form"	30	++++	++++	+++
<u>Haemaphysalis punctata</u>	12	++	-	-
<u>Rhipicephalus sanguineus</u>	20	+++	+	+
<u>Ornithodoros moubata</u>	31	+	-	-
<u>Ornithonyssus bacoti</u>	77	NR	NR	-
<u>Xenopsylla cheopis</u>	13	NR	NR	-
<u>Aedes aegypti</u> (Liverpool strain)	26	-	-	-
NR Not recorded at this time interval		- ... 0	+ ... <5	Mean number of Parasites per arthropod.
		++ ... 5-10	+++ ... 10-20	
		++++ ... >20		

be seen on day 30. Two attempts were made to infest striped mice with Rhipicephalus appendiculatus but ticks failed to attach on both occasions.

Amongst arthropods not belonging to the family Ixodidae, only the argasid tick Ornithodoros moubata ingested microfilariae. In ticks that were dissected immediately after feeding a few active microfilariae were seen within the gut, but no developmental stages were found at 7 and 30 days after infection. Ornithonyssus bacoti, Xenopsylla cheopis and Aedes aegypti did not ingest microfilariae.

#### Development of A. globulosa in H. leachii ticks

The development of A. globulosa in the Nairobi and Machakos biotypes of H. leachii was studied by dissection and histological techniques. Nymphal ticks were infected by allowing them to feed on infected striped mice while confined to the ears in feeding capsules (see Chapter 3). Because of the long period required for these hard ticks to engorge (2-4 days), the duration of infection on the date they were examined was considered as the number of days since they initially attached. However, a number of ticks were removed from the host 1 and 2 days after the start of feeding so that information on the early development of A. globulosa in the vector could be obtained. In studies on the later development of the parasite, only ticks that had fed to repletion and detached naturally by the third day after attachment were used. Infected ticks were stored at 28°C and 80% relative humidity as previously described in Chapter 3.

a) Studies made by dissection

Live ticks were dissected in 0.85% saline on 1, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20 and 22 days post infection. Between 5 and 22 ticks were examined at each time interval. In dissections of recently engorged ticks, 0.1% saponin was added to the saline solution which caused rapid lysis of red blood cells without apparent harm to the filarial larvae. Ticks were dissected around the margin of the alloscutum and the body gently prised into two. Observations were made on living larvae within tick tissues and on those that had emerged into the medium. The tissues were then thoroughly teased and a search made for all stages of developing larvae under the X 100 magnification of a compound microscope. These were counted and subsequently preserved by transferring them on the tip of a cat's whisker to 70% alcohol containing 10% glycerine. The alcohol was allowed to slowly evaporate leaving worms to clear in pure glycerine. The larvae were then mounted in a drop of fresh glycerine on a microscope slide for detailed examination.

The morphology of larvae was studied using a Nikon phase microscope: Worms were measured and representative stages drawn with the aid of a camera lucida attachment.

Observations

Over half the ticks examined in this study (64% of 153) contained developing larvae of A. globulosa. Both sexes of H. leachii supported the infection with a mean of 7.8 larvae per infected tick. Details of the worm recoveries and stages of larval development in ticks

examined 1-22 days post infection are given in Table 20. A total of 766 larvae were recovered and examined and their growth, in terms of length and width, is depicted graphically in Figure 21: All widths referred to in the text were measured at the excretory cell. Measurements of the major internal structures of microfilariae are given in Table 14, and for larvae at the first moult, second moult and infective stage in Table 21. Drawings of the various larval stages are presented in Figures 22-27.

Development and location of *A. globulosa* in the vector (see Table 20)

The microfilariae of *A. globulosa* required 13-18 days to mature to infective stage larvae. On the first day after tick attachment, examination of the midgut revealed that some microfilariae had already been ingested before the uptake of blood had begun. The few microfilariae seen in the gut were exsheathed and highly active. Others were present outside the gut, buried in epidermal cells underlying the cuticle: Microfilariae in this situation were difficult to see and made only feeble movements.

On day 3, when most ticks were detaching, greater numbers of larvae were seen. As the midgut of freshly engorged ticks could not be extracted undamaged, it was not possible to determine the exact proportion of worms which escaped the blood meal naturally. However, only a few active and exsheathed microfilariae were seen in the blood which showed vigorous movements of the cephalic hook. Most larvae were present outside the gut and many were buried within the epidermis. Of 173 larvae recovered on day 3, 84 were still morphologically identical to microfilariae, while the remainder had reached the early

do most larvae have been seen?



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*No tick  
larvae seen  
tick?*



**TABLE 20**

The development of *A.globulosa* in the vector: Percentages of each larval stage present at various times post infection.

Days after tick attachment	No of ticks	Stage of development.							TOTAL LARVAE
		MICROFILARIA	1st LARVA	1st MOULT	2nd LARVA	2nd MOULT	EARLY 3rd LARVA	INFECTIVE LARVA	
1	6	100	0	0	0	0	0	0	11
3	22	84	16	0	0	0	0	0	173
4	5	100	0	0	0	0	0	0	12
6	7	64	36	0	0	0	0	0	55
8	10	19	79	2	0	0	0	0	67
9	7	14	29	55	1	0	0	0	78
10	3	23	45	25	7	0	0	0	69
11	6	15	8	0	77	0	0	0	13
12	6	0	0	0	96	4	0	0	45
13	15	2	0	0	25	40	31	2	48
14	6	0	0	0	10	20	40	30	10
15	11	0	0	0	12	6	76	6	17
16	9	1	0	0	4	3	63	29	70
17	5	0	0	0	0	0	30	70	10
18	20	0	0	0	0	0	8	92	39
20	10	0	7	0	0	0	0	93	14
22	5	0	0	0	0	0	3	97	35
<b>TOTAL</b>	<b>153</b>								<b>766</b>

first-stage of development.

Between 4-9 days after infection the number of microfilariae remaining in the midgut steadily declined. Those seen after day 6 showed no movement and had presumably died in the rapidly digesting blood meal. Most first-stage larvae appeared in ticks from 3-10 days post infection. Throughout this period, a proportion of these worms was always recovered from the epidermal cell layer. However, most larvae were not found within cells, although the trauma of dissection may well have dislodged them from intracellular sites.

Larval development in some ticks was slow, or possibly retarded, and microfilariae were commonly seen as late as 10 days post infection. On day 6, 64% of 55 worms were still morphologically identical to microfilariae; however, by day 8 this figure had dropped to 19% of 66 larvae.

Worms undergoing the first moult were seen 8-10 days post infection. The peak of moulting occurred on day 9 when 55% of 78 worms were at this stage. Seven moulting larvae were seen still within the epidermal cell layer where their erratic movements caused the thin overlying plasma membrane to stretch considerably.

Most second-stage larvae occurred in ticks 10-13 days post infection. Between days 10-12, the proportion of larvae at this stage rose from 7% of 69 worms to 96% of 45. A number of second-stage larvae were seen tightly coiled within large fat cells immediately beneath the epidermis: Here they appeared to fill the entire volume of the cell and their active movements caused marked changes in cell shape. The majority of second-stage larvae were not observed in intracellular sites, but again it is believed that they may have emerged from cells ruptured during dissection.

Worms undergoing the second moult were seen 12-16 days post

infection. The peak of moulting occurred on day 13 when 40% of 48 larvae were recorded at this stage. A few moulting worms were still enveloped in the plasma membrane of tick fat cells but the majority of larvae were found free in the dissecting fluid where they showed vigorous movements.

Early third-stage larvae were first observed 13 days after infection. These could be distinguished from infective third-stage forms by their thicker appearance and less active movements. Early third-stage worms were most numerous on days 15 (76% of 13 larvae) and 16 (63% of 70 larvae) but later their numbers rapidly declined.

The proportion of infective third-stage larvae present in ticks rose progressively between 13-18 days after infection. On day 18, 92% of 39 worms were fully developed to the infective stage. However, their number rose slightly to 97% of 35 worms by day 22. All third-stage larvae were recovered from the haemocoel. Those that had reached the infective stage were extremely active and occurred in all regions of the tick including a few in the legs and chelicerae.

Throughout the course of infection in almost all the ticks examined, much asynchronous development was observed amongst the larvae. For example, at 10 days after infection 3 ticks contained 69 larvae of which 23% were microfilariae, 45% were first-stage larvae, 25% were at the first moult and 7% were at the second stage. It seems likely that this asynchrony in development may be attributed in part to the long duration of tick feeding, since microfilariae were ingested over a 3 day period.

FIGURE 21 Growth of *A. globulosa* in the vector

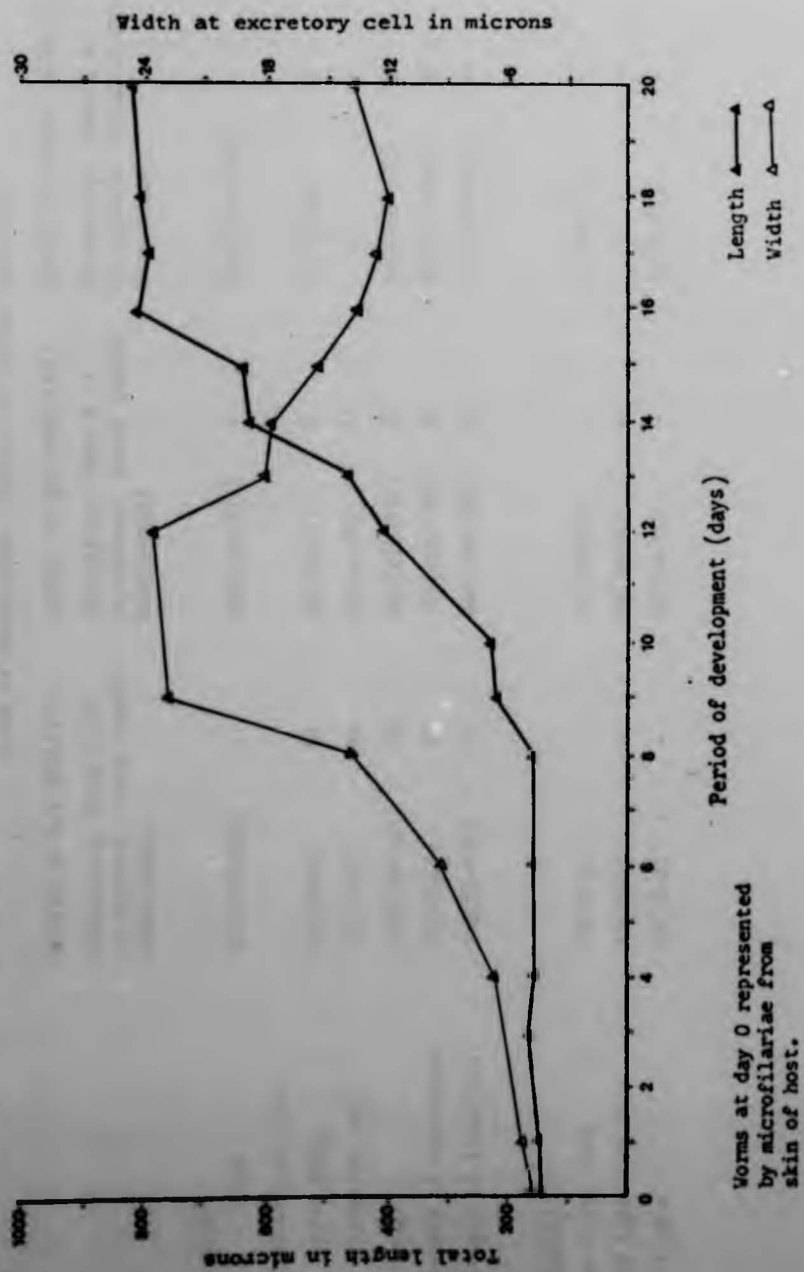


TABLE 21Measurement of Ackertia globulosa larvae from Haemaphysalis leachii.

	Stage of development (number of larvae measured)					
	LARVAE AT 1st MOULT(18)		LARVAE AT 2nd MOULT(20)		INFECTIVE STAGE LARVAE(20)	
	Dimensions in microns Mean(range)	Mean % of total length	Dimensions in microns Mean(range)	Mean % of total length	Dimensions in microns Mean(range)	Mean % of total length
<u>LENGTHS</u>						
Total	218(163-269)	-	448(344-557)	-	812(713-1016)	-
Anterior end to:-						
nerve ring	32(20-49)	15	32(16-57)	7	72(57-98)	9
excretory cell	53(39-59)	24	64(49-82)	14	-	-
anus	185(134-229)	85	407(295-516)	91	748(648-926)	92
Length of oesophagus	79(65-92)	36	136(107-197)	31	213(180-246)	26
Length of intestine	110(82-147)	51	264(164-328)	59	517(439-597)	64
<u>WIDTHS</u>						
At nerve ring	20(14-26)	-	21(16-26)	-	13(10-16)	-
At excretory cell	22(16-28)	-	24(16-30)	-	-	-
At anus	20(16-27)	-	22(14-30)	-	12(9-15)	-

Morphology of larvae during development (Table 21; Figures 21-27)

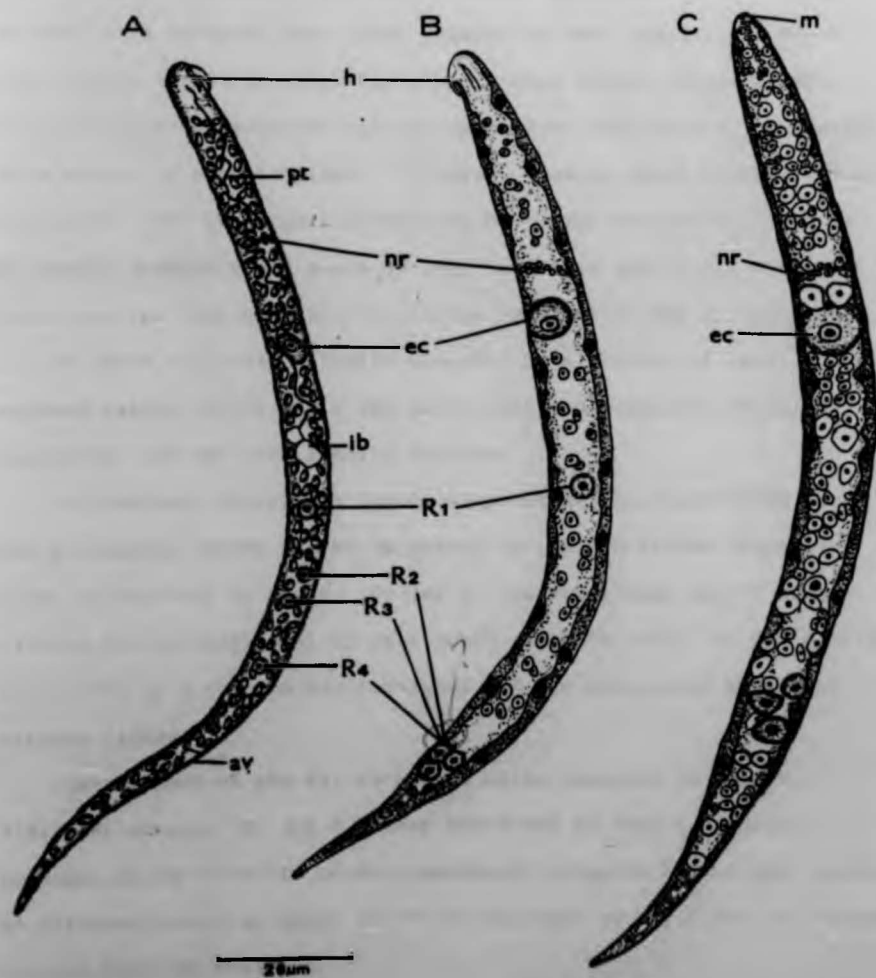
The microfilaria (1-3 days post infection; Figure 22A)

A description of the sheathed microfilaria, as extracted from host skin, is given in Chapter 4.

The exsheathed microfilaria recovered from ticks was slender with an evenly tapered tail and rounded head. It measured 147 microns long by 5.4 microns wide, based on specimens preserved in alcohol-glycerine. The cuticle was finely striated. The individual nuclei of the nuclear column were tightly packed. At the anterior extremity, a large cephalic hook with associated "Mundegebilde" could be seen. The cephalic space, inner body and anal vesicle appeared as clear zones in the nuclear column. A dark, refractile pharyngeal thread stretched from the cephalic space to the inner body. The nerve ring was difficult to distinguish but in some specimens could be seen as a band of minute nuclei suspended in a clear space. Below it, a large excretory cell with prominent nucleus and nucleolus filled almost half the width of the body. The RI cell was large and distinct, visible immediately below the inner body, but the 3 remaining R cells (R2, R3 and R4) were only poorly differentiated in most specimens.

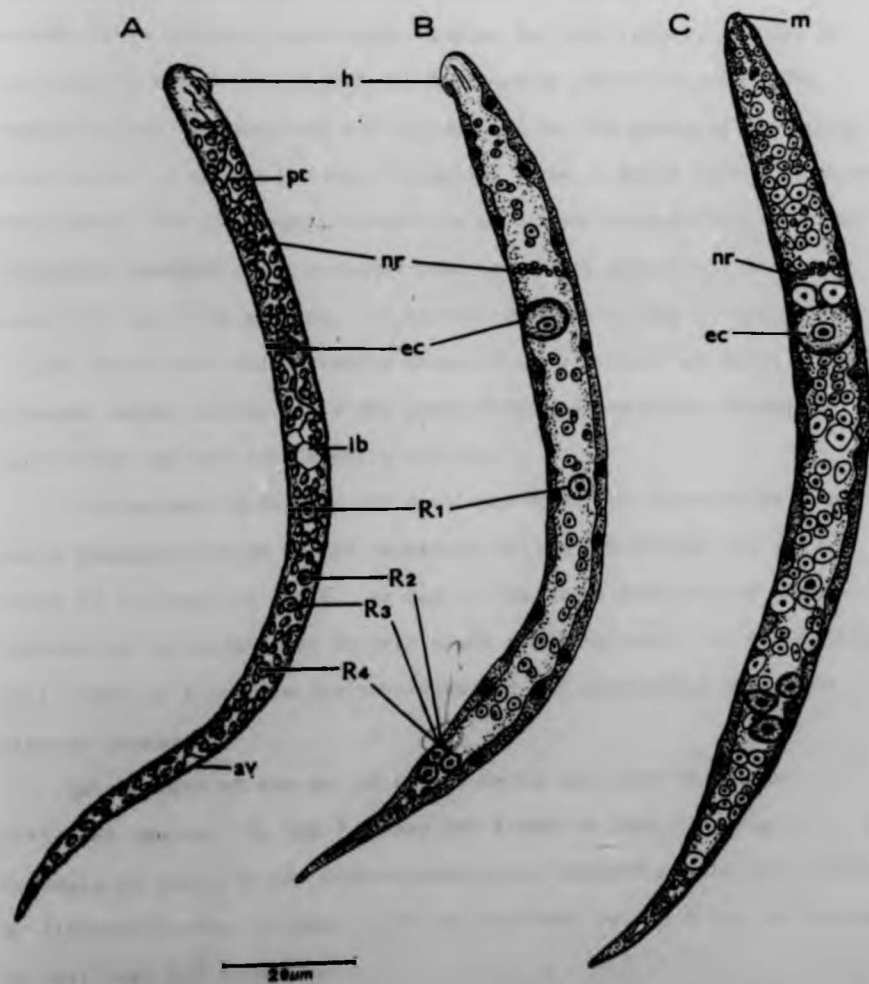
Early first-stage larva (3-6 days post infection; Figure 22B and C, Figure 23A)

Early first-stage larvae showed little change in length but a marked increase in width. At the end of this stage they measured 167 microns long by 11.4 microns wide. Larval growth appeared to be due



The early first-stage : A Exsheathed microfilaria on day 1 ( lateral view ). B First-stage larva on day 3 ( lateral view ). C First-stage larva on day 6 ( dorsal view ).





The early first-stage : A Exsheathed microfilaria on day 1 ( lateral view ). B First-stage larva on day 3 ( lateral view ). C First-stage larva on day 6 ( dorsal view ).

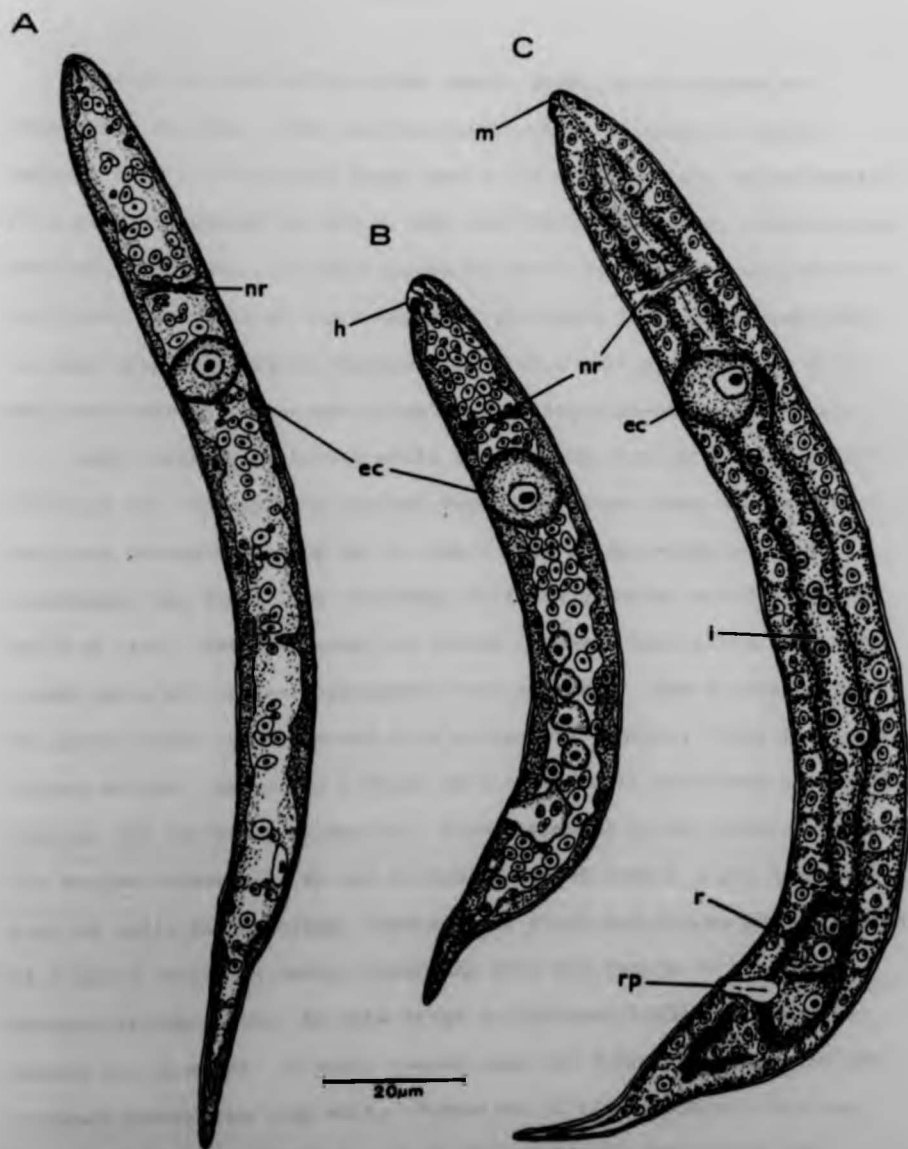


mainly to an increase in cell size and the pseudocoel contained large, scattered cells with prominent nuclei. The increase in width was not uniform and some larvae showed a bulge at the excretory cell from which they tapered to either end. The tail retained its narrow, microfilarial appearance and the cuticular striations, which faded as the larva widened, were still visible in this region. By day 6 the head of the first-stage larva had become rather pointed. The cephalic hook was retained and was opposed at the anterior extremity by a series of minute spines. In dorsal view, a small mouth could be discerned. The pharyngeal thread was no longer visible but a column of small, rounded cells could be seen tracing a path 10-12 microns long from the oral opening. In larvae examined on day 3, the position of the nerve ring was virtually obscured by a cluster of small, closely aligned cells, but by day 6 the nerve ring had assumed a granular appearance and was more readily visible.

A prominent feature in the development of the first-stage larva was a dramatic growth of the excretory cell which filled the entire width of the body by day 6. By day 3, the inner body was no longer visible but an individual R1 cell could still be seen. On day 6 this cell could no longer be distinguished and had presumably undergone mitotic division.

Development of the R2, R3 and R4 cells appeared to follow a different course. By day 3, these had fused to form a discrete triangle of cells in the region previously occupied by the anal vesicle. No differentiation of these cells was apparent by day 6 but an increase in cell size had occurred.

**FIGURE 23** DEVELOPMENT OF ACIERTIA GLOBULOSA IN TICKS



The late first-stage : **A** First-stage larva on day 6 ( ventral view ). **B** First-stage larva on day 8 ( lateral view ). **C** First-stage larva on day 9 ( lateral view ).

Late first-stage larva (6-9 days post infection: Figure 23B  
and C)

Towards the end of the first larval stage, worms became progressively stouter. This was the result of an increase in width between days 8 (14 microns wide) and 9 (22 microns wide) unaccompanied by a marked increase in length (159 and 218 microns long, respectively). However, throughout the development of first-stage larvae no reduction in length was observed (that depicted in Figure 23B being exceptional in this respect). It is therefore concluded that A. globulosa does not pass through a "sausage stage" in its development in the vector.

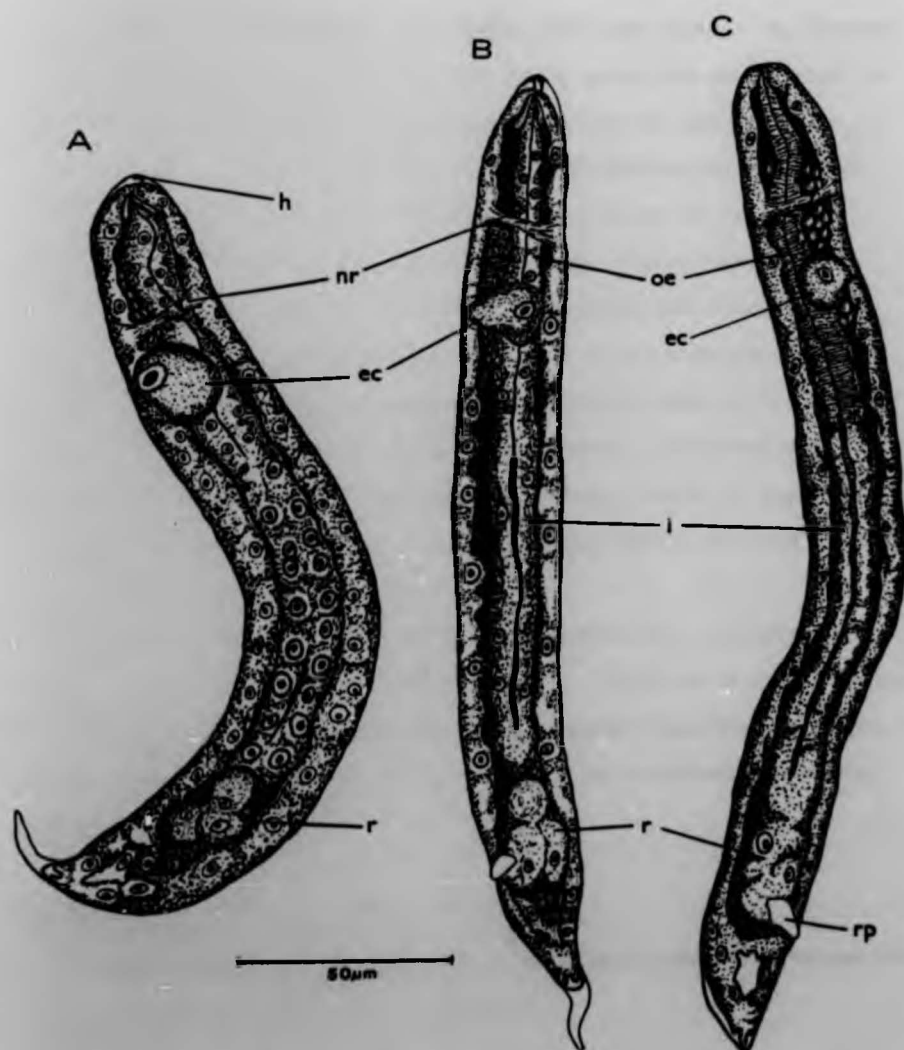
Late first-stage larvae still retained the narrow tail of microfilariae and tapered to a pointed head. The nerve ring was less conspicuous on day 8 than by day 9 when a clearly discernable nerve commissure was seen. The excretory cell was granular and had reached maximum size. The pseudocoel of larvae on day 8 was filled with large round cells but little organization was apparent. The fusion of R2, R3 and R4 cells had coalesced into a discrete vesicle; this formed the future rectum. Above it, a chain of 5 large cells stretched anteriorly towards the excretory apparatus; these appeared to be precursors of the future intestine. By day 9 it was evident that a rapid organization of cells had occurred. The enteric tract had formed and consisted of a solid column of cells stretching from the rectum to within 20 microns of the mouth. At this stage no differentiation of the oesophagus was apparent. A small rectal plug had formed but this did not protrude beyond the body wall. Formation of the hypodermis had been initiated and this appeared as a border of tissue underlying the cuticle.

Larvae at the first moult (9-10 days post infection: Figure 24A and B)

Larvae were stout on entering the first moult and measured 218 microns long by 22 microns wide (Figure 24A). These were comparable in size to the most advanced first-stage larvae. During the process of moulting, separation of the first-stage cuticle could be seen at anterior and posterior ends; the hook remained attached to the cuticle and was shed at the moult. Moulting larvae underwent rapid development while still invested in the first-stage cuticle. In some, elongation in length, differentiation of the gut and development of a body cavity had progressed to a marked extent before moulting was complete (Figure 24B). Retention of the cuticle during the second stage of development was not observed. Differentiation of the gut into oesophagus and intestine became apparent, but muscular and glandular regions of the oesophagus had not yet developed. The gut occupied 87% of the total length and stretched from a well formed rectum to the oral opening. 70% of the length of the gut comprised the intestine. There was a small, non-protruding rectal plug. In larvae completing the moult, a double row of cell nuclei running along the length of the gut became separated by the formation of a gut lumen.

During the moult, the progressive development of a body cavity was observed working anteriorly from the region around the rectum. Suspended in the body cavity in the vicinity of the nerve ring were clusters of ganglionic cells. Towards the end of the moult, a reduction in the size of the excretory cell became apparent.

**FIGURE 24** DEVELOPMENT OF ACKERTIA GLOBULOSA IN TICKS



The first moult and second-stage : A First-stage larva entering the moult, day 9 ( lateral view ). B Larva at first moult, day 9 ( lateral view ). C Second-stage larva on day 10 ( lateral view ).

Second-stage larva (10-13 days post infection: Figure 24C)

Growth of larvae during the second stage was rapid. The process of elongation begun at the end of the first moult was accelerated so that worms measuring 230 microns long on day 10 had reached 416 microns long by day 12. However, increase in girth had stabilised so that larvae that measured 23 microns wide on day 12 appeared relatively slender compared with shorter first-stage worms.

Second-stage larvae had a characteristically blunt appearance at the anterior and posterior end. The tip of the tail bore 5 small caudal appendages. The gut showed a considerable degree of differentiation: Striations were visible on the muscular oesophagus and a well-defined gut lumen had developed within the full length of the intestine. However, the lumen stopped short of the rectum which remained blocked by a rectal plug.

A noticeable change in second-stage larvae was a progressive reduction in the size of the excretory cell. This was still large at the end of the second stage but by then occupied less than half the width of the body. Definition of the hypodermis increased markedly during the second stage.

Larvae at the second moult (13-14 days post infection: Figure 25A and B)

Larvae entering the second moult were long and thick measuring 448 microns long by 24 microns wide. The second-stage cuticle separated from the body at anterior and posterior ends (Figure 25A) and then detached along the full length of the body, leaving the larva invested



in a loose fitting sheath (Figure 25B). Within this sheath were large brown globules of an irregular shape. During the moult there appeared a short refractile thread at the anterior extremity of the larva connecting it to the old cuticle. As the cuticle was shed, the thread was extruded from the oesophagus: In early third-stage larvae a vestige of this thread was frequently seen emerging from the buccal cavity.

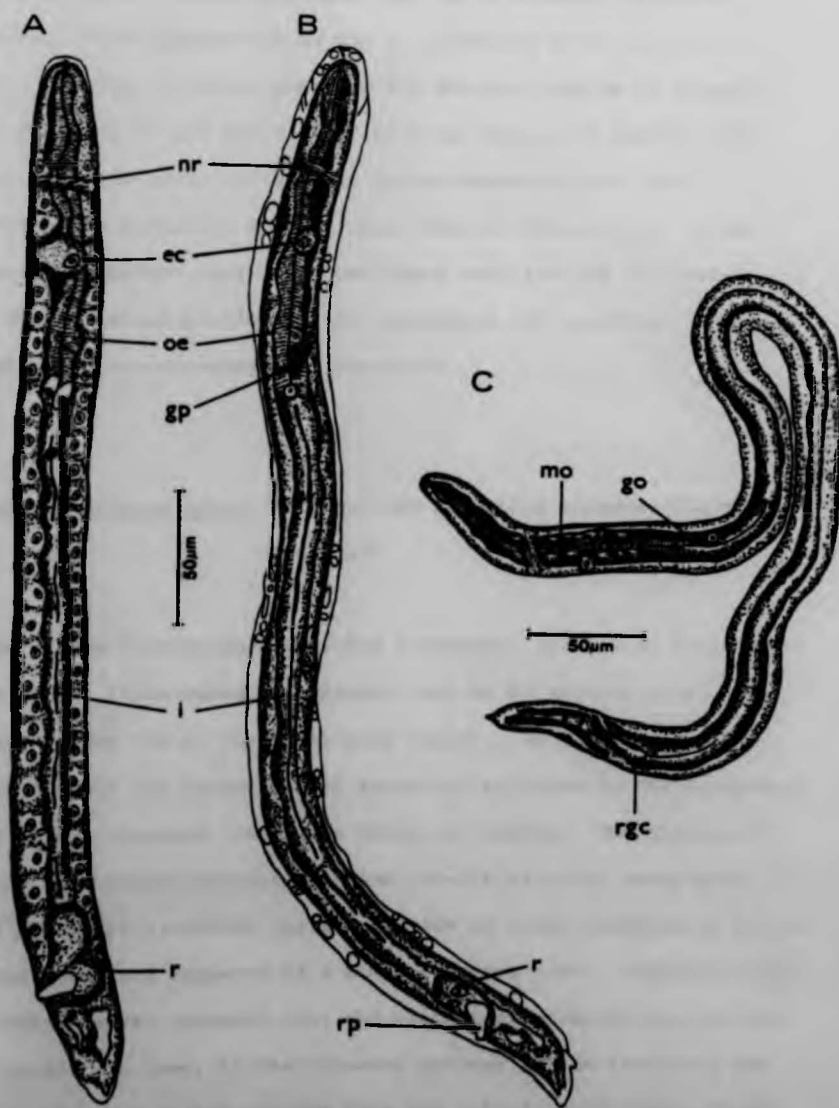
Larvae continued to develop while invested in the second-stage cuticle. Before moulting was complete, a small buccal cavity had formed at the anterior end and the gut had come to occupy 90% of the total body length. The oesophagus, which comprised 34% of the gut length, was greatly expanded posterior to the excretory cell but as yet had not developed a defined glandular region. Growth of the intestine had been slower than for the oesophagus. It now comprised 66% of the overall gut length with a well developed lumen. However, this remained discontinuous at the rectum which bore an enlarged rectal plug protruding from the anus.

The nerve ring was situated 32 microns from the anterior end and appeared to contain granulo-fibrinous material. Below it was a greatly reduced excretory cell. The genital primordium was seen for the first time and appeared as an ovoid cluster of small cells, situated immediately anterior to the oesophageal-intestinal junction. The hypodermis was little changed from that in advanced second-stage larvae.

Early third-stage larva (14-18 days post infection: Figure 25C)

Immediately after the second moult, larvae underwent rapid elongation and became progressively narrower. Those that had recently moulted

**FIGURE 25** DEVELOPMENT OF *ACKERTIA GLOBULOSA* IN TICKS



The second moult and third-stage : A Second-stage larva entering the moult, day 12 ( lateral view ). B Larva at second moult, day 13 ( lateral view ). C Early third-stage larva on day 14 ( lateral view )

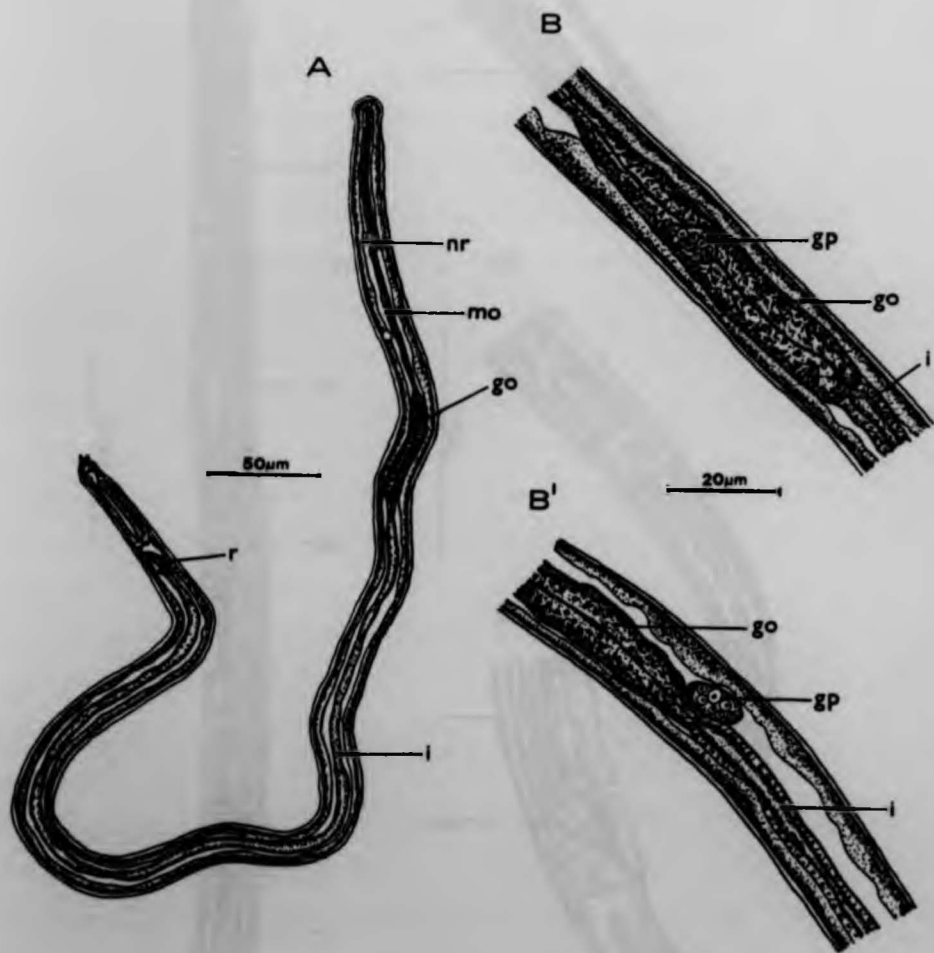


could readily be distinguished from "mature" third-stage larvae. Gross examination showed them to be shorter and stouter than infective forms, and in living specimens they had a somewhat granular appearance: This appeared to be due to retention of intestinal contents. On day 14 larvae measured 633 microns long by 18 microns wide, but by day 17 had reached 796 microns long by 13 microns wide. In other respects early third-stage larvae resembled the later, fully developed infective stage forms. Even in the earliest third-stage worms, a patent rectum had developed with loss of the rectal plug. The posterior portion of the oesophagus was glandular. Only the vestige of an excretory cell remained.

Infective-stage larva (18 days post infection onwards: Figures 26 and 27)

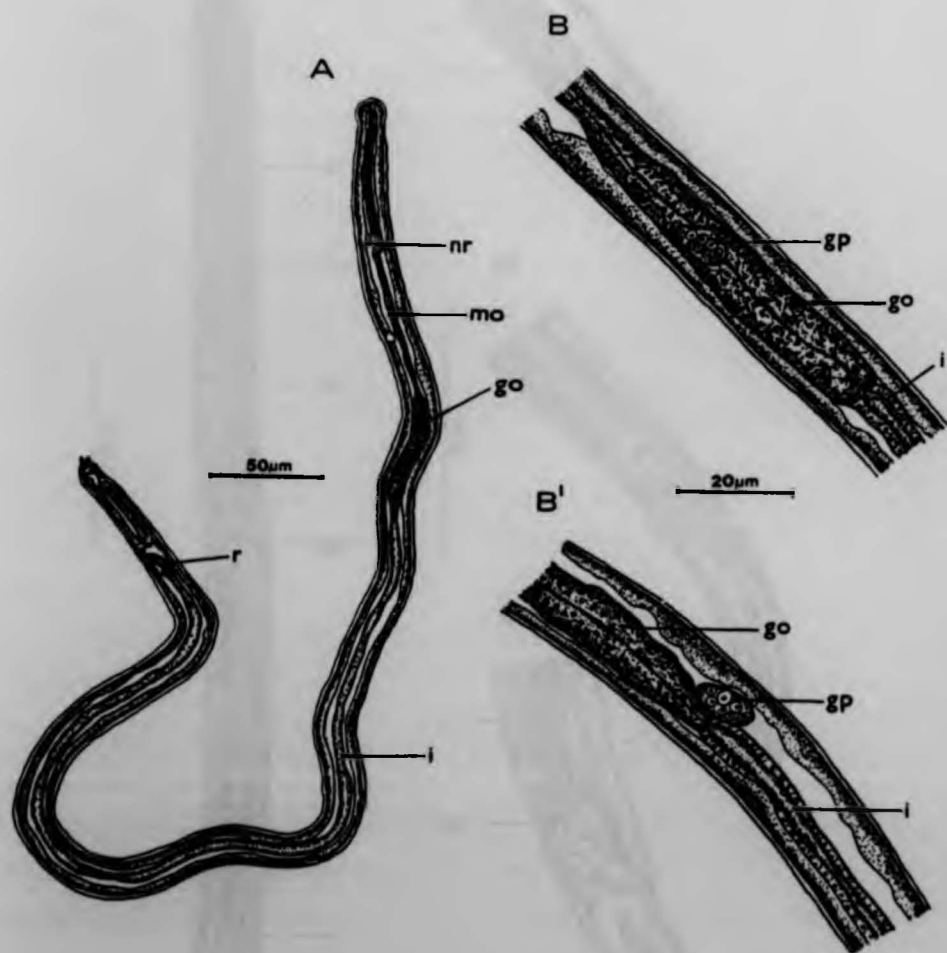
The mature third-stage larva was a slender, filariform organism (Figure 26A). It measured 812 microns long by 13 microns wide. The gut extended for 92% of the total body length. In the period since the second moult the intestine had elongated relative to the oesophagus so that it now occupied 72% of the total gut length. The oesophagus consisted of a narrow muscular portion (54-81% of total oesophageal length) and broad glandular portion (19-46% of total oesophageal length), while the intestine appeared as a simple, narrow tube. Examination of 20 infective larvae revealed that the genital primordium occupied one of two sites: In some, it was situated mid-way in the region of the glandular oesophagus (161 microns from the anterior extremity, Figure 26B), while in others it occurred at, or immediately posterior to, the oesophageal-intestinal junction (237 microns from the anterior extremity,

**FIGURE 26** DEVELOPMENT OF ACKERTIA GLOBULOSA IN TICKS

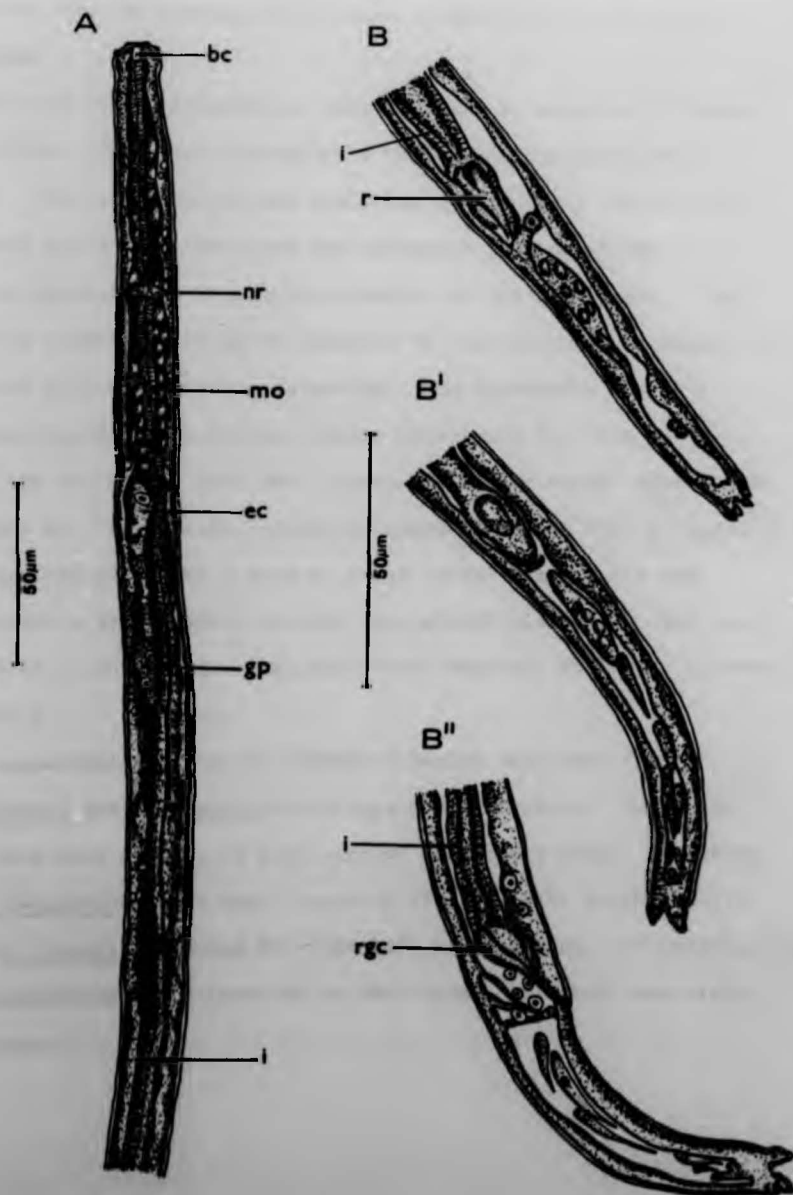


The infective stage : A Infective larva on day 18 ( lateral view ).  
 B Detail of a "female" larva showing the position of the genital primordium. B' Detail of a "male" larva showing the position of the genital primordium.

**FIGURE 26** DEVELOPMENT OF ACKERTIA GLOBULOSA IN TICKS



The infective stage : A Infective larva on day 18 ( lateral view ).  
 B Detail of a "female" larva showing the position of the genital primordium. B' Detail of a "male" larva showing the position of the genital primordium.



The infective stage : A Detail of the head of the infective larva. B Detail of the tail ( ventral view ). B' Detail of the tail ( dorsal view ). B'' Detail of the tail ( lateral view ).

Figure 26B<sup>1</sup>). This probably represents a sex difference between larvae with females possessing the more anteriorly placed genital primordium.

The head of the third-stage larva was almost invariably bulbous (Figure 27A). This bore 2 rings of 4 circumoral papillae and 2 amphids. The buccal cavity was small and cylindrical. Clusters of ganglionic cells stretched from the atrophied remains of the excretory apparatus to a position anterior to the nerve ring. The nerve ring itself was situated anterior to the glandular oesophagus, 72 microns from the anterior extremity. The hypodermis was well organized into discrete lateral chords underlying the thin cuticle.

At the tip of the tail were 3 large, ear-like caudal appendages, alternated by 3 far smaller caudal appendages (Figure 27B, B<sup>1</sup> and B<sup>11</sup>). The rectum was served by a pair of large rectal gland cells and emptied into a simple anus situated on a slight elevation. The tail measured 63 microns long. The anal ratio (Wharton, 1957) was between 5.5 and 6.5.

A comparison was made of infective larvae collected from R. sanguineus and H. leachii at 30 days post infection. Worms collected from both species of tick were of comparable size: 15 larvae from R. sanguineus had a mean length of 795 (734-825) microns while 15 from H. leachii measured 810 (698-934) microns long. No abnormalities in worm morphology were observed in the larvae collected from either tick species.

b) Histological studies on *A. globulosa* in ticks

Three to 12 ticks were fixed on each of 16 occasions at 1, 2, 3, 5, 7, 8, 10, 12, 13, 14, 16, 20, 22, 29, 36 and 70 days post infection. Two uninfected ticks were also included as controls for observations on the histopathology of infection.

Ticks were fixed in Dubosc-Brazil for 24 hours, dehydrated in alcohol, and transferred to Supercedrol (B.D.H. Ltd.) for 3-7 days. These were then vacuum embedded in paraffin wax and serially sectioned in sagittal plane at 5 microns thickness. The sections were stained in haematoxylin and eosin.

Observations

Over half the ticks examined by histology (69% of 75) contained developing larvae of *A. globulosa*.

During the first 24 hours of attachment, scarcely any blood was ingested by ticks but many microfilariae had been taken up. Some were present in the lumen of the midgut while others were found crossing the gut epithelium in various regions of the body. At 1 and 2 days post attachment some sections of microfilariae were seen within the columnar cells of the gut epithelium, lying parallel to the basement membrane. Here they appeared to traverse at least 2 epithelial cells before emerging into the haemocoelic space around the gut.

Microfilariae that had escaped from the midgut entered the layer of epidermal cells underlying the cuticle. Here they penetrated the layer of cells at any point, and by piercing intercellular membranes came to lie within the bed of epidermal cells. Within this tissue it

was observed that individual microfilariae occupied several cells to accommodate their size (Plate 25). Some microfilariae entering the epidermis within the first 48 hours were seen with part of their length free in the haemocoel, which they presumably had to cross. However, by the third day after tick attachment the gut caecae had been distended with blood to such an extent that migration of the parasites occurred directly from the gut wall into the epidermis. On the fifth and eighth days after infection the migration of microfilariae appeared to have ceased and those remaining in the gut were trapped in blood meals which showed signs of partial digestion.

Larvae in the epidermis on days 3 and 5 were of greater diameter than microfilariae. Evidence of growth was also given by the occasional appearance of mitotic figures in developing larvae. Up to 10 days after infection a clear zone was seen around larvae in the cytoplasm of epidermal cells which was rarely observed around microfilariae. However, it is not known whether this cavity was the result of larval digestion of host tissue or merely a shrinkage artefact due to fixation.

During the first 2 days of tick attachment, epidermal cells were columnar in appearance and underwent many mitotic divisions. Despite the increase in the number of cells, these became stretched by the third day of feeding as the softened alloscutum began to swell. This meant that microfilariae and developing larvae present in the epidermis towards the end of engorgement were highly constricted within the tissues. By day 8, however, epidermal cells had reverted to their original shape which allowed for the girth of late first-stage larvae (Plate 26).

Much of the substance of epidermal cells appeared to be destroyed in the region of developing worms. As early as day 3, the presence of microfilariae in the epidermis displaced cell nuclei within the



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*leachi*

10  $\mu$ m

Plate 25. *Microfilaria* of *A. globulosa* in the epidermis of the tick vector, *H. leachi*, 1 day after infection.



30  $\mu$ m

Plate 26. Late first-stage larva of *A. globulosa* in the epidermis of *H. leachi*, 8 days after infection.

cytoplasm. Many nuclei in parasitised cells of infected ticks were pyknotic in character. As larvae grew in length, interconnecting cell membranes within the epidermis were progressively obliterated. Nevertheless, plasma membranes above and below the larvae usually remained intact up to day 8, holding them in place within the disrupted tissue.

Between 8-10 days post attachment much reorganization was evident in pharate nymphs. Epidermal cells showed a marked proliferation and were larger than in the unfed nymph and could be seen to secrete the new cuticle external to the epidermis. Internally, the fat body of nymphs became apparent as clumps of cells underlying the epidermis. The development of an exuvial cavity commenced on day 8 and progressed to day 13, when most ticks moulted. 12-5-18  
day 1

At 8 days post infection some larvae remained within the intact layer of proliferating epidermal cells; others were seen emerging from the epidermis into the bed of underlying fat cells (Plate 27). By day 10, all larvae appeared to be in the process of breaking out of the epidermis. In some instances the entire length of a larva could be traced through serial sections, coiled tightly within an individual fat cell (Plate 28); However, others remained with portions of their length in both epidermis and fat cells. In the main, larvae in the fat body occurred in those cells immediately beneath the epidermis.

Between 10-13 days post infection larvae in the fat cells showed signs of rapid development: In some sections of worms a clearly defined gut and body cavity could be distinguished. Parasitised fat cells suffered severe damage. In ticks 12 and 13 days after infection much of the cytoplasm of these cells had been destroyed and only the remains of cell nuclei were left (Plate 29). However, as with earlier larval stages in the epidermis, those in the fat body caused only local damage.



30 μm

Plate 27. Late first-stage larva of A. globulosa leaving the epidermis to enter the fat body of H. leechii, 8 days after infection.



30 μm

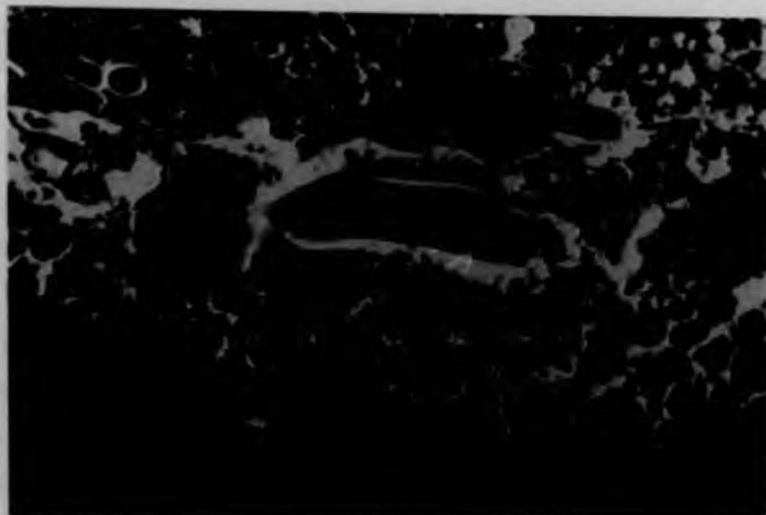
Plate 28. Second-stage larva of A. globulosa coiled within a fat cell of H. leechii, 10 days after infection.

The majority of ticks examined 14 and 16 days after infection had already moulted. The appearance of moulted ticks was quite different from that of the pharate nymph. Little evidence remained of the previous blood meal and the gut caecae had contracted to a size seen in unfed ticks. At 14 days post infection, a number of worms had left the fat cells and entered the haemocoel. Sections of larvae were seen between branches of the gut caecae and in spaces between the gut and cells of the fat body (Plate 30). From 16-70 days post infection all larvae were observed in the haemocoel.

Few abnormalities were observed in the development of A. globulosa in H. leachi. Only in one instance was larval development in the fat body witnessed before day 8: This was in the case of a tick examined 5 days post infection. Possibly this worm was a precocious second-stage form as in the same tick others were developing normally in the epidermis. Two ticks examined 22 and 29 days post infection contained larvae in the muscle fibres of the legs. These worms appeared to be third-stage forms and the muscles they occupied were somewhat necrotic. Whether the larvae had actually developed in the muscle fibres or merely penetrated them during their migrations through the haemocoel could not be determined: However, no earlier developmental stages were observed in the muscles of infected ticks.

#### Summary of the histopathology of parasitised tissues

A. globulosa inflicted only moderate damage to its arthropod host at the intensities of infection seen in this study. Nevertheless, severe local damage to the tissues was inevitable in view of the large size of larvae relative to the tick cells they parasitised.



30  $\mu$ m

Plate 29. Fat cell of H. leachii occupied by a second-stage larva showing the disruption of cell structure, 12 days after infection.



30  $\mu$ m

Plate 30. Third-stage larva of A. globulosa in the haemocoel of H. leachii, 14 days after infection.

The epidermis of nymphal ticks showed remarkable powers of regeneration after infection: Infected ticks examined from day 16 onwards revealed no signs of damage to the epidermal cell layer. This was probably because many larvae had already left the epidermis for the fat body by the tenth day after infection before the proliferation of cells in this tissue reached a peak. Therefore, the repair and replacement of damaged epidermal cells could readily be accomplished following previous infection. However, the recovery of fat cells after infection could not be assessed as it was observed that these normally underwent degenerative changes in fasting, uninfected ticks.

#### Spatial distribution of *A. globulosa* in the vector

##### a) Distribution of developing larvae in fasting ticks

From the histological examination of infected ticks, the distribution of first, second and third stage larvae was plotted within various regions of the arthropod body (Figure 28). This was done by making camera lucida drawings of ticks cut in sagittal section and marking the positions of sections of larvae. Only sections of worms were counted as in many cases it proved impossible to trace whole parasites with certainty. The positions of first, second and third stage larvae were recorded in randomly selected tick sections at 2-8, 9-13, and 20-70 days post infection respectively.

b) Distribution of infective larvae in feeding ticks

Twelve nymphal ticks were given the opportunity to reattach to a rodent, 42 days after feeding on a heavily infected striped mouse. These were removed in batches of 4 after 6, 48 and 72 hours reattachment to the host and processed for histological examination. The distribution of third-stage larvae was then assessed as described above.

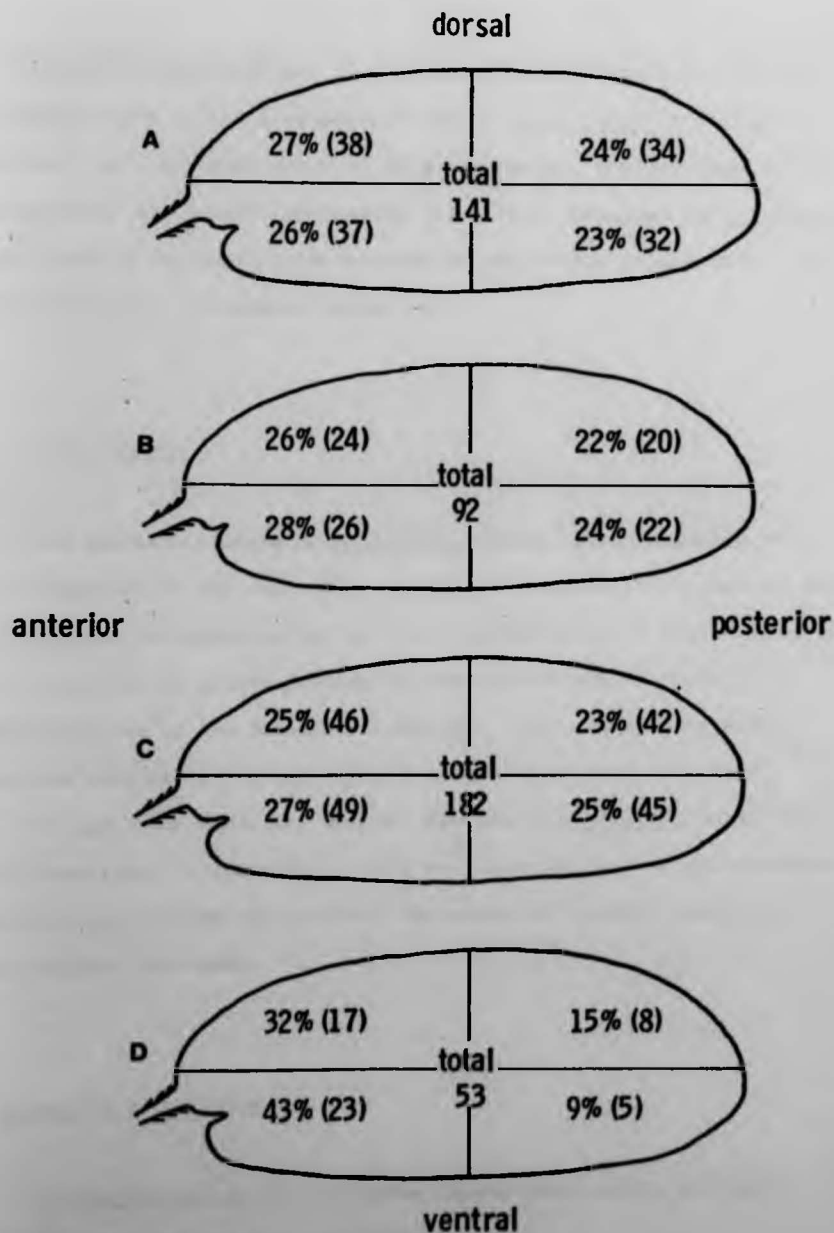
Results

The camera lucida drawings made of sectioned ticks were divided into 4 by bisecting them with lines along horizontal and vertical axes. For each stage of larval development, the cumulative number of worm sections present in each sector of the tick was determined and expressed as a percentage of the total number of sections counted. The results of this study are depicted diagrammatically in Figure 28.

All stages of A. globulosa were randomly distributed in fasting ticks. However, the distribution of infective larvae in ticks examined after 6 hours reattachment to the host appeared to be significantly different. In these cases, most larvae tended to be present in the anterior region of the body, especially around the salivary alveoli and ducts. Unfortunately, no worms were seen outside the haemocoel so the route of escape of infective larvae from feeding ticks could not be elucidated. As no worms were found in any of the 8 nymphs examined after 48 and 72 hours reattachment, it may well be that the escape of infective larvae occurs relatively early in the process of feeding.



**FIG. 28** DIAGRAMMATIC REPRESENTATION OF SAGITTAL SECTIONS THROUGH <sup>209</sup>  
H. LEACHII TICKS SHOWING SPATIAL DISTRIBUTION FOR SECTIONS  
 OF A. GLOBULOSA LARVAE



Distribution of : A First stage larvae in epidermis, 2-8 days post infection. B Second stage larvae in fat body and epidermis, 9-13 days post infection. C Third stage larvae in haemocoel, 20-70 days post infection. D Third stage larvae 6 hours after reattachment of tick to host.



### Responses of the vector to *A. globulosa*

Earlier in this chapter it was shown that different biotypes of *H. leachii* vary in their susceptibility to *A. globulosa* infection. To study the underlying cause of this phenomenon, the Machakos (highly susceptible) and Nairobi (partially refractory) biotypes of *H. leachii* were examined for possible mechanisms of resistance to infection using dissection and histological techniques.

#### Observations

The Machakos biotype of *H. leachii* showed little evidence of a host reaction and the main cause of worm death appeared to result from the trapping of microfilariae in the coagulating blood meal. This did not constitute an active process of destruction and accounted for relatively few of the parasites ingested. Only rarely were microfilariae encountered in blood meals after 3 days post infection.

On the other hand, the Nairobi biotype of *H. leachii* exhibited much resistance to infection. This was directed against all developmental stages of the parasite and was mediated through encapsulation and cellular reactions.

#### Encapsulation reactions

The encapsulation of developing larvae began early in their migration through the body of ticks.

Reactions against microfilariae appeared to be of 2 distinct

types. The first was directed against microfilariae while still in the blood meal. In tick sections examined 3 days after infection, a proportion of microfilariae were found to be surrounded by small plaques of pigmented material. These appeared to coalesce around the worms, encapsulating them in a tightly fitting tube. However, some microfilariae seemed able to escape encapsulation: Empty tubes containing no microfilariae, but of equivalent diameter (5 microns), were seen scattered in the blood meals of several ticks.

The second type of reaction to microfilariae was confined to those in the process of crossing the haemocoel. In sections of ticks 3-16 days after infection microfilariae were seen enveloped in a thick wall of host material. As histochemical stains were not employed the precise nature of this material could not be determined; however, in sections stained with haematoxylin and eosin it appeared a bright yellow. Microfilariae encapsulated in this second type of reaction were effectively stopped from reaching intracellular sites. In some cases, whole worms became enclosed in the host material. In others, sections of microfilariae were found half buried in the epidermis but with the portion of body trailing in the haemocoel thickly encapsulated.

Larvae provoked a less marked, but no less frequent host reaction after entering intracellular sites of development. Between 8 and 22 days after infection, a proportion of worms had become surrounded by concentric rings of pigmented material. In its extreme form, several layers of host material were laid down around the developing parasites. As a number of encapsulated worms were still present in the epidermis as late as 22 days after infection, it may well be that these had been killed or their development arrested. An alternative hypothesis could be that reactions occurred around dead parasites.

On entering the haemocoel, third-stage larvae provoked an

encapsulation reaction similar to that elicited by microfilariae. In sections of ticks 16-29 days after infection, larvae were observed that were surrounded by a thick wall of host material (Plate 31). Worms recovered from ticks at dissection revealed that the encapsulating material enveloped them completely. A morphological examination of these larvae showed that the integrity of worm structures had broken down: This was confirmed in sections of larvae examined histologically (Plate 31).

#### Cellular reactions

Associated with the encapsulation of parasites in the haemocoel was a marked infiltration of haemocytes. In transverse sections of larvae in ticks, these were observed encircling the worms.

In many instances, however, the concentration of haemocytes around third-stage larvae was unaccompanied by encapsulation (Plate 32). Layers of haemocytes 3 cells deep were commonly seen surrounding the parasites. However, unlike the case of encapsulated worms, those surrounded by haemocytes alone did not appear necrotic.

There seems little doubt that encapsulation and cellular reactions seen in Nairobi ticks were the result of infection with A. globulosa. No accumulations of haemocytes or deposits of pigmented material were observed except around filarial larvae. Nevertheless, the resistance of Nairobi ticks to A. globulosa was far from absolute. In some ticks, no reactions whatever were observed, while in others only a proportion of the larvae present had been encapsulated. In general, however, ticks that reacted to developing worms did so to the majority present. No attempt was made to quantify these effects.

30  $\mu$ m

Plate 31. Encapsulation of a third-stage larva of Aglobulosa in the hamocoel of H. leechii (Nairobi biotype), 22 days after infection. Note also the accumulation of hamocytes around the parasite.

30  $\mu$ m

Plate 32. Third-stage larva of Aglobulosa surrounded by hamocytes in the hamocoel of H. leechii (Nairobi biotype), 16 days after infection.

### The infectivity of third-stage larvae

Third-stage larvae were described earlier in this chapter and differentiated into "early" and "infective" forms on morphological grounds. Larvae of the latter type were far more vigorous.

In Nairobi ticks it was noticed that a large proportion of third-stage larvae failed to develop to typically slender, infective forms. In some ticks, 100% of the worms present as late as 30 days after infection were still early third-stage larvae. After an equivalent period of infection in Machakos ticks, less than 5% of the worms remained at this stage.

To see if differences in the appearance and motility of third-stage larvae had a bearing on their infectivity to rodents, 2 groups of jirds were inoculated with "early" and "infective" third-stage worms. All of the parasites were derived from Nairobi ticks 30 days after infection so that sufficient numbers of both types of larvae could be obtained from the same batch of infected ticks.

Four jirds were given 35, 37, 36 and 38 early third-stage larvae, and a second group of 4 were given 30, 30, 32 and 43 infective-stage larvae. The parasitological success of the infections in these animals was monitored by examining them by skin-snip for microfilariae, 60-200 days after infection. Animals which failed to develop patent infections within 200 days were autopsied.

### Results

Patent infections developed in 3 of 4 jirds inoculated with infective form larvae but none of 4 given early third-stage larvae.

On autopsy, no worms were recovered from any of the "negative" animals. This result suggests that the change seen in larvae as they develop from early to infective third-stage worms in the haemocoel of the vector is an indication of them acquiring the capacity to infect their definitive host.

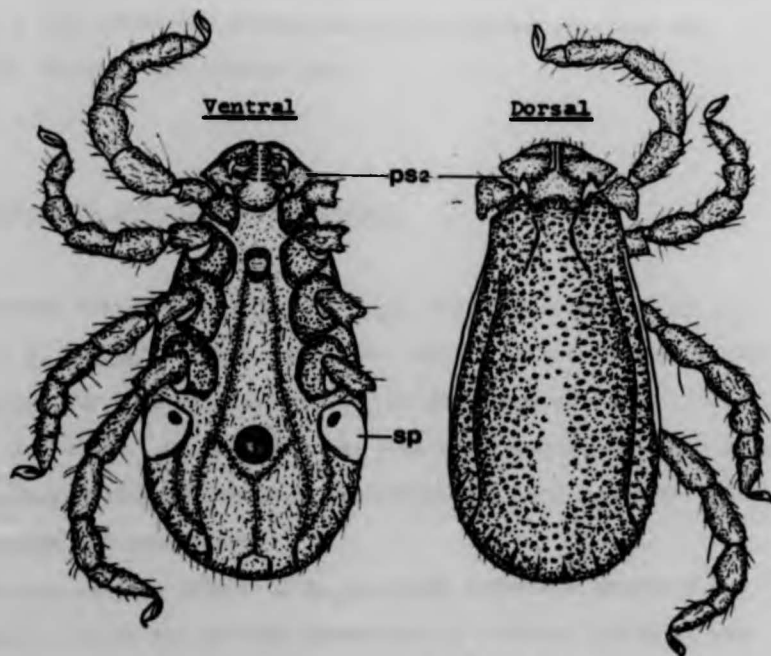
#### Morphology of the Nairobi and Machakos biotypes of *H. leachii*

As Nairobi and Machakos ticks exhibited different susceptibility to *A. globulosa* infection, an attempt was made to find morphological characters that might be used to differentiate the 2 biotypes.

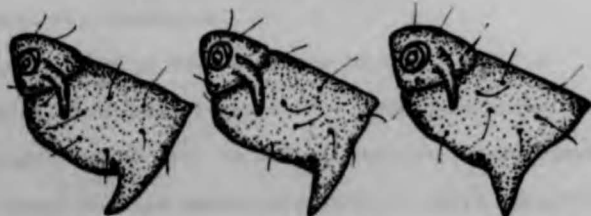
Hoogstraal (1975 - personal communication) suggested that the direction of the postero-external spur of palpal segment 2 might be a useful clue in distinguishing various forms of *H. leachii* and that the shape of the spiracular plates might provide an additional lead. To explore the possibility of using these features, 20 male ticks of each biotype were examined.

#### Results (Figure 29)

The results of this study were disappointing as biotypes of *H. leachii* from Nairobi and Machakos could not be differentiated on the characters examined. Differences were found in the shape of spiracular plates but these showed as much variation within each biotype as between them. A similar picture emerged with the direction of the postero-external spur on palpal segment 2: The spur was directed either posteriorly, posteromedially, or in almost every



Variation in direction of the basal spur on  
palpal segment 2 (ps2)



Variation in shape of the spiracular plate (sp)





intermediary position. Because of the inconsistency in both of these characters, neither could be used to differentiate H. leachii ticks. However, a more extensive search for distinguishing features was beyond the scope of the present work.

#### Gross effects of infection on H. leachii

Moderate infections of A. globulosa were well tolerated by all stages of H. leachii. Ticks fed on the ears of infected rodents with 5-417 mff/mg survived equally well and no mortality could be attributed to infection. Of the few ticks that died within one month after feeding, many contained few or no microfilariae. The cause of this mortality was not ascertained.

Only one adverse effect of A. globulosa infection in ticks was identified. During the routine dissection of infected ticks it was noticed that those containing large numbers of infective larvae were often markedly undersized. To investigate this phenomenon further, the following experiment was performed.

Nymphal stages of H. leachii were confined to the ears of a striped mouse (A17) with 64 mff/mg of ear skin. Detaching ticks were collected 2-4 days later. These fell into 2 categories: Most nymphs fed to repletion and had a swollen appearance while a small proportion (less than 8%) were hardly enlarged, having failed to engorge sufficient blood to distend the alloscutum. Intermediate sizes were not observed.

Undersized ticks were collected with an equivalent number of replete individuals chosen at random as the nymphae detached. Each tick was then weighed immediately and dissected in water to count



microfilariae. The number recovered from each nymph was recorded.

The maximum number of ticks that could be fed together on a single host was around 50. Because only 3-4 of these might be expected to show a reduced intake of blood, it was necessary to pool results obtained from 5 infestations. These were performed on the same animal over 70 days. During this period, the microfilarial density in ear skin showed no appreciable change (64 mff/mg - 65 mff/mg).

### Results

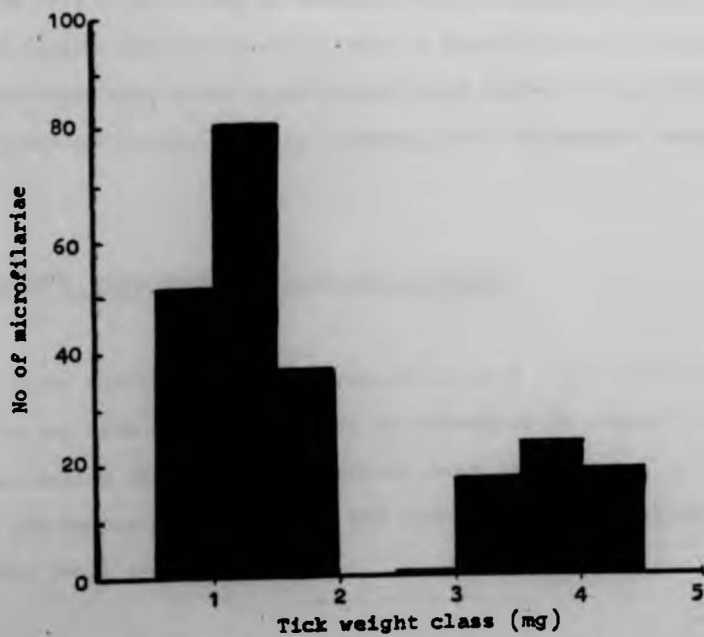
A total of 16 undersized nymphs together with 16 controls (fully engorged ticks) were examined. The results of this experiment are given below (Table 22) and have been plotted as a histogram in Figure 30.

TABLE 22

<u>Tick weight class</u> (milligrams)	<u>No. ticks</u> <u>examined</u>	<u>Mean no. microfilariae</u> <u>ingested (range)</u>
0.5 - 0.9	3	52 (16 - 112)
1.0 - 1.4	11	81 (3 - 327)
1.5 - 1.9	2	37 (22 - 52)
2.0 - 2.4	0	-
2.5 - 2.9	1	0
3.0 - 3.4	5	17 (0 - 31)
3.5 - 3.9	8	23 (0 - 68)
4.0 - 4.4	2	19 (15 - 22)

**FIGURE 30**

Relationship between the number of microfilariae ingested and tick weight, immediately after an infective feed.



Undersized ticks weighed less than half that of controls but contained many more microfilariae than normally replete individuals. The mean number of microfilariae ingested by the 2 categories (i.e. ticks weighing between 0.5 - 1.9 mg and 2.5 - 4.4 mg) was 71 and 19, respectively. An inversely proportional relationship was found between the weight of ticks immediately after feeding and the number of microfilariae ingested. The coefficient of correlation was  $-0.39$  ( $0.05 > P > 0.01$ ).

From this study it may be concluded that engorgement of blood is not a prerequisite for the ingestion of microfilariae. In addition, it appears that some ticks which ingest large numbers of microfilariae before blood imbibition is fully underway, fail to complete engorgement.

#### Dynamics of *A. globulosa* infection in *H. leachii*

In order not to put infected rodents at risk, tick infestations had to be set with an upper limit of 200 larvae or 50 nymphs. However, some information could still be obtained about the dynamics of infection in the vector population with the proviso that this was based on relatively small samples.

##### 1) The conversion of microfilariae to infective larvae

Larvae of the Machakos biotype of *H. leachii* were fed on the ears of an infected striped mouse with 35 mff/mg of ear skin. Engorged ticks detaching 4 days later were collected. A sample of 20 was dissected immediately after feeding and the number of microfilariae

Undersized ticks weighed less than half that of controls but contained many more microfilariae than normally replete individuals. The mean number of microfilariae ingested by the 2 categories (i.e. ticks weighing between 0.5 - 1.9 mg and 2.5 - 4.4 mg) was 71 and 19, respectively. An inversely proportional relationship was found between the weight of ticks immediately after feeding and the number of microfilariae ingested. The coefficient of correlation was  $-0.39$  ( $0.05 > P > 0.01$ ).

From this study it may be concluded that engorgement of blood is not a prerequisite for the ingestion of microfilariae. In addition, it appears that some ticks which ingest large numbers of microfilariae before blood imbibition is fully underway, fail to complete engorgement.

#### Dynamics of *A. globulosa* infection in *H. leachii*

In order not to put infected rodents at risk, tick infestations had to be set with an upper limit of 200 larvae or 50 nymphs. However, some information could still be obtained about the dynamics of infection in the vector population with the proviso that this was based on relatively small samples.

##### 1) The conversion of microfilariae to infective larvae

Larvae of the Machakos biotype of *H. leachii* were fed on the ears of an infected striped mouse with 35 mff/mg of ear skin. Engorged ticks detaching 4 days later were collected. A sample of 20 was dissected immediately after feeding and the number of microfilariae

ingested was recorded. Thirty-nine days later, when microfilariae had developed to third-stage larvae, a further sample of 20 ticks was dissected: The number of infective-stage larvae these contained was recorded. The efficiency of the vector in supporting development of microfilariae through to third-stage larvae was then estimated using the following equation:-

$$\frac{\text{Number of infective larvae developing}}{\text{Number of microfilariae ingested}} \times 100$$

Larval ticks ingested a mean of 4.5 (0-16) microfilariae; 39 days later they contained a mean of 3.3 (0-10) infective larvae. The efficiency of conversion of microfilariae to third-stage larvae was 73%.

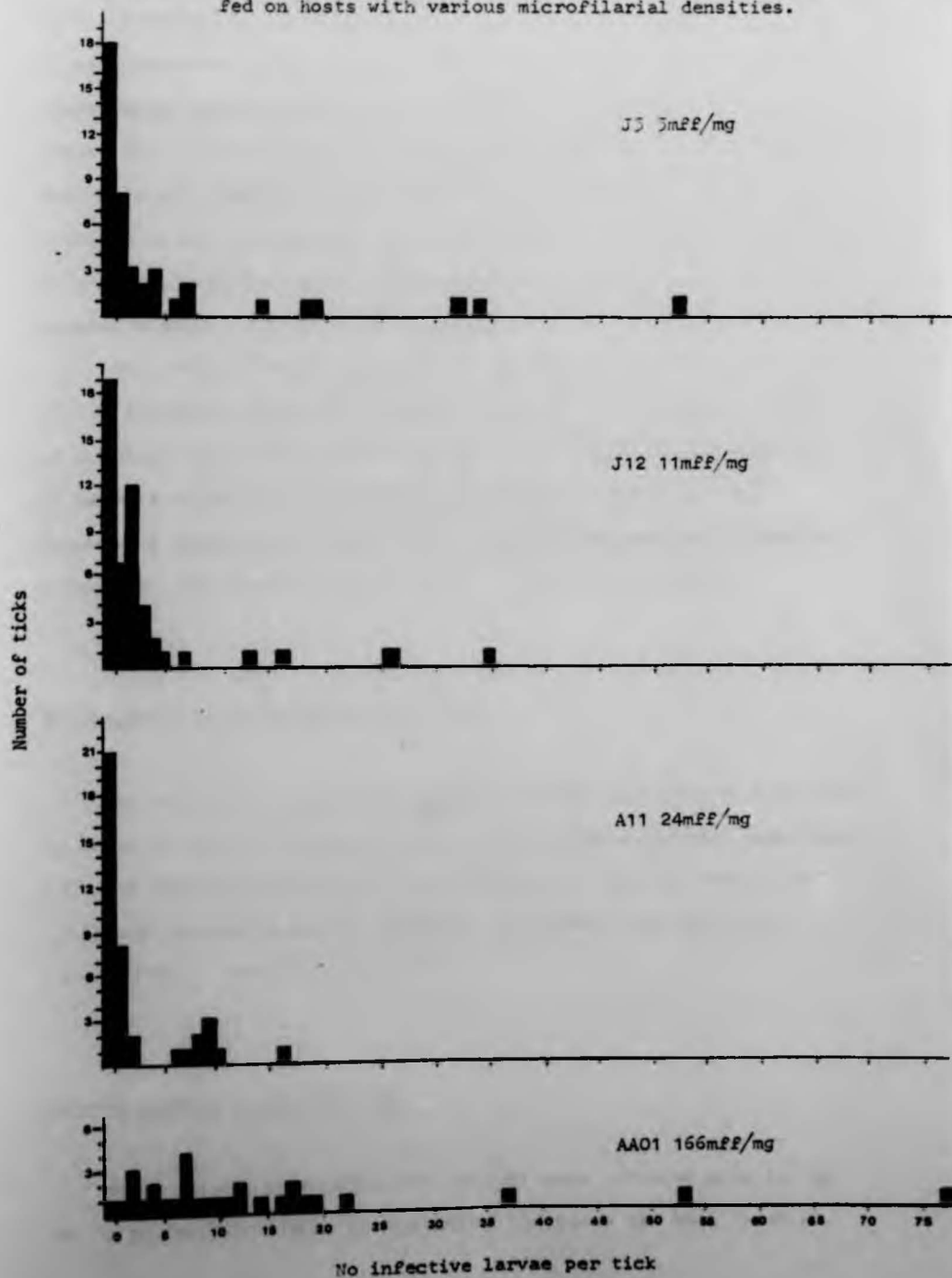
2) Frequency distribution of infective larvae in populations of ticks fed on hosts with different microfilarial densities

Nymphs of the Machakos biotype of H. leachii were fed on the ears of 4 infected rodents with microfilarial densities in ear skin of 5, 11, 24 and 116 mff/mg. Thirty days later, between 30 and 50 ticks from each animal were dissected and the number of infective larvae per tick was recorded.

The results of this work are given in Figure 31. When ticks fed on animals with 5, 11 and 24 mff/mg, 58, 62 and 48% of them respectively contained infective larvae 30 days later. When the microfilarial density was higher (116 mff/mg), 97% of the ticks harboured third-stage parasites.

All 4 groups of ticks contained a wide range in number of infective larvae. However, most of those fed on hosts with low microfilarial

**FIG. 31** Frequency distributions of infective larvae in ticks fed on hosts with various microfilarial densities.



levels subsequently contained few or no third-stage worms. Thus, ticks infested on rodents with 5, 11 and 24 mff/mg of ear skin harboured means of 5.1 (0-52), 3.1 (0-35) and 2.4 (0-16) infective larvae per tick respectively, 30 days after infection. This rather unusual result stems from the fact that in 2 of the samples of ticks examined a few individuals contained exceptionally high worm numbers. In general, however, progressively fewer ticks contained progressively greater numbers of third-stage larvae.

Ticks fed on the animal with 116 mff/mg harboured a mean of 13.6 (0-77) infective larvae 30 days after infection. In this population of nymphae, the frequency distribution of infective larvae appeared to be more normally distributed about the mean (see Figure 31). However, a proportion of ticks still contained exceptional numbers of parasites: The maximum recovered from a single tick was 77.

#### Transmission of infective-stage larvae

The infective larvae of A. globulosa remained viable in ticks for at least 125 days. Thirty-two third-stage larvae recovered from ticks 125 days after infection were subcutaneously inoculated into a jird (J13) and produced a patent infection, indicating that they had retained their infective capacity.

#### Natural process of transmission

Three striped mice (A34, A35 and A36) were infested with 49, 48 and 52 H. leachii nymphs, 42 days after the ticks had been infected.

All of these nymphs were derived from the same batch of ticks infected as larvae on a single striped mouse. A sample of 20 nymphs dissected 42 days after infection contained a mean of 3.3 larvae per tick.

Engorged nymphs were recovered from each animal and immediately dissected; the number of infective larvae remaining in the ticks was recorded. The development of patent infections in striped mice was assessed by examining skin snips taken from 75 days post infection.

### Results

All 3 striped mice developed patent infections within 80-96 days of infestation with infected ticks.

Very few engorged nymphs contained infective larvae after the "transmission feed". The efficiency of transmission of third-stage larvae (L3) from infected nymphs to the definitive host was estimated using the equation:-

$$\frac{\text{Mean no. L3 in nymphs after transmission feed}}{\text{Mean no. L3 in nymphs before transmission feed}} \times 100$$

The results are presented in Table 23.

TABLE 23

<u>Host</u>	<u>No. infective larvae in ticks</u>		<u>% output of infective larvae from ticks</u>
	<u>Before feed</u> (Mean (S.E.))	<u>After feed</u> (Mean (S.E.))	
A34	3.3 (0.8)	0.2 (0.1)	93
A35	3.3 (0.8)	0.4 (0.1)	87
A36	3.3 (0.8)	0.2 (0.1)	94



From these data it was estimated that between 87 and 94% of the infective larvae in nymphal ticks were transmitted to rodents while attached to the host. It is therefore concluded that transmission of A. globulosa infective larvae is achieved through the bites of infected ticks and that the majority of larvae escape from the vector during a single feed.

#### DISCUSSION

As with all the authenticated reports of filarial development in the vector, that of A. globulosa begins after microfilariae penetrate an intracellular site. Sheathed microfilariae taken up by feeding ticks exsheath in the gut, migrate through the gut epithelium and enter epidermal cells within 24 hours of ingestion. Here they occupy several cells to accommodate their size. During the next 9 days, first-stage larvae grow in the epidermis and undergo the first moult; then they migrate out of epidermal cells and enter underlying fat cells. Within fat cells, worms develop as second-stage larvae until day 13 when the second moult occurs. Third-stage larvae appear in the haemocoel 13-14 days after infection and over the next 3-8 days continue to mature to infective-stage parasites. These migrate randomly through the haemocoelic spaces of the tick and may remain viable for long periods in the interval before the next blood meal.

A wide variety of developmental stages were observed in ticks on each day after infection. In part, this asynchrony in larval development is attributed to the long period of tick attachment since microfilariae were ingested throughout the 3 days of feeding. However, even when the vectors are ones which feed within minutes, the

development of certain filarial species may be highly asynchronous (Williams, 1961; Schacher and Khalil, 1968). This suggests that individual rates of larval growth may also be responsible for some asynchrony in development.

The life-cycle of A. globulosa in its intermediate host bears many similarities to that of A. marmotae (described by Ko, 1972b). Both species develop in hard ticks and are exceptional in that larvae occupy epidermal cells in early development. In Ixodes cookei, Ko (1972b) observed that the microfilariae of A. marmotae appeared in the epidermis during the first week of infection and that some degree of development took place within this site. He also reported that microfilariae penetrated cells of the fat body where they continued to develop to the second moult. Ko (1972b) concluded that the limited growth of larvae in the epidermis indicated that development may not be completed in this site. In the present study, the microfilariae of A. globulosa were never observed in the fat body; only those that had passed through the first moult left the epidermis to penetrate fat cells.

In the arthropod host it is unusual for filarial parasites to develop in more than one intracellular site, since site specificity is normally characteristic for each species (Lavoipierre, 1958b; Hawking and Worms, 1961; Nelson, 1964). Development may occur in the muscles (Noë, 1908; Blacklock, 1926; Buckley, 1938; O'Connor and Beatty, 1938; Worms et al., 1961), malpighian tubules (Wharton, 1959; Kartman, 1953; Frank, 1964), or fat body (Connal and Connal, 1922; Highby, 1943; Mackerras, 1953; Webber, 1955; Nelson, 1961, 1962; Spratt, 1972). Bain (1967) noted that Dipetalonema viteae will develop in both the muscles and salivary glands of O. tartakovskyi. However, it is believed that this is the first report of a movement of larvae from

one intracellular site to another during the normal course of development of a filarioid in its vector.

The timing of migration of larvae to the epidermis and subsequently to fat cells is an interesting aspect of the vector-parasite relationship. In this connection, observations made by Balashov (1972) on the physiological events in feeding and fed ixodid ticks may help to appreciate the reasons why larvae develop in two separate sites. Microfilariae penetrate the epidermis at a time when protein synthesis in this tissue is proceeding at an intense rate. Before the new tick cuticle is deposited, a large accumulation of glycogen fills the entire basal vacuolated zone of epidermal cells. As ticks approach the moult, glycogen levels in the epidermis fall rapidly, and second-stage larvae leave this tissue to enter fat cells which are reaching maximum size. This suggests that the movement of larvae may well be related to their nutritional requirements. In first-stage larvae, which lack a fully-formed gut, feeding may be accomplished by absorption of nutrients through the larval cuticle, as suggested by Nelson (1964). In later stages, when the gut has differentiated, food may be taken up directly via the mouth. For example, Kan and Ho (1971) suggested that the larvae of Breinvia sergenti ingest mitochondria and membranous material from the fat cells they occupy in Aedes togoi; Beckett and Boothroyd (1970) produced conclusive evidence that the larvae of Brugia pahangi feed on mitochondria in the muscle fibres of Aedes aegypti. Nelson (1961) found that the larvae of Dipetalonema mansonbahri failed to develop in the fat cells of fleas unless food reserves in the insect's fat body were continually replenished by repeated blood meals.

The movement of A. globulosa within its hard tick vector may also be related to the fact that there are few tissues which remain stable enough in the pharate nymph to support the development of a large

parasite for the full 14 day period of the intracellular phase.

In the present study it was observed that microfilariae in the arthropod midgut showed vigorous movements of the cephalic hook. Similar observations have been made by Esslinger (1962) and Nelson (1964) who suggested that this structure is used by microfilariae to penetrate host cells as they escape from the gut lumen. With A. globulosa, microfilariae migrated through gut epithelial cells before emerging into the haemocoel. This sojourn of worms through the gut epithelium also occurs during the passage of B. pahangi and Setaria labiatopapillosa microfilariae into the haemocoel of mosquito hosts (Esslinger, 1962; Bain and Brengues, 1972). Nevertheless, the phenomenon may not be common to all filarial species as Ko (1972b) reported that the microfilariae of A. marmotae migrated between epithelial cells when traversing the gut.

Most filarial parasites are known to have a sausage stage during development in the arthropod. However, this stage is absent in A. globulosa as it also has shown to be in D. grassii, D. mansonbahri, Macdonaldius oschei, Stephanofilaria stilesi, D. viteae, D. setariosum, and A. marmotae (Noé, 1908; Nelson, 1961; Frank, 1964; Hibler, 1966; Bain, 1967; Worms, 1970; Ko, 1972b). Buckley (1955) suggested that the presence or absence of a sausage stage in development is related to the species of arthropod that serves as the vector and is absent in tick and flea transmitted filarioids. Of the 8 species listed, 6 are transmitted by ticks, 1 by fleas and 1 by horn flies. The absence of a sausage stage was regarded as a primitive character by Chabaud (1954) and by Bain (1967).

Although microfilariae of A. globulosa were taken up from rodents by both argasid and ixodid ticks, development took place in only two species of the latter family. This is not surprising as the specificity

of filarial parasites for their intermediate hosts is well recognised and few are transmitted by more than one family of arthropods (Hawking and Worms, 1961; Nelson, 1964). Only Dipetalonema viteae, transmitted by hard and soft ticks (Chabaud, 1954) and D. reconditum by fleas and lice (Nelson, 1962) are generally accepted to be exceptions to this rule. Onchocerca volvulus will develop in mosquitoes as well as blackflies, but only after the former have been infected artificially (Zielke et al., 1977; Zielke, 1977).

In the Onchocercidae, the specificity of some of the species for their arthropod hosts may be so marked that development will only occur in certain strains of a single vector (Nelson, 1964). The influence of genetic and other factors on vector susceptibility to parasitic infections is a feature common to a variety of vector-parasite relationships (reviewed by Macdonald, 1967) and amongst the filarial worms has been intensively studied for mosquito-transmitted forms (e.g. Kartman, 1957; Hawking and Worms, 1961; Macdonald, 1962; McGreevy et al., 1974).

From the present study it was apparent that differences also exist in the susceptibility of various biotypes of H. leachii to infection with A. globulosa. Refractory forms of this tick manifest their resistance to developing larvae with encapsulation and cellular reactions. Encapsulation reactions, which are a common feature of many filarial infections in mosquitoes, have been the subject of reviews by Brug (1932), Lavoipierre (1958b), and Esslinger (1962). In ticks, Bain (1967) noticed that encapsulation of D. viteae larvae was common in the abnormal host, Ornithodoros erraticus, but rare in the natural host, O. tartakovskyi. However, Frank (1964) observed reactions to Macdonaldius oschei in its natural vector Ornithodoros talage.

Cellular reactions to filariae in acarines have previously been reported by Hughes (1950) and by Freer (1953). In mites, these authors observed that the microfilariae of L. carinii may be phagocytosed by large cells in the gut lumen. Yoeli et al. (1958) also observed cellular and encapsulation reactions to Dirofilaria immitis larvae after injecting microfilariae into caterpillars. However, the accumulation of haemocytes around larvae of A. globulosa in ticks bore a closer similarity to reactions described in mosquitoes infected with Foleyella or Brugia (Schacher and Khalil, 1968; Beckett and Macdonald, 1971).

Many third-stage larvae in ticks that elicited strong cellular reactions failed to develop to the infective stage. These remained as early third-stage forms and were morphologically distinct from infective stages. They also lacked the capacity to develop further after injection into rodents. Interestingly, Bain (1969) similarly divided larvae of O. volvulus into early and late third-stages and suggested the former were not infective.

In contrast to partially refractory biotypes of H. leachii, those that were fully susceptible to infection supported the development of most ingested microfilariae (73%) to infective-stage larvae. Ticks, unlike many simuliids, ceratopogonids and mosquitoes, lack two important mechanical barriers to infection with filarial worms, the pharyngeal armatures and peritrophic membrane (Duke and Lewis, 1964; Eichler, 1973; Bryan et al., 1974; Omar and Garms, 1975; Mellor, 1975). This may account for the relatively high proportion of microfilariae that successfully escaped from the midgut in H. leachii compared with the numbers that escape in many insect vectors.

Another important distinction between acarines and many free-flying insects as vectors of filarial worms is that both sexes of the



former group feed on blood while only the females in the latter group. This means that filarial parasites transmitted by acarines are able to exploit a far greater proportion of the vector population for the transmission of infection. In the present study it was found that H. leachii ticks of either sex ingested microfilariae and supported development. Similarly, both sexes of Ornithonyssus bacoti support development of L. carinii (Nelson, 1964) and either sex of Ornithodoros moubata serve as vectors of D. viteae (personal observation). Interestingly, Townson (1975) showed that B. pahangi will develop in the males of Aedes aegypti if infected artificially by injecting microfilariae. The microfilariae of Onchocerca gutturosa will also develop in males of Simulium ornatum after injection into the thorax (personal observation). It therefore appears that feeding habits, rather than hormonal factors, are the principal reason why male simuliids and mosquitoes fail to participate in the transmission of filarial infections.

Pathological effects of filarial larvae on arthropods have been the subject of much interest and extensive study (reviewed by Lavoipierre, 1958b; Hawking and Worms, 1961; Nelson, 1964). This work has revealed that damage to the tissues occupied by developing larvae is an almost universal phenomenon amongst the vectors of filarial worms, affecting muscle fibres, fat cells and malpighian tubules.

In H. leachii ticks infected with A. globulosa, first and second stage larvae caused severe local damage to epidermal and fat cells; however, the epidermis showed a remarkable ability to regenerate after infection. Ko (1972b) found little sign of damage to the cells of Ixodes cooki caused by A. marmotae. On the other hand, muscle fibres of soft ticks infected with D. viteae may be completely destroyed (Chabaud, 1954; Bain, 1967; Worms, 1970; Londono, 1976a). This damage

may impede the mobility of ticks and inhibit their ability to moult and ingest blood (Chabaud, 1954; Londono, 1976a).

H. leachii nymphs fed on infected striped mice ingested many microfilariae before blood was taken up. This suggests that a significant proportion of the worms were ingested along with host tissue fluid, the uptake of which normally precedes blood imbibition in ixodid ticks (Sutton and Arthur, 1962; Chinery, 1964). However, some nymphs that took up exceptionally large numbers of microfilariae during the early stages of feeding failed to complete engorgement as the alloscutum did not swell. Balashov (1972) has noted that specialized epidermal cells, the so-called dermal glands, play an important role in the complicated cuticular transformations that occur from the start of tick feeding to the end of moulting. Cuticular growth is associated with enlargement and acceleration of epidermal cell synthetic activity. It may therefore be that the large numbers of microfilariae which invaded the epidermis of heavily infected nymphs interfered with the normal processes of cuticular changes necessary to allow complete engorgement.

Worms et al. (1961) reported that Ornithodoros tartakovskyi died as a result of overwhelming numbers of D. viteae larvae but did not succumb to heavy infections of D. setariosum (Worms, 1970). Mortality in Ornithonyssus bacoti infected with L. carinii was found to be due to the escape of infective larvae from the mite while feeding, rather than to the presence of larvae in the mite while fasting (Williams and Kershaw, 1961). In the present study no mortality amongst ticks could be attributed to infection, either while the worms developed or during their subsequent transmission.

The infective larvae of A. globulosa remained viable in ticks for at least 125 days. This is in keeping with other tick-transmitted



species since D. viteae will survive for 203 days in O. tartakovskyi (Worms et al., 1961) and up to 729 days in O. moubata (personal observation). Third-stage larvae of D. setariosum live for 121 days in O. tartakovskyi (Worms, 1970). The ability of ticks to survive long periods of starvation (especially among the Argasidae) may well be an important factor in the longevity of these filariae. Bosworth and Ewert (1971), working with Brugia malayi, showed that third-stage larvae from Aedes togoi were as infective to cats after 30 days as after 14 days: However, few mosquitoes survived for more than one month. Therefore, the life-span of the vector might be a critical factor in the survival and longevity of various filarial larvae.

With many species of filarial worms in their arthropod hosts it has been observed that third-stage larvae spontaneously congregate near the mouthparts in readiness for transmission (Noé, 1901; Blacklock, 1926; Steward, 1937; Jordan, 1959; Laurence and Pester, 1961; Nelson and Pester, 1962; Frank, 1964; Benach and Crans, 1973; Ho et al., 1974; Spratt, 1974). However, the infective larvae of A. globulosa were randomly distributed in fasting ticks. Only after the nymphs had attached to rodents and begun to feed did larvae migrate towards the mouthparts to escape from the arthropod. This also appears to be the case with the larvae of L. carinii in Ornithonyssus bacoti, Loa loa in Chrysops silacea and D. viteae in Ornithodoros tartakovskyi (Williams, 1948; Gordon and Crewe, 1953; Lavoipierre, 1958a; Bain, 1967; Londono, 1976a).

As ixodid ticks feed for several days, the transmission of larvae may be relatively slow. With A. marmotae, Ko (1972b) reported that infective larvae escaped from Ixodes cooki throughout the full 7 days of tick attachment. However, with A. globulosa it was found that 87-94% of the third-stage larvae in H. leachii nymphs escaped from these

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ticks during the first 24 hours after attachment. In the case of mosquito-transmitted filariae, the escape of larvae must be achieved far more rapidly. Recently, Ho and Lavoipierre (1975), working with B. pahangi, found that 91% of the larvae in Aedes togoi were transmitted by these insects during a single feed of which 57% escaped within 5 seconds.

In summary, susceptible forms of H. leachii ticks proved excellent vectors of A. globulosa. Seventy-three percent of the microfilariae ingested developed to infective stage larvae in the Machakos biotype. Most third-stage worms were subsequently transmitted when nymphs reattached to rodent hosts. Infected ticks suffered few adverse effects and mounted little host reaction to developing larvae. In contrast, less susceptible forms of H. leachii inhibited the normal development of many larvae. It therefore appears that strain differences in the H. leachii complex may well play an important role in the transmission of A. globulosa in nature.

## CHAPTER 6

THE ACTION OF DIETHYLCARBAMAZINE ON THE MICROFILARIAE OF  
A. GLOBULOSA IN EXPERIMENTALLY INFECTED RODENTS

INTRODUCTION

It has long been recognized that research on the chemotherapy of filarial infections in man requires suitable experimental infections in laboratory animals. Studies on the life-cycle, maintenance and host-parasite relationships of A. globulosa in rodents have been reported in previous chapters and provide a number of criteria to judge the value of this infection as a possible model for onchocerciasis and streptocerciasis. However, an important step to determine the value of this parasite for chemotherapeutic studies was to test a drug of known microfilaricidal action against the microfilariae of A. globulosa in vivo. In this chapter the results of a preliminary trial using diethylcarbamazine (DEC) in experimentally infected rodents are reported. This drug was an obvious choice as it is widely recognized as an effective microfilaricide in man against Onchocerca volvulus (Mazzotti and Hewitt, 1948; Hawking, 1950; Burch and Ashburn, 1951; W.H.O., 1966; Duke, 1968a; Nelson, 1970; Duke and Anderson, 1972a; W.H.O., 1972; Hawking, 1973; Anderson et al., 1976a) and Dipetalonema streptocerca (Wanson et al., 1950; Colbourne, 1952; Duke, 1957a; Meyers et al., 1972).

Diethylcarbamazine was first shown to be active against filarial worms by Hewitt et al. (1947) working with Litomosoides carinii in cotton rats and for more than 25 years it has remained the most

important drug in the treatment of human filarial infections (Hawking, 1973; W.H.O., 1974; Lämmler et al., 1975). For this reason, much attention has been paid to the activity of this compound against a variety of filarial parasites of man and animals and amongst the many publications on this subject, comprehensive reviews have been produced in recent years by Hawking (1973), Lämmler et al. (1975) and Sasa (1976). The only other drug currently available that is recommended by the World Health Organization for the treatment of onchocerciasis is suramin (W.H.O., 1972, 1975). In contrast with DEC, suramin kills all stages of O. volvulus in the definitive host including the developing larvae (Duke, 1974a), adult worms and microfilariae (Van Hoof et al., 1945, cited by Wanson, 1950; Burch and Ashburn, 1951; W.H.O., 1966, 1972; Duke, 1968b; Nelson, 1970; Hawking, 1973; Lämmler et al., 1975; Anderson et al., 1976b).

While DEC and suramin have proved of much value in ameliorating disease in selected cases of onchocerciasis (Nelson, 1970) and suramin has been used for many years in mass treatment campaigns in Venezuela (W.H.O., 1972), it is generally considered that neither drug is suitable for wide-scale use against onchocerciasis (Nelson, 1970; W.H.O., 1972; Hawking, 1973; Anderson et al., 1976a and b). Both can cause severe side-effects which are unpleasant and sometimes dangerous so patients must be treated under close medical supervision. Adverse reactions accompanying the death of parasites may be particularly marked with DEC (Mazzotti, 1948; Hawking, 1950, 1955; Nelson, 1970; Duke and Anderson, 1972a; W.H.O., 1972) and although the toxicity of this drug is low (Hawking, 1973), Omen (1969) has reported fatalities amongst Ethiopian patients who were in a poor general state of health at the time of treatment. On the other hand, suramin is highly toxic and there have been several fatalities due to exfoliative dermatitis

and renal complications (Van Hoof et al., 1947; Nelson, 1955; Diaz, 1957; Satti and Kirk, 1957; Budden, 1959; Anderson et al., 1976b).

There is an urgent need for new and safe drugs for the treatment of onchocerciasis but chemotherapeutic studies are handicapped by the lack of a convenient laboratory model for screening. Therefore, the action of drugs on skin microfilariae in animals has received little attention. Thienpoint and Biche (1957) claimed some success using DEC to treat 2 cows with dermal onchocerciasis but Mazzotti (1952) reported that this drug was ineffective against the microfilariae of Onchocerca cervicalis in horses. Nevertheless, Thomas (1958) found that a case of erysipeloid reaction in an infected horse improved after drug treatment which he suggested was due to the destruction of microfilariae in the skin. Cello (1970) also reported that DEC kills the microfilariae of O. cervicalis in horses and recommended its use in conjunction with corticosteroids for the treatment of ocular disease. More recently, Denham and Mellor (1976) used cows infected with Onchocerca gutturosa to test a promising new filaricidal compound (compound "E" - Freidheim) and evaluate this host-parasite system as a possible tertiary screen for drugs. The results of this work encourage further efforts to be made to use animal species of Onchocerca for chemotherapy trials. Useful studies have also been made by Duke (1974a, b and c) on the action of drugs in chimpanzees infected with the human parasite, O. volvulus.

There are obvious advantages in a screening system that employs a smaller host, but the only chemotherapeutic studies on skin microfilariae in rodents have been those of Eichler (1970 - Ph.D. thesis, Univ. of London) and Al Zubaigy (1973 - Ph.D. thesis, Univ. of London). Both of these workers found that the microfilariae of O. gutturosa transfused into laboratory mice were killed by DEC. However, there

was a high natural mortality of microfilariae in the abnormal host and Eichler (1970) considered this system too insensitive for routine use.

A number of workers have used in vitro techniques to examine the action of drugs on filarial worms (Hawking, 1940; Rose et al., 1944; Otto and Maren, 1949; Hawking et al., 1950; Natarajan et al., 1973, 1974; Rogers and Denham, 1976 - unpublished) but these were not used in the present study as the principle aim was to determine the effect of treatment on microfilariae in the skin. Moreover, present evidence indicates that DEC is inactive against filarial worms in vitro (Hawking, 1973; Sasa, 1976).

#### MATERIALS AND METHODS

Two striped mice and 4 jirds that had been experimentally infected with A. globulosa and developed patent infections were used in this trial. Each jird received a single inoculation of 50 infective larvae and the striped mice were infested with infected ticks. Because of the difficulty of maintaining A. globulosa in the laboratory it was not possible to infect more animals for a larger trial, or controls from the same batch of infective larvae. Instead, the course of microfilarial densities in the 4 treated jirds were compared with those in 7 untreated jirds, and microfilarial levels in the 2 treated striped mice were compared with those in a third striped mouse (A34) which had also been exposed to infected ticks but did not receive treatment (see Chapter 3, Figures 2 and 3 for the course of infection in these animals).

To assess microfilarial densities in the infected rodents, jirds



were biopsied from the ear from day 60 post infection and striped mice from day 70. Thereafter, skin-snips were taken at 10 day intervals. By 110 days post infection, all animals had a rising level of microfilariae in the skin and large biopsies (5-10 mg.) were taken from both ears of each rodent to determine the "pre-treatment" microfilarial density. The course of DEC was then begun. In the present study, the dihydrogen citrate salt of diethylcarbamazine ("Barocide", Burroughs Wellcome and Co.) containing 50% by weight of active base was used. Each rodent was given a daily oral dose of 300 mg./kg. body weight of DEC citrate in water for 5 consecutive days. Five uninfected jirds were treated as controls. Twenty-four hours after the first dose of drug, a large skin biopsy was taken from the ear of each rodent for histological examination. This was fixed in 10% formol saline and routinely stained in haematoxylin and eosin. After each dose of drug, all animals were kept under close observation for 6 hours and any response to treatment was noted: samples of 20-80 cu.mm. of urine and 30 cu.mm. of tail blood were collected from infected rodents and examined in a counting chamber for microfilariae. This was done as it has been shown that in human cases of onchocerciasis, microfilariae enter the blood and urine during DEC therapy (Fuglsang and Anderson, 1974; Duke *et al.*, 1975). Twenty-four hours after the end of treatment, infected rodents were skin-snipped from the ear (10-20 mg. biopsies) and the density of microfilariae recorded (mf./mg.). Microfilarial levels were subsequently measured at weekly intervals for one month following treatment and on day 70, when each rodent was autopsied. Female worms recovered on autopsy were examined by phase microscopy to check for any abnormalities in the development of microfilariae in utero (previously described in Chapter 4).



RESULTS

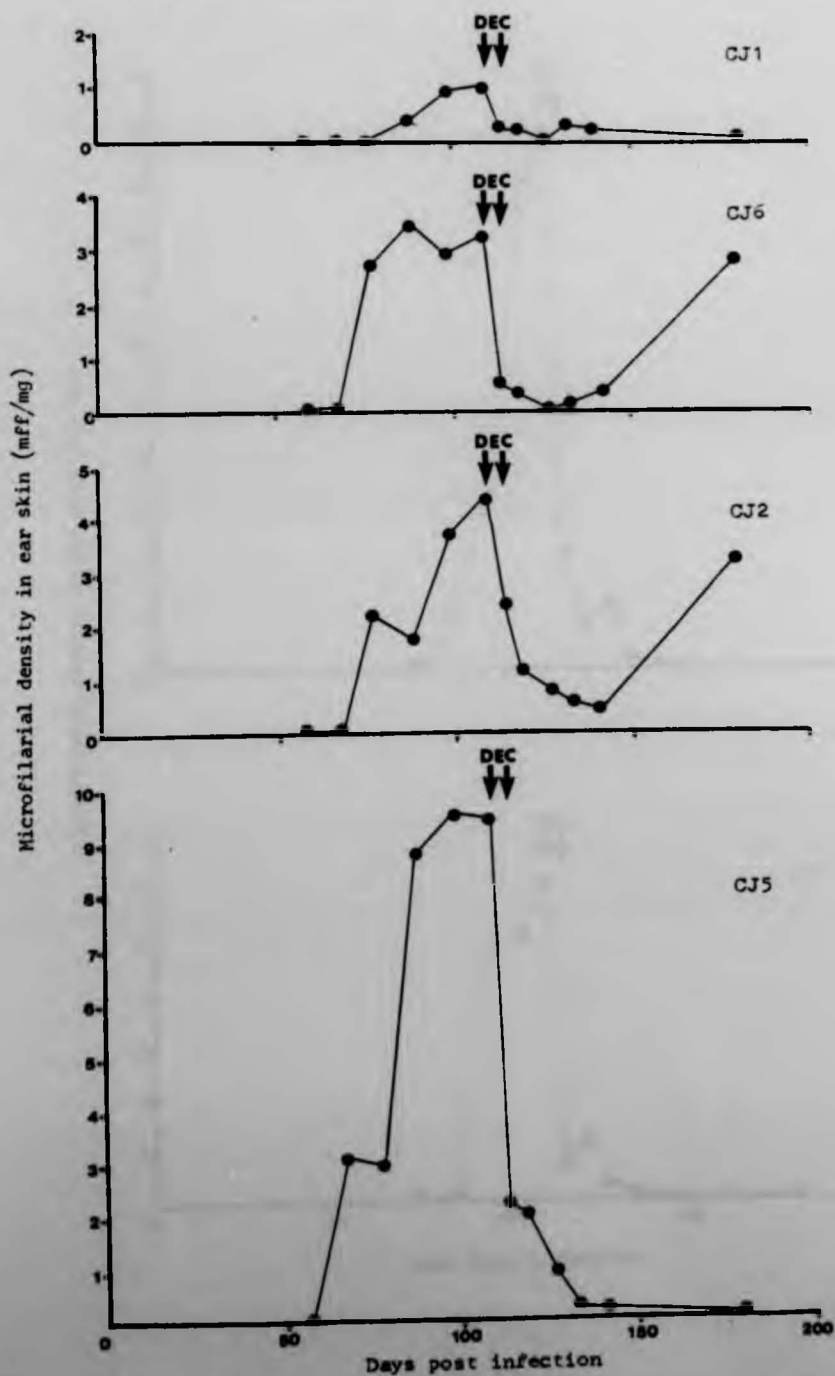
The course of microfilarial densities before and after treatment with DEC are presented in Figure 32 for jirds and Figure 33 for striped mice.

Twenty-four hours after the final dose of drug, a sharp reduction occurred in the number of microfilariae emerging from skin snips in all 6 infected rodents. Microfilarial densities fell by 78% and 88% of pretreatment levels in the 2 striped mice and by 72 (45-84)% in the 4 jirds. Over the following month, the levels of microfilariae in all 6 animals continued to decline. By the end of this period the residual density of microfilariae had fallen to 0% and 2% of pretreatment levels in the striped mice and to 11 (2-20)% in the jirds. In contrast, microfilarial densities in 6 of 7 untreated jirds and the untreated striped mouse either rose or remained stable during the equivalent period of infection (days 110-140 post infection): In the remaining jird, there was a gradual decline in the level of microfilariae lasting from day 119 to 238 after infection. No microfilariae were found in urine or blood samples taken from infected rodents during the period of treatment.

DEC was well tolerated by uninfected jirds at the dosage used in the present study and provoked no adverse reactions. However, infected rodents showed much distress during treatment and scratched themselves vigorously. This response occurred in all 6 infected hosts but developed earlier and with greater intensity in the more heavily infected individuals. Thus CJ5 and A35 which had 10 microfilariae per milligram of skin (mf./mg.) became extremely agitated and began to scratch around the head and neck within 3 hours of the first dose of DEC. In CJ5 this resulted in the loss of hair from behind the ears and from part of the head (Plate 33). Subsequent

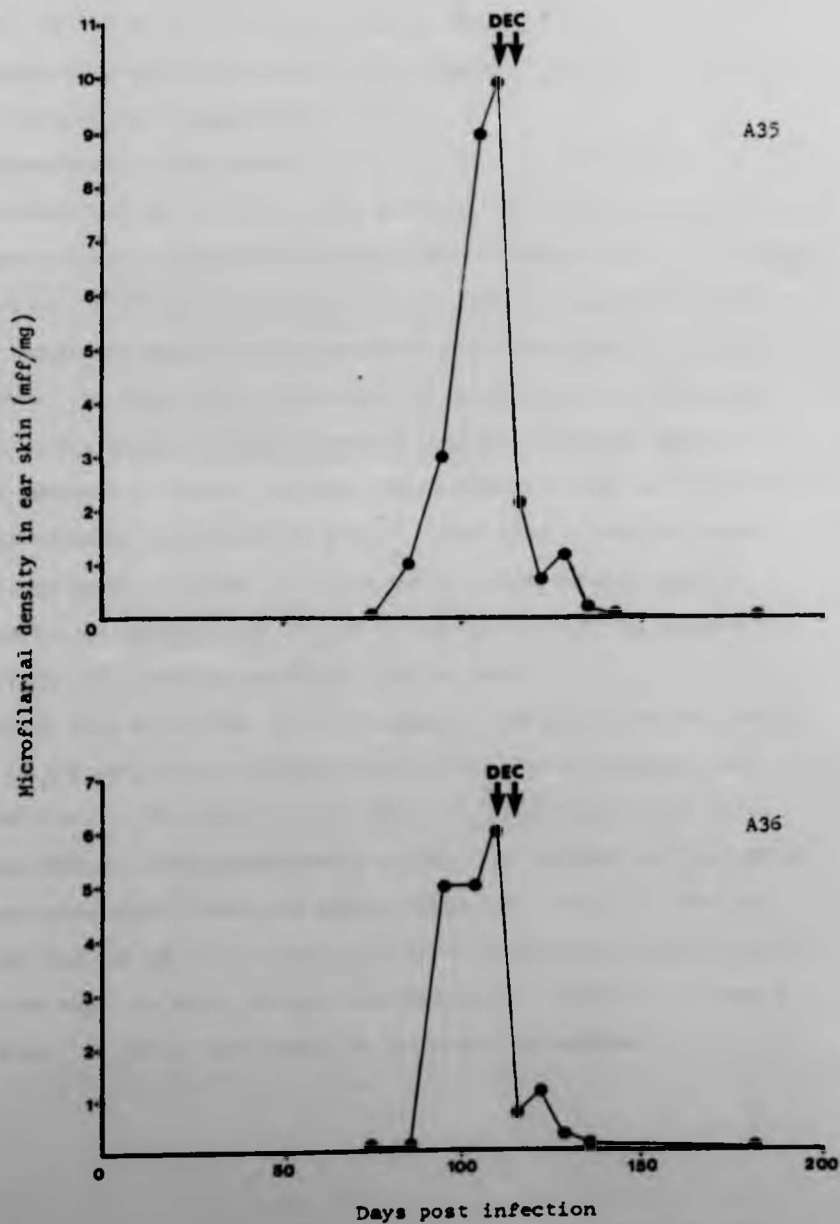
FIGURE 32

Course of microfilarial densities in ear skin of experimentally infected jirds before and after administration of diethylcarbamazine citrate (DEC)



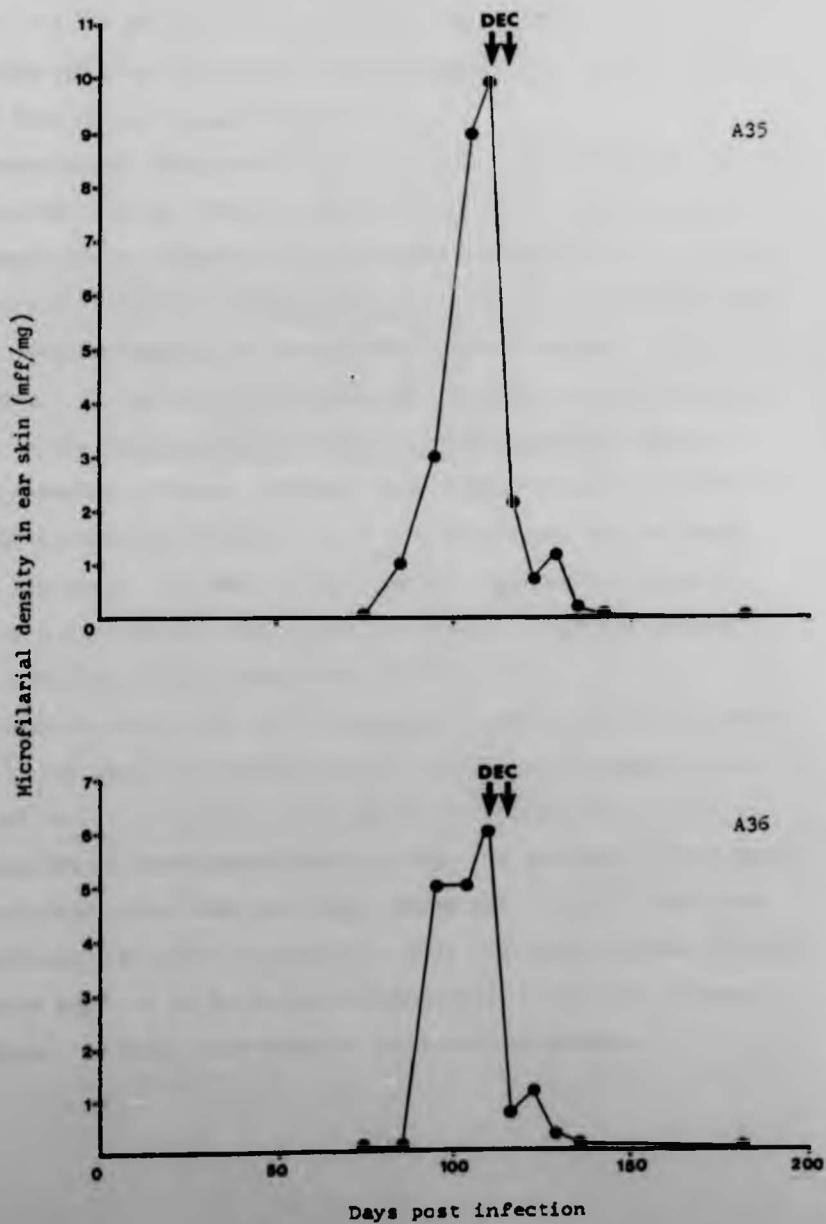
**FIGURE 33**

Course of microfilarial densities in ear skin of experimentally infected striped mice before and after administration of diethylcarbamazine citrate(DEC).



**FIGURE 33**

Course of microfilarial densities in ear skin of experimentally infected striped mice before and after administration of diethylcarbamazine citrate (DEC).



doses of the drug provoked intense scratching to resume within 15 minutes and last for several hours. Following the second dose of DEC, CJ6, CJ2, and A36 with 3-6 mff./mg. showed a similar but milder response. By the fourth day of treatment, CJ1 with 1 mff./mg. also showed signs of irritation and began to scratch. Scratching ceased within 3 days of the end of treatment.

A histological examination of skin biopsies from infected rodents 24 hours after the first dose of DEC revealed focal accumulations of inflammatory cells. These occurred in all 6 treated rodents and were more numerous in the most heavily infected animals. The inflammatory reaction consisted mainly of neutrophils, with eosinophils and a few histiocytes. In many instances these foci of cells surrounded microfilariae in the dermis (Plate 34) but in others, no microfilariae could be detected in serial sections taken through sites of inflammation. Some microfilariae were found in all of these animals free of local cellular reactions. No foci of inflammatory cells were seen in 5 uninfected jirds treated with DEC or in infected jirds and striped mice that had not received treatment (see Chapter 4).

Seventy days after the end of treatment, the microfilarial levels in both of the striped mice and 2 of the 4 jirds were extremely low (0-0.1 mff./mg.). In 2 jirds, the number of microfilariae had risen to 73% and 88% of their pretreatment levels. On autopsy, both of these jirds contained viable male and female worms and a single female was recovered from one of the striped mice (A35). All the females contained embryonated eggs and fully formed microfilariae, apparently undamaged by the drug. No worms were found in the remaining animals.



Plate 33. Evidence of scratching in a jird infected with *A. globulosa*, 24 hours after diethylcarbamazine therapy. Note the bald patch of fur directly behind the ears.



30  $\mu$ m

Plate 34. Inflammatory reaction around a microfilaria of *A. globulosa* in the skin of an infected jird, 24 hours after diethylcarbamazine therapy. The reaction consists of mononuclear, eosinophils and histiocytes.

## DISCUSSION

In spite of the small number of animals used in this trial several interesting parallels emerged between the effect of DEC on A. globulosa infections in rodents and that on O. volvulus and D. streptocerca infections in man. The results of this study strongly suggest that DEC is active against the microfilariae of A. globulosa in jirds and striped mice, as it is against those of most filarial parasites including O. volvulus and D. streptocerca (Hawking, 1973). In all of the rodents given DEC the microfilarial level dropped sharply during treatment, while in untreated hosts it either rose or remained stable during the equivalent period of infection. Nevertheless, the action of DEC was relatively slow and not all the microfilariae were destroyed. By the end of treatment their numbers had been reduced to 24% of the pretreatment level and continued to decline for a month until only 8% remained. This result clearly illustrates the need to study the course of an infection for some time after treatment so that slow or delayed effects of a compound may be expressed; especially as a slow acting microfilaricide might well offer the best means of reducing the microfilarial load in onchocerciasis patients while minimising the reactions associated with the death of parasites.

The recovery of viable adult worms from treated rodents and repopulation of the skin with microfilariae suggest that DEC has little or no macrofilaricidal action on A. globulosa. It is generally considered that the adults of O. volvulus are also resistant to DEC (Hawking, 1950; Mazzotti, 1951b; Duke, 1957b, 1968a; W.H.O., 1972) although Meyers et al. (1972) suggested that the adults of



D. streptocerca may be killed by this drug.

DEC had no effect on the microfilariae of A. globulosa in utero and appeared to produce no lasting suppression of microfilariae in the skin: In 2 hosts the adult worms had replenished the skin with fresh microfilariae within 70 days of treatment. In onchocerciasis patients treated with DEC, the destruction of microfilariae in the skin similarly evokes no lasting protection (Hawking, 1950; Duke, 1957b, 1968a; Nelson, 1970; W.H.O., 1972; Anderson et al., 1976a). According to Duke (1957b, 1968a), microfilarial densities build up immediately after treatment and may reach from 30% to over 100% of their pretreatment levels within a year.

Several workers have investigated the mode of action of DEC (recently reviewed by Sasa, 1976) and it is generally agreed that this drug does not kill filarial worms by its direct action but mediates in their destruction by the host defence system. Hawking et al. (1950) and Hawking (1952) suggested that DEC modifies microfilariae in such a way that they become susceptible to phagocytic cells, but the alteration produced by this drug remains obscure. Blood-dwelling microfilariae are trapped and destroyed by fixed macrophages in the liver (Hawking et al., 1950; Woodruff, 1951; Taylor, 1960b; Duke, 1960) while those of O. volvulus and D. streptocerca are attacked by cells in the skin (Hawking, 1952; Meyers et al., 1972).

The treatment of rodents infected with A. globulosa provoked an acute inflammatory reaction in the skin which was very similar to that described by Hawking (1952) in onchocerciasis patients treated with DEC. Microfilariae in the skin of untreated rodents elicited no histological reaction (see Chapter 4) but within 24 hours of the administration of DEC an intense focal infiltration of cells developed around many of the parasites in the dermis. This reaction was characterized by neutrophils,

eosinophils and histiocytes which suggests that immune complexes had formed in the skin as a manifestation of an immediate hypersensitivity response (Osler, 1963; Gall and Coombes, 1968). Since the foci of inflammation were more widespread than the microfilariae themselves, it would appear that the antigenic stimulus for this reaction was not restricted to somatic microfilarial antigen and may have included products released from damaged parasites.

Possibly DEC acts on the microfilariae of A. globulosa in a similar fashion to that against O. volvulus microfilariae in man. Hawking (1952) proposed that this drug "opsonizes" the parasites in the skin causing them to be attacked and destroyed by wandering phagocytes. Foreign protein released during the destruction of microfilariae then excites an immediate hypersensitivity reaction in the pre-sensitized host. Presumably rodents become sensitized to the microfilariae of A. globulosa during the patent infection, but the efferent arc of the immune response is blocked until the parasites are recognized as foreign during drug treatment. It is interesting to note that Eichler (1970), working with proxy hosts, found that the death of O. gutturosa microfilariae in mice treated with DEC provoked no histological reaction. On the other hand, Al Zubaidy (1973) showed that mice could elicit an immediate hypersensitivity reaction to these parasites if they were first sensitized with an adult worm extract. These findings lend support to the view that the inflammatory response associated with the death of microfilariae in the skin is due to an immunological process requiring prior sensitisation, rather than to a toxic reaction as suggested by Rodger (1962).

Some microfilariae of A. globulosa did not elicit local cellular reactions during treatment and it seems probable that these correspond with the worms that emerged from fresh skin snips taken after treatment.

Hawking (1952) observed a similar phenomenon in onchocerciasis patients treated with DEC and suggested that a proportion of microfilariae may be innately more resistant to the effects of this drug: He postulated that such microfilariae might be the recent progeny of adult worms or derived from some reservoir within the host where they are less readily affected by the drug. Fuglsang and Anderson (1974) noted that the microfilariae of O. volvulus persisted in the blood of patients even after 2 weeks of DEC therapy and suggested that they may be less vulnerable in the vascular system than in the skin. The microfilariae of A. globulosa are released into the arterial system from adult worms and circulate in the blood to reach their final site in the skin (see Chapter 4), so it may be that these had continued to "seed" the skin with fresh parasites while established microfilariae were still being killed.

Cellular reactions in the skin of treated rodents were accompanied by an acute pruritic response, manifested by the irritation and scratching shown during treatment. Pruritis developed in even the most lightly infected rodent, although its intensity was a reflection of the weight of infection before treatment. It is also significant that most of the irritation appeared to emanate from the ears and head which is the region of highest microfilarial density in striped mice and jirds (see Chapter 4). These observations imply that the sudden death of microfilariae in the skin during drug treatment gave rise to an acute pruritic response in rodents infected with A. globulosa. This is of much interest as the development of itching in human subjects given DEC is highly characteristic in cases of O. volvulus infection (known as the Mazzotti reaction) and may occasionally occur in patients infected with D. strantocerca (Mazzotti, 1948; Hawking, 1950; Duke, 1957a and b; Meyers et al., 1972). This response to treatment is the basis of

a sensitive diagnostic test for onchocerciasis (Mazzotti, 1948; Hawking, 1950; Nelson, 1970) and is accompanied in man by the clinical appearance of swelling and oedema of the skin, erythrema, a fine papular rash, febrile reactions and other systemic symptoms suggestive of an acute allergic reaction (Mazzotti, 1948; Hawking, 1950; Nelson, 1970; W.H.O., 1972, 1975; Duke and Anderson, 1972a). It is these reactions associated with the mass destruction of microfilariae in the skin that makes the use of DEC unpopular for the treatment of onchocerciasis. In jirds and striped mice no gross skin changes were observed, but it should be borne in mind that the infections in these animals were light compared with those that commonly develop in nature.

This discussion has sought to compare the effects of DEC on A. globulosa infections in rodents with those on O. volvulus and D. streptocerca infections in man. In the Conclusions section that follows, the results of this work are considered with those of the previous studies described in Part I, and the potential of A. globulosa in rodents as a laboratory model for studies on the chemotherapy and pathology of onchocerciasis and streptocerciasis is evaluated.

SUMMARY OF THE MAIN OBSERVATIONS AND CONCLUSIONS FROM THE STUDIES  
REPORTED IN PART I

A. PARASITOLOGICAL ASPECTS OF A. GLOBULOSA INFECTIONS

1. Ackertia globulosa, like most filarial worms, is a relatively host-specific parasite. This occurs in at least 4 species of rodents in the natural habitats in Kenya but could be transmitted to only a single laboratory rodent (the jird, Meriones unguiculatus) of 5 species tested.
2. In its principal host, the striped mouse, A. globulosa is a well-adapted parasite that infects a high proportion of both the males and females, lives and reproduces for the natural life-span of these rodents, and gives rise to no serious pathological conditions that might prejudice host survival.
3. The development of A. globulosa in the definitive host follows a similar course to that of other rodent filariae: The third moult occurs at around 10 days after infection, the fourth moult at 25 days after infection and the first appearance of microfilariae at 74-88 days.
4. The adult worms of A. globulosa are highly site-specific to the pulmonary arteries. Female worms release microfilariae into the bloodstream that travel via the general circulation to reach their final destination in the skin. Unlike several other filarial infections, the number of adult females present in the host appears to be a significant factor determining the microfilarial density.
5. In Kenya, Haemaphysalis leachii leachii is a natural vector of A. globulosa, and a relatively high proportion of the nymphs on wild rodents contain infective larvae. It seems that the dynamics

of transmission of this filarial parasite may be different from that of many free-flying insect transmitted species, being achieved by relatively few bites of the vector arthropod but compensated for by the high infection rate in ticks.

6. The microfilariae of A. globulosa exhibit behavioural characteristics to enhance transmission that are indicative of a highly-evolved host-parasite relationship between this filaria and its hard tick vector. These include the concentration of microfilariae in the ears of the host which correspond with a preferred biting site of H. leachii on rodents, and their accumulation beneath the mouthparts of feeding ticks during the 3 day period of tick attachment.
7. H. leachii ticks of various biotypes showed different susceptibilities to A. globulosa infection. Susceptible forms supported the development of most microfilariae to infective-stage larvae in 14-18 days, but partially refractory forms mounted encapsulation and cellular reactions that inhibited normal development of many larvae. Strain differences in the H. leachii complex may therefore play a significant role in the transmission of A. globulosa in nature.
8. As with other filarial parasites whose life-cycles are known, the development of A. globulosa in the arthropod vector is intracellular. However, unlike most other filarial species, the larvae of A. globulosa move from one intracellular site to another during development which is necessitated by the marked physiological and anatomical changes that occur in pharate ticks soon after feeding.
9. The third-stage larvae of A. globulosa are transmitted to rodents by the bites of infected ticks and the majority escape during a single feed. In nature only the immature stages of H. leachii feed on rodents while the adults feed on larger carnivores such as dogs. The successful transmission of A. globulosa infective larvae



to susceptible rodent hosts therefore depends on larval ticks acquiring the infection and nymphal ticks transmitting it. Since the larvae of H. leachii moult approximately 13 days after the blood meal and are ready to feed as nymphs around 7 days later, the development of A. globulosa from microfilaria to infective-stage larva in 18 days must be an important factor enabling H. leachii to serve as a vector of this rodent parasite.

B. PROSPECTS OF A. GLOBULOSA IN RODENTS AS A LABORATORY MODEL FOR ONCHOCERCIASIS

1. The microfilariae of A. globulosa cause no pathological conditions in the skin of rodents, but a few may enter the eyes of heavily infected animals which is an interesting parallel with Onchocerca infections.
2. The action of diethylcarbamazine against the microfilariae of A. globulosa and responses of infected rodents during the course of treatment merit further attention, as they suggest that this filarial infection could be particularly valuable as a primary chemotherapy screen for onchocerciasis and model to study mechanisms of Mazzotti reactions.
3. However, the maintenance of A. globulosa in the laboratory is a stumbling block that has not been satisfactorily resolved. As the prevalence of the parasite in striped mice is high within a very short distance of research centres in Nairobi, it is suggested that further work should be done in this enzootic region which will expedite both the chemotherapy studies and attempts to overcome difficulties of maintenance.

PART II

STUDIES ON TICK-BITE HYPERSENSITIVITY  
IN A RODENT HOST IN RELATION TO  
TRANSMISSION OF A TICK-BORNE FILARIAL WORM



CHAPTER 7INTRODUCTION

It is well known that the bites of many haematophagous arthropods provoke an acute allergic reaction in the mammals on which they feed. Such reactions are manifested by localised inflammatory changes in the skin which develop around the site of the arthropod bite within minutes, hours or days and which may occasionally be accompanied by a systemic reaction (Benjamini and Feingold, 1970). Many of the earlier studies on the host response to arthropod bites were confined to detailed clinical and histopathological descriptions of the feeding lesions produced by species of medical or veterinary importance (such as those reviewed by Frazier, 1969). However, in recent years increasing emphasis has been placed on investigations to elucidate the mechanisms involved in the hypersensitivity response to arthropod bites (reviewed by Benjamini and Feingold, 1970) and the effects of this response on the arthropods themselves (Trager, 1939a and b, 1940; Mellanby, 1943; Brumpt and Chabaud, 1947; Chabaud, 1950; Riek, 1956, 1962; Nelson and Bainborough, 1963; Arthur and Snow, 1967; Roberts, 1968a and b; Tatchell and Moorhouse, 1968; Nelson *et al.*, 1972; Allen, 1973; Bagnall and Rothwell, 1974; Bennett, 1974; Roberts and Kerr, 1976; Kemp *et al.*, 1976). Studies such as these have shown that hypersensitivity to arthropods is not only important from the viewpoint of the allergic manifestations as they affect the vertebrate host, but also from the standpoint of its influence on the arthropod population, because several species may suffer serious deleterious effects resulting from the host response to their bites.

As many of the blood-sucking arthropods that elicit hypersensitivity reactions also serve as vectors of parasitic organisms, adverse effects on their feeding and survival may have an important bearing on the transmission of infection. Moreover, the capacity of the vector to pick up infection from the host and the fate of parasites deposited back into the skin might also be affected by an inflammatory reaction at the bite site. Surprisingly, this aspect of the host response to arthropod bites (i.e. the possible role of arthropod-bite hypersensitivity in the transmission of arthropod-borne infections) has been largely ignored; yet the existence of a relationship between bite-hypersensitivity and parasite transmission could have important epidemiological implications for a number of serious infections (discussed by Gordon and Crewe, 1948; Gordon, 1958; Kartman, 1964; Benjamini and Feingold, 1970; Arthur, 1973a).

In order to test the hypothesis that the transmission of filarial parasites might be influenced by allergy to arthropod bites (as originally proposed by Gordon, 1958), an attempt was made in the present study to determine possible effects of tick-bite hypersensitivity in a rodent host (the jird, Meriones unguiculatus) on the dynamics of transmission of a tick-borne filarial worm. For this work the widely studied filarial parasite Dipetalonema viteae was used together with the argasid tick, Ornithodoros moubata. Maintenance of the parasite is described in Chapter 8 and the hypersensitivity experiments are reported in Chapter 9. However, before dealing with the specific experiments that this study entailed and the reasons for choosing the particular model infection employed, a brief review is included of those aspects of the host response to arthropod bites pertinent to the present investigation.

Background to the present work

Studies on the mechanisms involved in the host response to arthropods have been comprehensively reviewed by Benjamini and Feingold (1970). A feature of particular interest in the development of bite-hypersensitivity is that there appears to be a definite sequence of changing skin reactivity in hosts which are repeatedly exposed to the bites of the same, or closely related, blood-sucking arthropods. This phenomenon was originally recognised by Mellanby (1946) who found that human subjects exhibited a characteristic sequence of altered skin reactions when repeatedly bitten by Aedes aegypti. Initially, no reactions were produced in response to bites received by previous unexposed persons, but repeated exposures at frequent intervals resulted in the development firstly of delayed skin reactions (appearing 20-24 hours after biting) and later of additional immediate reactions (appearing within minutes of biting). After a further period of exposure to bites, these subjects gradually lost their delayed skin reactivity but continued to respond with immediate reactions for several months. Although none of Mellanby's original group ceased to produce immediate reactions within the period of observation, others who were bitten by many thousands of mosquitoes eventually entered a state of non-reactivity (described as "hypo-sensitivity") when they would no longer respond with either immediate or delayed skin reactions to Aedes bites (Mellanby, 1946).

This sequence of changing skin reactivity to mosquito bites (Mellanby, 1946) bears a close similarity to the classical "Jones-Mote" type hypersensitivity response of man and animals to injections of foreign sera into the skin (Mote and Jones, 1936). Furthermore, as a feature of the host response to arthropod bites it does not appear to

be restricted to the reaction of man to the bites of mosquitoes. Earlier reports by Kemper (1929) and by Theodor (1935) also imply that the skin reactivity of man may later with time in response to repeated exposures to bed-bugs and sandflies. In addition, the entire sequence of changing skin reactivity to arthropod bites has been demonstrated in experimental animals by McKiel (1959), working with mosquitoes, and by Benjamini *et al.* (1961) and Larrivee *et al.* (1964), working with fleas.

While it is known that the oral secretions of a number of arthropods (and in particular arachnids) contain toxic materials which can cause a marked skin reaction (Balashov, 1972; Arthur, 1973a), it is generally accepted that the sequence of changing skin reactivity resulting from repeated exposure to blood-sucking arthropods is a manifestation of an immunological process in the sensitised host to components of the arthropod saliva (Clements, 1963; Benjamini and Feingold, 1970). Several workers have shown that bite-hypersensitivity can be experimentally induced by injections of antigen prepared from whole arthropods or isolated salivary glands (Trager, 1939a and b; Gregson, 1942; Dubin *et al.*, 1948; McKiel, 1959; Benjamini *et al.*, 1960) and can be transferred with lymph node cells, and to some extent with serum, to animals with no history of previous sensitisation (Trager, 1939a; Bagnall and Rothwell, 1974; Bagnall and Doube, 1975; Roberts and Kerr, 1976; Wikel and Allen, 1976).

The hypersensitivity response evoked in sensitised animals may have a variety of effects on the arthropods themselves. On the one hand, fleas, mosquitoes and many rapidly feeding argasid ticks appear able to engorge normally on even highly reactive hosts (Trager, 1940; Mellanby, 1946; Dubin *et al.*, 1948; McKiel, 1959; Benjamini *et al.*, 1960; Alger and Cabrera, 1972; Balashov, 1972; Sutherland and Ewen,

1974; Kolstrup, 1974, pers. comm.), although Sutherland and Ewen (1974) reported that egg production may be lower, and Alger and Cabrera (1972) found that the death rate was higher, amongst mosquitoes which were fed on rabbits that had been immunised with mosquito extracts. On the other hand, hippoboscids, lice, Sarcoptes mites and several genera of ixodid ticks may suffer a variety of adverse effects on sensitised animals, which acquire a high degree of resistance to reinfestation (Trager, 1939a and b; Mellanby, 1943, 1944; Brumpt and Chabaud, 1947; Chabaud, 1950; Riek, 1956; Nelson and Bainborough, 1963; Arthur and Snow, 1967; Roberts, 1968a and b; Nelson et al., 1972; Balashov, 1972; Allen, 1973; Bagnall and Rothwell, 1974; Bennett, 1974). Acquired resistance to ixodid ticks has been particularly well documented and may be expressed by a lower infestation rate (Trager, 1939a; Gregson, 1942; Brumpt and Chabaud, 1947; Chabaud, 1950; Riek, 1956; Roberts, 1968a and b; Allen, 1973; Bagnall and Rothwell, 1974), reduced intake of blood (Trager, 1939a; Arthur and Snow, 1967), lower egg production (Arthur and Snow, 1967; Bennett, 1974), and higher mortality rate (Riek, 1956; Bagnall and Rothwell, 1974) amongst ticks which attach to hosts that have been previously infested.

Although a number of authors have commented on the possibility of a relationship between hypersensitivity to arthropod bites and the transmission of arthropod-borne parasites, there has been little attempt to test this hypothesis. The aim of this work, as stated earlier, was therefore to examine the various ways in which an allergic reaction to an arthropod vector might influence the transmission of a filarial worm. These included the possible effects of the host reaction on:

A. The vector population

- the ability of the vector to engorge normally on the host
- the survival and fecundity of the vectors after feeding

B. The transmission of infective larvae

- the escape of infective larvae from the vector to the host and their subsequent survival in the vertebrate tissues

C. The uptake of microfilariae

- the capacity of the vector to take up microfilariae from infected hosts
- the subsequent development of microfilariae to infective stage larvae

Of the many and varied infectious organisms transmitted by arthropods, filarial worms were considered a particularly interesting group for study with respect to the effect of the bite reaction on the transmission of infection. Unlike many arthropod-borne diseases, filarial infections typically required prolonged transmission of infective larvae for the development of patent infections in the definitive host (c.f. Hairston and De Meillon, 1968). Furthermore, the adult worms generally live and reproduce for many years so microfilariae will continue to be present in the vertebrate and available for transmission over a relatively long period (Nelson, 1966). During this interval the host response to the bite of the vector may be expected to change, as reported by Mellanby (1946). Therefore, microfilariae and infective larvae that pass through the feeding lesion of the arthropod vector will encounter a markedly different local environment during the act of transmission that will be governed by the type of skin reaction which is mounted by the host. As the transfer of parasites between the vector and the host is a particularly vulnerable stage in the life-cycle of filarial worms, demanding rapid physiological adjustments to the new environment, this might create a link between the success of transmission of microfilariae and infective larvae and



the nature of the host response to the salivary secretions of the vector.

The model of Dipetalonema viteae in the jird Meriones unguiculatus was chosen for this work for the following reasons:

1. In jirds the microfilariae of D. viteae are non-periodic (Hawking, 1967) which was a great asset in studies on the uptake of microfilariae by feeding ticks.
2. Jirds are relatively tame and can be handled without anaesthesia which avoided uncontrolled rises in peripheral microfilaraemia (Worms et al., 1961). Again, this simplified studies on the uptake of microfilariae by ticks.
3. D. viteae is transmitted by argasid ticks which elicit hypersensitivity reactions but remain able to engorge (Trager, 1940). This was considered essential in order to study the relationship between bite-hypersensitivity and the transmission of infection.
4. Soft ticks feed rapidly, as with vectors of human filarial parasites.
5. However, soft ticks, unlike mosquitoes, only feed from pools of extravasated blood which collect beneath the mouthparts (Lavoipierre and Riek, 1955; Balashov, 1972) and never directly from the lumen of capillaries (Gordon and Lumsden, 1939). The use of an arthropod which feeds in a consistent manner and inoculates its saliva into the dermal tissues was considered to be of vital importance in a study of the hypersensitivity response at the site of biting and the way this might influence the transmission of infection.



CHAPTER 8LABORATORY MAINTENANCE OF DIPETALONEMA VITAEAE

This chapter describes the techniques used in the routine maintenance of Dipetalonema vitaeae. The life-cycle of this parasite, as reproduced in the laboratory, is depicted in Figure 34. Details of specialised experimental procedures are included in Chapter 9.

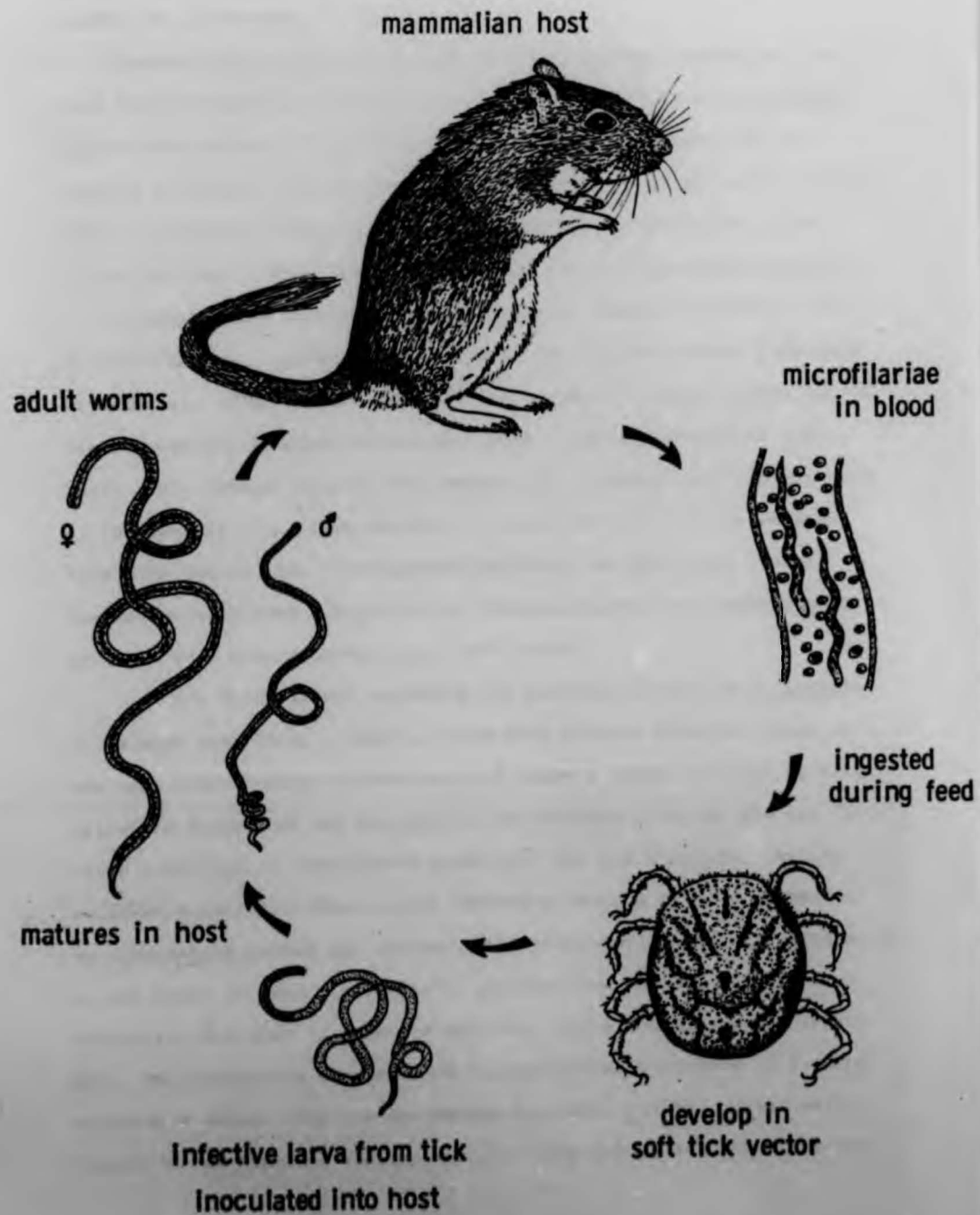
Maintenance of the soft tick vector

The stock of Ornithodoros moubata used in this study was derived from ticks received in 1972 from the Bayer Pharmaceutical Company, Germany. This strain was originally presented to the company in 1955 by Professor Enigk of Tierärztliche Hochschule, Hannover.

All stages of O. moubata were maintained in an insectary at 26°C and 80% relative humidity. Nymphal ticks were stored in 2 x 1 inch glass tubes, and adults in waxed paper drinking cups, which were sealed with covers of nylon gauze. The batches of ticks were held in white enamel trays laid within a water surround to prevent their accidental loss through escape and attack by ants.

Feeding of the stock colony was performed on rabbits and guinea-pigs. For feeding of adult ticks, a 4-5 kilogram New Zealand white rabbit was placed in a stout wooden restraining box so that only the head protruded. The ears were shaved with electric hairclippers and enclosed in rectangular polythene bags containing up to 400 ticks. These were allowed

FIG. 34 LABORATORY MAINTENANCE OF DIPETALONEMA VITEAE



90 minutes to complete engorgement and were then collected, sexed and paired off in batches of 50.

Female ticks commenced egg-laying 2 weeks after feeding and the eggs began to hatch 2-3 weeks later. The larval stage of O. moubata, unlike most members of the family Argasidae, is quiescent and non-feeding and remains within the egg-shell until the first moult (Davies, 1947). Therefore, emerging ticks were already at the first nymphal instar and were offered their first blood meal 10 days after hatching.

Nymphal ticks were fed on guinea-pigs at monthly intervals. To do this, the host was anaesthetised with an intraperitoneal injection of Nembutal, shaved around the torso with electric hairclippers, and a feeding capsule strapped to the back with a small, adjustable girth belt. This feeding capsule was similar, but larger, than the one used on the ears of jirds (see Chapter 3), consisting of a clear perspex tube with fitted lid, lined around the lower rim with foam rubber. Nymphae were allowed 2 hours in the feeding capsule to complete engorgement and were then removed with a soft brush.

In this study it was necessary for specific stages of O. moubata to be kept separately. Nymphal ticks only proceed from one stage to the next after taking a blood meal and since a number of them usually failed to engorge at any one feed it was necessary to isolate fed from unfed individuals. When large numbers of the early nymphal instars required separation, this became extremely tedious if done by hand so an alternative method was devised. Fed and unfed nymphs were separated on the basis of size difference by passing them through an apparatus originally designed to separate male and female mosquito pupae (Plate 35). This apparatus consisted of 2 glass plates held face to face by adjustable screws with a wedge-shaped gap between them. Great variability in the taper of the gap could be obtained by adjustment of the



Plate 35. Apparatus for separating fed and unfed ticks.



Plate 36. Tick required for infection with O. morsitans.



Plate 35. Apparatus for separating fed and unfed ticks.



Plate 36. Bird prepared for infestation with Q. leishmani.

screws. The mixture of fed and unfed ticks was suspended in water and poured into the gap at the top of the plates. Running water was used to flush these through the apparatus and the screws adjusted so that only the small, unfed nymphs passed unimpeded out of the bottom of the plates. By readjustment of the screws, fed ticks that had been trapped behind could then be washed down. Ticks were collected in trays placed beneath the apparatus and dried on filter paper. Their immersion in water caused no deleterious effects and made the handling of a large number of active nymphs much easier. The separation of ticks by sieving proved impractical, as it was impossible to prevent them clinging to the mesh and to each other.

Various reports on the maintenance of O. moubata (reviewed by Hoogstraal, 1956) indicate that the number of nymphal stages through which the ticks pass before reaching maturity may vary between different strains. With the colony used in this work, most males appeared at the fourth moult and females at the fifth.

#### Production of infective larvae

Jirds infected with D. viteae, and with 5-7 microfilariae per cu.mm. of tail blood, were used to infect ticks. If hosts with microfilaraemias much in excess of this figure were used, many of the engorged ticks died. Lower microfilarial levels gave rise to poor yields of infective larvae.

Ticks were infected at the third nymphal instar. This stage was chosen for its moderate size which permitted jirds to be infested with up to 40 nymphs at a time in safety, while the average intake of microfilariae (and hence the yield of infective larvae) still remained high.

The method used to infest jirds with O. moubata (Plate 36) was similar to that described for guinea-pigs, only a smaller feeding capsule was used. Unlike guinea-pigs, jirds were not anaesthetised. This avoided uncontrolled rises in the number of circulating microfilariae (Worms et al., 1961). After an infective blood meal, nymphs were stored as described for those of the stock colony. Under these conditions, D. viteae microfilariae required 28 days to complete development to the infective stage.

#### Recovery of infective larvae

Infective larvae remained viable in ticks for up to 729 days. However, larvae for use in experimental work were collected only from ticks given an infective feed 30-60 days previously. The nymphs received no intervening meals.

To obtain a large number of infective larvae, 20-30 infected ticks were dissected with fine forceps in 10-15 ml of medium 199. The tick tissues, together with emerging infective larvae, were then poured into the top of a Baermann apparatus. This apparatus consisted of a glass funnel, supported by a retort stand, into which was placed a sieve of 75 micron pore size. A short length of rubber tubing, attached to the stem of the funnel, was clamped off with artery forceps. The funnel was filled with enough medium 199 to cover the mesh of the sieve, care being taken to draw out any air bubbles trapped beneath the mesh with a Pasteur pipette.

After half an hour, infective larvae had migrated down the funnel and were tapped off into a small petri dish by collecting approximately 20 ml of medium. The worms were counted under a dissecting microscope



and transferred by pipette to watch glasses containing a minimal amount of medium 199. Care was taken to avoid damaged or sluggish infective larvae. Worms were allowed to settle and were then loaded into 1 ml disposable syringes ready for inoculation.

#### Infection of jirds with *D. viteae* infective larvae

The jirds used in this work were obtained from animal suppliers (Bantin and Kingham, Hull) and laboratory bred stock. These were housed in standard 15 x 9 inch rat cages and fed proprietary rodent pellet (Diet 86, Dixon Ltd., Ware) with water supplied by licking bottles. Up to 6 jirds could be housed per cage, but care had to be taken to introduce all the animals into new quarters simultaneously to avoid severe fighting.

Male jirds were used for infection with *D. viteae*. Each animal was given a single, subcutaneous inoculation of 50 infective larvae into the back or axia of the hind legs. Larvae were injected with between 0.3-0.5 ml of medium 199, using a 1 ml disposable syringe coupled to a 21G x 1.5 inch needle. When an accurate count was required of the number of larvae inoculated, those left in the syringe after injection were flushed out and their number deducted from that of the original inoculum.

#### Estimation of microfilaraemia in jirds infected with *D. viteae*

Most jirds developed patent infections 45-60 days after inoculation

with infective larvae, and the level of microfilariae in the peripheral circulation was then estimated from counts made in blood collected from the tail of unanaesthetised animals. After cutting the tail and encouraging bleeding, 20 cu.mm. of blood was drawn up into a Gold Line graduated pipette. This was immediately discharged into a counting chamber containing 150 cu.mm. of phosphate buffered saline (P.B.S.) with 0.1% saponin.

The counting chamber (described by Denham et al., 1971) consisted of a large microscope slide etched with a grid of parallel lines 3 mm. apart. The grid, which assisted the counting of microfilariae, was framed by thin glass strips stuck down with D.P.X. (R. Lamb, London) to form a well 23 x 15 x 1 mm. in size. Counts of microfilariae were made under the X 40 magnification of a dissecting microscope with critically directed sub-stage illumination.

Although water is normally used as the diluent in counting chambers (see Denham et al., 1971), the microfilariae of D. viteae, unlike some other filarial species (e.g. Brugia pahangi), die in water. As microfilarial counts were easier to perform when the parasites remained active, P.B.S./saponin was preferred for use with D. viteae. This diluent caused rapid lysis of red blood cells, enabling microfilariae to be seen, while the parasites remained motile for at least half an hour.

CHAPTER 9HYPERSENSITIVITY EXPERIMENTSI. Character and ontogeny of the hypersensitivity response to tick bite in jirds

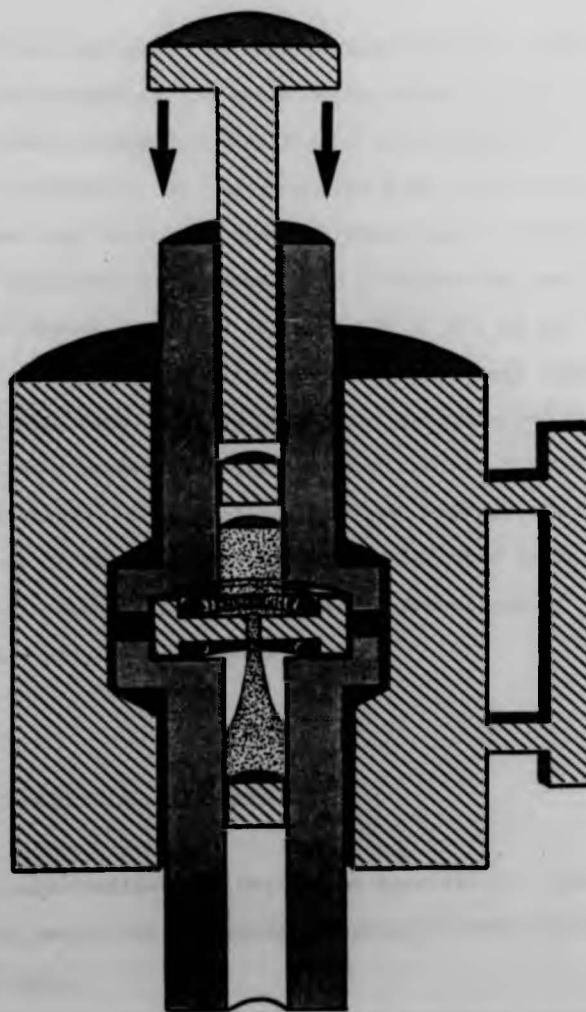
In order to assess the significance of hypersensitivity to tick bite on the transmission of D. viteae, it was first necessary to establish that jirds could be sensitised to the bite of O. moubata. Two methods were employed in an attempt to induce such sensitisation. The first involved the repeated infestation of jirds with nymphal ticks while the second consisted of immunising jirds with an extract derived from the salivary glands of adult ticks. The sensitisation of jirds to tick bite was then assessed by their footpad response to tick salivary gland extract, the histopathology of the bite reaction and the presence of serum antibodies detected in double gel diffusion and passive cutaneous anaphylaxis tests.

MATERIALS AND METHODSPreparation of antigen

1,500 adult ticks were dissected in phosphate buffered saline (P.B.S.) and the salivary glands collected. These were washed in 3

changes of P.B.S., transferred to 1 ml of sterile distilled water and pipetted into the chamber of a Hughes X-press (Figure 35) which had been chilled overnight at  $-20^{\circ}\text{C}$ . The X-press was then returned to the deep freeze ( $-20^{\circ}\text{C}$ ) for a further 3 hours before the frozen suspension of salivary glands was crushed. This was done by applying pressure to the plunger of the X-press with the aid of a fly-press which forced the frozen material through a narrow hole in the partition that divides the press into 2 chambers. To ensure that the antigen was thoroughly ground, the suspension of salivary glands was forced from one chamber to the other 40 times and the X-press chilled briefly at regular intervals throughout the crushing process. The press was then dismantled and the salivary gland suspension transferred in a frozen state to a 30 ml specimen bottle containing 15 ml of sterile distilled water. This was maintained at  $4^{\circ}\text{C}$  while the contents were stirred for 24 hours with a magnetic stirrer, transferred to centrifuge tubes, and spun for 5 minutes at 3000 revs. per minute on a Piccolo centrifuge. The supernatant was drawn off, lyophilised in an Edwards EF03 freeze drier and the dried residue stored in sealed tubes at  $4^{\circ}\text{C}$ .

As required, the lyophilised antigen was reconstituted with sterile P.B.S. (pH 7.2). From this, a sample was removed and the amount of protein contained in the mixture was estimated using either the photocalorimetric method described by Lowry *et al.* (1951) or by direct measurement on a clinical refractometer (Cosmo type R308). The quantity of P.B.S. used for reconstitution was then adjusted to achieve the desired concentration of protein in the final solution.



CROSS-SECTIONAL VIEW OF THE APPARATUS IN USE

#### Footpad test

0.01 ml of salivary gland extract containing 25 µg protein was injected into the footpad of the right front leg of each jird using a 0.1 ml microlitre syringe 710 (Hamilton - Boneduz Schweiz) coupled to a 26 G x ½ inch needle. At the same time 0.01 ml of sterile P.B.S. was injected into the footpad of the left front leg to serve as control. Footpad thickness was measured with a micrometer (Mercer, St. Albans, England) before injection and at 1, 3, 6, 24, 48 and 72 hours after injection. At each time interval, 3 readings were taken of each footpad and the mean nett swelling of the antigen-injected footpad (i.e. the difference between antigen-injected and P.B.S.-injected footpads) was recorded. This swelling was then expressed as the percentage change in thickness of the antigen-injected footpad from its own original thickness to permit a better comparison of footpad swellings between animals of various sizes.

#### Detection of antibodies in jird sera

Jird sera were examined for antibodies specific for tick salivary gland antigen in double gel diffusion and passive cutaneous anaphylaxis (P.C.A.) tests.

Ouchterlony double diffusion in gel was performed as described by Crowle (1961) using 3 x 1 inch microscope slides covered with 2 ml of 1% Special Agar Noble (Difco) in 0.15 M sodium chloride solution. 0.1% sodium azide was added as preservative. Wells of 2 or 4 mm diameter were cut 3 mm from a central well containing tick salivary gland extract (10 mg protein per ml) and the test and control sera

introduced into the outer wells. Only neat sera were used. Tests were performed in triplicate and controlled with normal jird serum and P.B.S. in the outer wells. As an additional control, tests were run in which P.B.S. was used to replace antigen in the centre well. Slides were incubated in a humid chamber for up to 7 days to allow for maximum development of precipitin lines: They were then washed in P.B.S., fixed with methanol, and examined over an illuminated box.

P.C.A. tests were performed using a method modified from that described by Jarrett and Stewart (1972). 0.05 ml of test and control sera were injected intradermally into the shaved backs of albino jirds or Hooded Lister rats. Forty-eight hours later, the recipients were injected intravenously with 0.25 ml of tick salivary gland antigen containing 2.5 mg protein, together with 0.5 ml of 1% Evans blue. After injection, animals were kept under observation for 6 hours to allow for the development of blue weals at the sites of injected sera. The procedure for demonstrating passive cutaneous anaphylaxis was controlled by running a parallel series of tests with the sera of jirds and Lister rats that had been immunised with the widely used allergen, ovalbumin. Animals were immunised with 1 mg ovalbumin (Sigma Grade V) combined with 100 mg aluminium hydroxide gel (as adjuvant) and the sera collected 3 weeks later. This was stored at  $-70^{\circ}\text{C}$  and used in P.C.A. tests under identical conditions to the other test sera.

#### Histology of the bite reaction

To study the histopathology of the bite reaction, jirds were infested with 20 third-stage nymphs on a shaved portion of the left flank 24 hours prior to autopsy, and again on the right flank 6 hours



prior to autopsy. Skin biopsies were taken at autopsy from both of the bite-sites and from a control site on the flank where no ticks had attached. These were fixed in 10% formal saline or Carnoy's fixative, processed, and cut at 5  $\mu$ m for staining with haematoxylin and eosin. Selected sections were also stained with carbol chromotrope for eosinophils (Lendrum, 1944), acidic toluidine blue for mast cells (Smith and Atkinson, 1956) or methyl green and pyronin Y for plasma cells.

#### Sensitising jirds by repeated infestation

70 jirds of either sex were randomly divided into 2 equal groups. For 10 weeks, animals of the first group (R1) were repeatedly infested at weekly intervals with 25 third-stage nymphs confined to the back in a feeding capsule (see Chapter 8). Animals of the second group (R2) were not infested and served as controls. Before each infestation, a sample of 3 jirds was removed at random from each group. These were bled from the tail for serum and their footpad response was measured. The sera from animals of each group were pooled separately and stored in 0.5 ml aliquots at  $-70^{\circ}\text{C}$ . One week after the final infestation the 5 remaining jirds of each group were bled, footpad tested, and infested with ticks so that skin biopsies could be taken for a histological examination of the bite reaction.

#### Sensitising jirds by immunisation

In order to determine the most suitable immunising dose of

salivary gland antigen for sensitising jirds to tick-bite, a preliminary experiment was performed with 12 randomly selected jirds. These were divided into 4 equal groups and given 5 weekly immunisations with 0.25 ml salivary gland extract containing 0 (P.B.S. control), 5, 50 or 500  $\mu$ g protein. The salivary gland extract was emulsified with an equal volume of Freund's complete adjuvant (F.C.A., Difco Laboratories, U.S.A.) and injected subcutaneously into the nape of the neck with a 1 ml tuberculin syringe coupled to a 21 G x 1.5 inch needle. One week after the final immunisation, the jirds were bled to obtain sera and footpad tests were performed.

All jirds given inoculations of 500  $\mu$ g of salivary gland antigen died within 7 days of the first immunisation, presumably due to the high toxicity of the extract. However, those that received 5 or 50  $\mu$ g dosages of antigen tolerated the 5 inoculations as well as controls. The results of footpad tests performed on these jirds are given in Table 24.

TABLE 24

Footpad response of jirds to tick salivary gland extract  
after immunisation with the homologous antigen

Immunisation	Mean nett swelling of antigen-injected footpad (as a percentage of its original thickness) at various times after injection					
	1 hr	3 hr	6 hr	24 hr	48 hr	72 hr
5 x 5 $\mu$ g antigen	7	5	13	19	15	12
5 x 50 $\mu$ g antigen	10	18	22	26	37	39
P.B.S. control	2	1	0	1	0	1

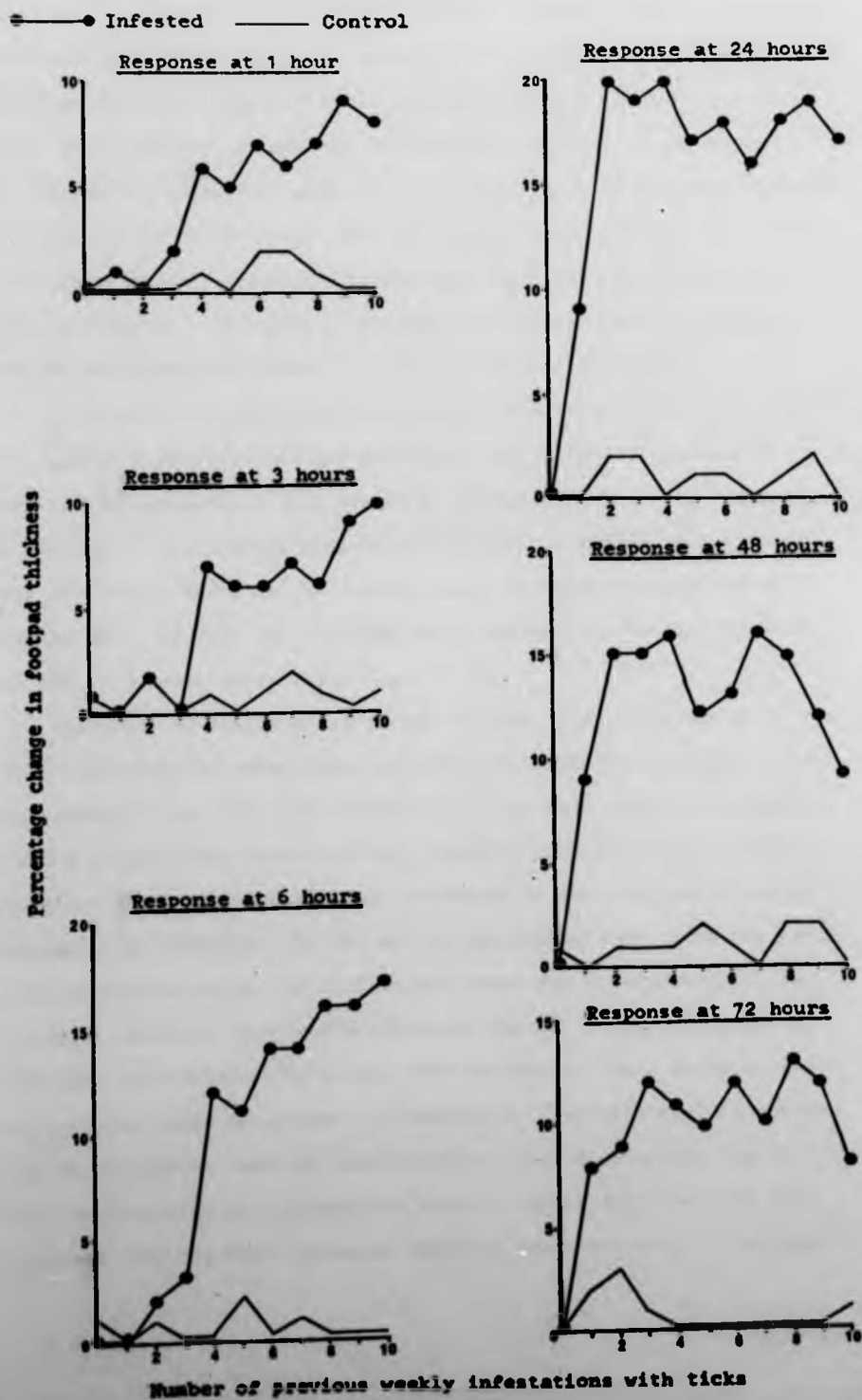
Both surviving immunised groups developed significant footpad swellings compared with controls, but jirds sensitised with the larger dosage of antigen (50 µg per injection) produced a more vigorous footpad response. In Ouchterlony tests, sera from these animals gave rise to 3 strong lines of precipitation. In contrast, sera from jirds immunised with 5 µg doses of antigen produced only 2 faint reaction bands, and control sera produced none. None of these sera gave rise to reaction weals in P.C.A. tests. In view of these results, all further immunisations of jirds with tick salivary gland extract were made using a regime of 5 weekly inoculations of 50 µg doses of antigen.

To study development of the hypersensitivity response in immunised animals, 40 jirds of either sex were randomly divided into 2 equal groups and immunised with either salivary gland extract (group M1) or P.B.S. (group M2) as described above. Before each inoculation, 3 jirds were removed at random from each group, bled for serum and footpad tested. One week after the final immunisation, the 5 remaining jirds of each group were bled, footpad tested and infested with ticks so that skin biopsies could be taken for a histological examination of the bite reaction.

#### Results

The results of footpad tests performed on jirds throughout the course of both sensitisation procedures (Figures 36 and 37) revealed that these animals could readily be sensitised to the bite of O. moubata by either natural (repeated infestation) or artificial (immunisation) means. Jirds of the control groups (R2 and M2) failed to respond in footpad tests to salivary gland extract and produced no

**FIGURE 36** Footpad response in jirds to tick salivary gland antigen 277 after repeated infestation with ticks



more than a 3% change in footpad thickness. However, jirds repeatedly infested with ticks (group R1) developed a marked delayed footpad response within one week of their initial exposure to ticks (Figure 36). This reaction, manifested by footpad swellings at 24, 48 and 72 hours after injection, gave rise to a mean nett increase in footpad thickness of 9% at 24 hours. Following the second and each subsequent exposure to ticks, repeatedly infested jirds produced a far stronger delayed response. Throughout this period, the mean nett increase in footpad thickness was around 20%, 24 hours after injection.

In contrast to the delayed response to salivary gland extract, the immediate reaction did not develop until jirds had received 4 previous infestations. This reaction, manifested by footpad swellings at 1, 3 and 6 hours after injection, steadily increased in magnitude over the next 6 weeks as the animals were successively reinfested (Figure 36). By week 10, the mean nett increase in footpad thickness was 17% at 6 hours after injection.

The onset of immediate reactions on week 4 was accompanied by the first appearance of detectable levels of precipitating antibody. In Ouchterlony tests, sera from animals that had been exposed to between 4 and 9 infestations gave rise to 2 lines of precipitation: These reactions bands grew progressively stronger as the immediate response increased in intensity. By the end of 10 infestations, 3 strong lines were produced by serum run against tick salivary gland extract: In contrast, none were obtained with normal serum. In P.C.A. tests, no reactions were produced by either test or control sera, or by serum derived from jirds immunised with ovalbumin. Nevertheless, it appears that the technique used for demonstrating passive cutaneous anaphylaxis was satisfactory because the sera of Lister rats that had been immunised with ovalbumin produced distinct reaction weals in recipient

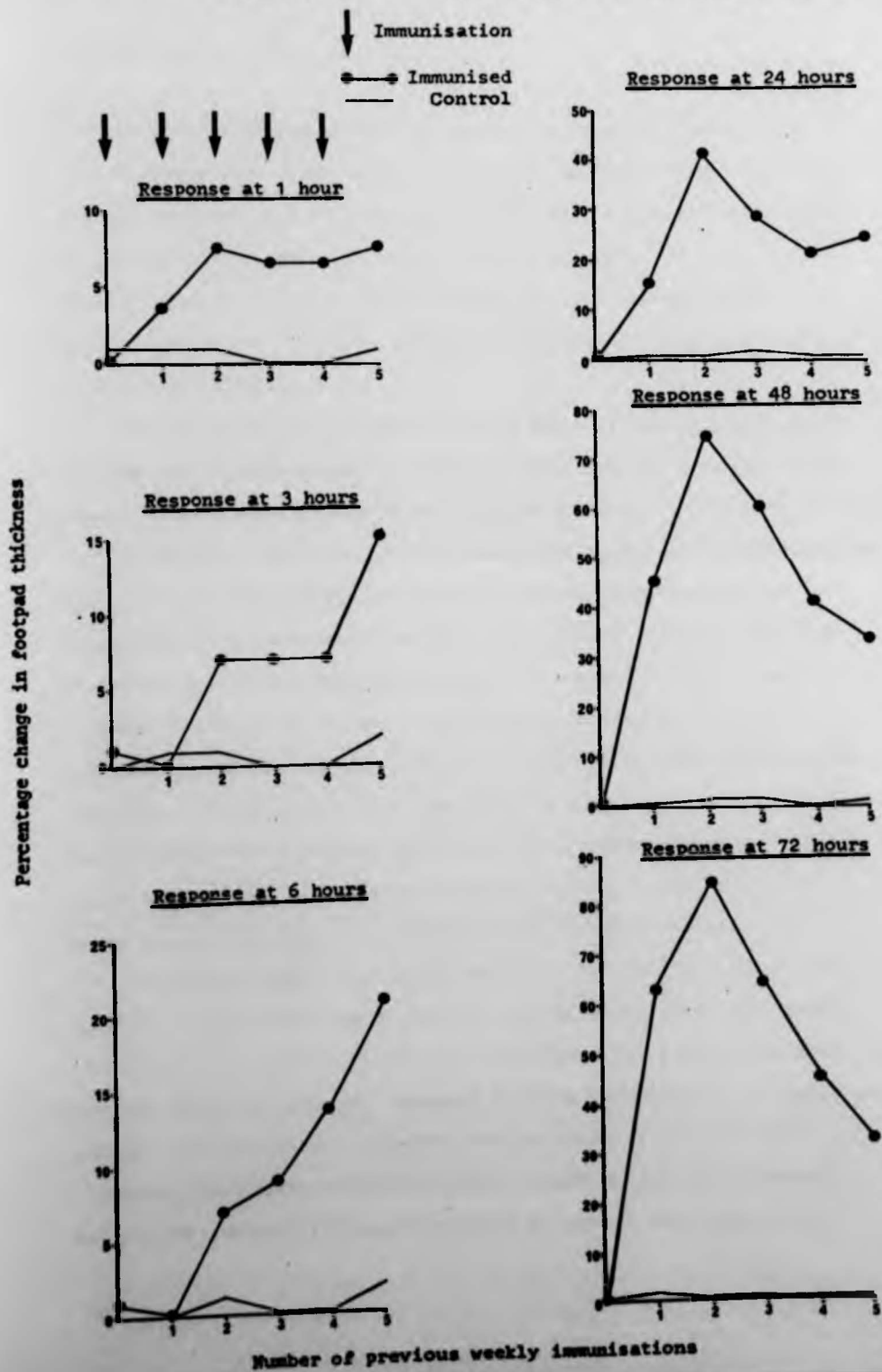
rats intravenously injected with the homologous antigen.

Jirds immunised with salivary gland antigen showed a similar pattern of footpad response to that seen in repeatedly infested animals, although the response was greater and more rapidly produced in the immunised group (Figure 37). Initially, antigen-injected jirds showed no response in footpad tests to salivary gland extract: However, within one week of the first immunisation these had developed a strong delayed response manifested by a mean nett increase in footpad thickness of 64% at 72 hours. The delayed response reached a peak following the second immunisation when the mean nett swelling of antigen-injected footpads was 85% at 72 hours. However, during the course of the next 3 immunisations the intensity of the delayed response fell: By the end of this period the mean nett increase in footpad thickness was only 32% at 72 hours.

In immunised jirds, the immediate response to tick saliva developed after 2 inoculations of salivary gland extract. Over the next 3 weeks this response became stronger as jirds received 3 further immunisations. By week 5, the mean nett increase in footpad thickness was 21%, 6 hours after injection.

In Ouchterlony tests, sera from control animals of group M2 failed to react with tick salivary gland extract. However, antibody was first detected in the sera of immunised jirds one week after the initial immunisation by the development of a single faint line of precipitation. With the onset of immediate reactions on the following week, 2 strong reaction bands were obtained with immunised jird serum. After the full course of immunisation this serum produced 3 strong lines against salivary gland extract. In P.C.A. tests, however, no reactions were produced by either test or control sera.

**FIGURE 37** Footpad response in jirds to tick salivary gland antigen 280  
after repeated immunisation with the homologous antigen





Histopathology of the bite reaction

In non-sensitised animals or those sensitised by immunisation skin biopsies from areas where no ticks had attached showed no histological abnormalities. In these "control sites", animals sensitised by repeated infestation exhibited a mild generalised cellular infiltration composed of neutrophils, lymphocytes and a large number of intact mast cells. Characteristically, the mast cells occurred around dermal blood vessels.

The histological appearance of skin where ticks had fed 6 hours earlier was markedly altered. Cellular infiltrations occurred in the dermis of both sensitised and non-sensitised jirds but were more intense in the sensitised animals. In non-sensitised jirds, bite-reactions were mild and were characterised by a small circumscribed haemorrhage surrounded by a cellular infiltration of neutrophils, with a few eosinophils and scattered intact mast cells. In sensitised jirds, foci of inflammation were far larger, consisting of an extensive zone of haemorrhage, oedema and cellular infiltration (Plate 37). Neutrophils were again predominant in this reaction but were accompanied by a larger number of eosinophils and mast cells, together with a few plasma cells (Plate 38). In sections stained in acidic toluidine blue it could be seen that many of the mast cells had degranulated.

Twenty-four hours after ticks had fed, the bite-reaction in non-sensitised jirds consisted of a much reduced haemorrhagic zone infiltrated solely by polymorphonuclear leucocytes. Few eosinophils remained and some of the neutrophils appeared to have degenerated. In sensitised animals the haemorrhage and oedema was similarly reduced and many neutrophils had pyknotic nuclei, but eosinophils were still numerous and the inflammatory focus had enlarged to contain many more cells

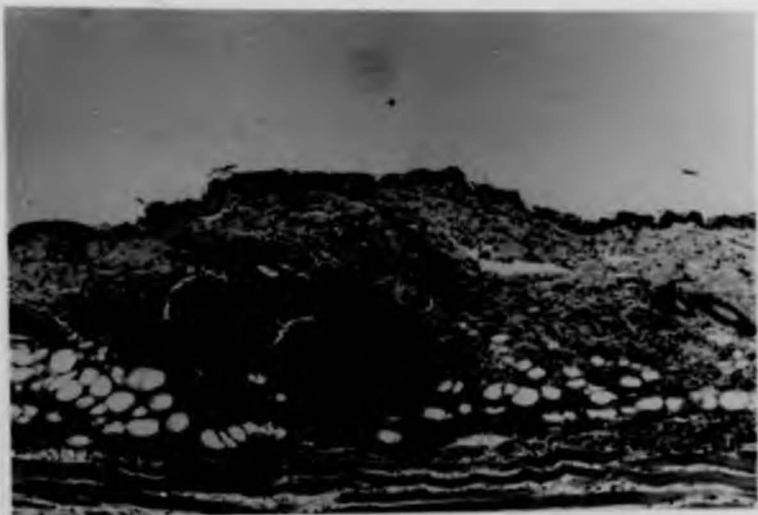
200  $\mu$ m

Plate 37. Reaction to the bite of *O. moubata* in a sensitised jird, 6 hours after tick feeding. Note the extensive area of haemorrhage, oedema and cellular infiltration, and the raised veil of skin seen in transverse section.

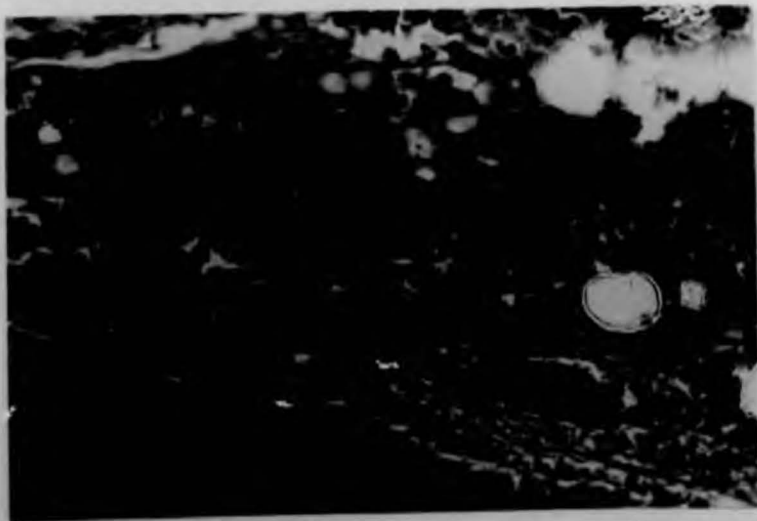
30  $\mu$ m

Plate 38. Detail of Plate 37 showing the marked infiltration of polymorphonuclear cells.

200  $\mu$ m

Plate 37. Reaction to the bite of *O. moubata* in a sensitised jird, 6 hours after tick feeding. Note the extensive area of haemorrhage, oedema and cellular infiltration, and the raised weal of skin seen in transverse section.

30  $\mu$ m

Plate 38. Detail of Plate 37 showing the marked infiltration of polymorphonuclear cells.

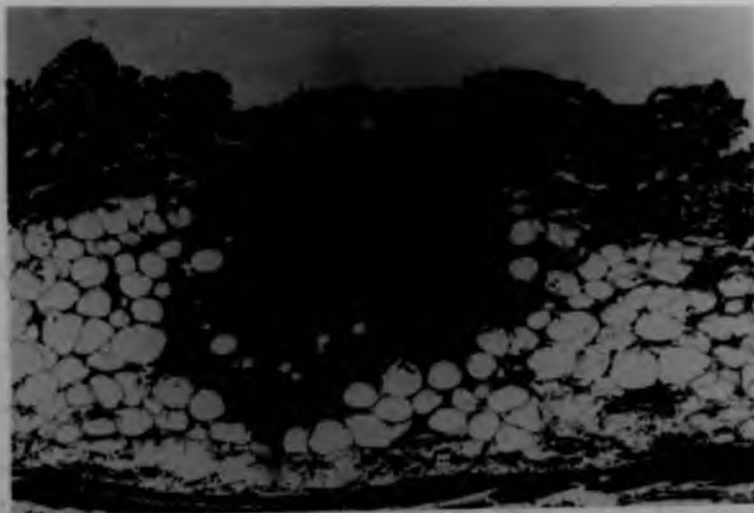
200  $\mu$ m

Plate 39. Reaction to the bite of *D. morbitans* in a sensitised jird, 24 hours after tick feeding. Note the increase in the number of inflammatory cells from the 6 hour response shown in Plate 37.

30  $\mu$ m

Plate 40. Detail of Plate 39 showing the mixed cellular response of polymorphonuclear cells and lymphocytes.

(Plate 39). In part, this was due to a monocytic infiltration of lymphocytes which had been recruited to the remaining polymorphonuclear leucocytes to give a mixed cellular response (Plate 40).

#### Conclusions from Section I

- 1) In jirds, the salivary secretions of O. moubata are both antigenic and toxic.
- 2) Toxicity was manifested by a mild polymorphonuclear infiltration of the skin in jirds first exposed to the bite of ticks and by the death of animals which received large doses (500 µg) of salivary gland protein.
- 3) Antigenicity was manifested by a sequence of cellular and humoral responses to tick saliva.

<u>STAGE</u>	<u>FOOTPAD RESPONSE</u>	<u>ANTIBODY RESPONSE</u>
I	None	None
II	24 hr response only	None
III	6 and 24 hr responses	First detected
IV	6 hr response increasing 24 hr response decreasing	Antibody level increasing

This sequence of changing reactivity to salivary secretions is similar to that reported by Benjamini et al. (1961) for guinea-pigs repeatedly infested with fleas.

- 4) The histopathology of the bite-reaction reported in this section supports the view that jirds mounted immediate (Arthus-type) and delayed hypersensitivity responses to tick saliva (see Dienes and Mallory, 1932; Gell and Hinde, 1954; Gell and Benacerraf, 1961 for

descriptions of the characteristic histology of immediate and delayed hypersensitivity reactions).

5) Jirds sensitised to tick bites by immunisation developed a vigorous immediate hypersensitivity response after 5 inoculations of salivary gland extract. As only the immediate reaction to bites could be expected to influence the transmission of D. viteae (i.e. a response provoked while the vector is still feeding), this sensitising procedure was used in all subsequent studies on the effect of the bite-reaction on transmission of the parasite.

## II. The effect of tick-bite hypersensitivity on the vector population

Having established that jirds could be sensitised to the bite of O. moubata, the next step was to determine whether ticks feeding on these rodents were affected by the host response. Accordingly, 2 experiments were performed in which ticks were fed on sensitised and non-sensitised jirds and the following parameters measured.

- a) The percentage of ticks engorged
- b) The weight of blood ingested (Experiment A only)
- c) The mortality amongst engorged ticks
- d) The time required to moult after feeding
- e) The fecundity of female ticks (Experiment B only)

### MATERIALS AND METHODS

A counted number of ticks were confined to the shaven back of each jird in a feeding capsule (described in Chapter 8) and allowed 3 hours to attach and engorge. All ticks were removed and those which appeared unfed were examined under a binocular microscope to confirm the absence of fresh blood in the midgut. The percentage of ticks engorged was then recorded. In Experiment A, each batch of ticks was weighed on an Oertling electronic balance (with a sensitivity of 0.1 mg) before the blood meal, and those that had engorged were reweighed after feeding. The mean fed and unfed weight of ticks and mean weight of blood ingested were then calculated.

In a preliminary experiment it was found that the weight of blood ingested by third-stage nymphs was directly proportional to their unfed weight (see Appendix 1). Inevitably, ticks used in the present study



varied in size and so to compare the weights of their blood meals it was necessary to convert these values into a form that eliminated the variable of unfed weight. To do this, a sample of ticks was fed on "normal" jirds, their individual unfed weights plotted against the weight of blood ingested, and a form of "calibration curve" constructed (see Appendix 1). From this could be determined an expected size of blood meal for ticks of known unfed weight and a ratio calculated of the observed/expected blood meal weights. This ratio was then used to compare the sizes of blood meal taken by ticks in Experiment A.

Fed ticks were retained in an insectary (under conditions described in Chapter 8) and examined daily at noon so that the number which were dead or moulting could be recorded. To determine the time interval between feeding and moulting the cumulative percentage of ticks that had moulted on each day following the blood meal was plotted and the period required for 50% of the population to moult, calculated. This was considered more satisfactory than taking a mean value as it eliminated the bias that entered the mean from the occasional tick in which the moult was delayed. In Experiment B, female ticks were retained after feeding and placed individually in holding tubes. Twenty-eight days were allowed for these to lay eggs and the number laid then determined by weight.

The feeding and post-feeding behaviour of ticks may be affected by a number of intrinsic and extrinsic factors. In order to reduce the variability in tick behaviour caused by factors other than the host response to tick-bite, the following precautions were observed.

- 1) All infestations were carried out under identical conditions of heat, light and humidity. After feeding, all ticks collected for study were also maintained under identical conditions.
- 2) The outbred jirds used in these experiments were randomly divided

into groups to minimise the influence of host genetic factors in the predisposition of ticks to feed on each group.

3) Jirds were not anaesthetised during infestation to avoid lowering of body temperature, but ticks were confined to feeding capsules to prevent the host disturbing engorgement.

4) All ticks were derived from the same stock colony and those refusing to feed during an experimental infestation were discarded (i.e. not returned to stock).

5) Ticks were starved for between 30 and 80 days prior to feeding. Within these limits, the percentage of ticks that engorged was unrelated to the period of starvation before the blood meal (see Appendix 2). Nevertheless, all ticks used on a given week (for test and control feedings) were starved for the same period of time prior to infestation.

#### Experimental designs

##### Experiment A

In this experiment, the influence of tick bite hypersensitivity on one specific stage of O. moubata (the third nymphal stage) was studied.

60 jirds of either sex were randomly divided into 3 groups. Jirds of the first group (5 animals) received 10 successive weekly infestations with 25 third-stage nymphs. Jirds of the second group (5 animals) were immunised with tick salivary gland antigen (as described in section I) and infested 7 days after each of the 5 immunising doses with 25 third-stage nymphs. Each week, 5 jirds were taken from the third group (50 animals) and infested on that one occasion with 25 third-stage nymphs derived from the same stock as those used in each of the test infestations. These jirds represented a non-sensitised population of

hosts to serve as a control for the tick populations used during each week of the experiment.

#### Experiment B

In nature, argasid ticks such as O. moubata frequently live in the resting places of their hosts and may therefore reattach to the same individual throughout their life-cycle. This experiment was designed to study the influence of tick-bite hypersensitivity on all stages of O. moubata and the cumulative effect of the bite reaction on ticks raised from egg to adult on the same individual host.

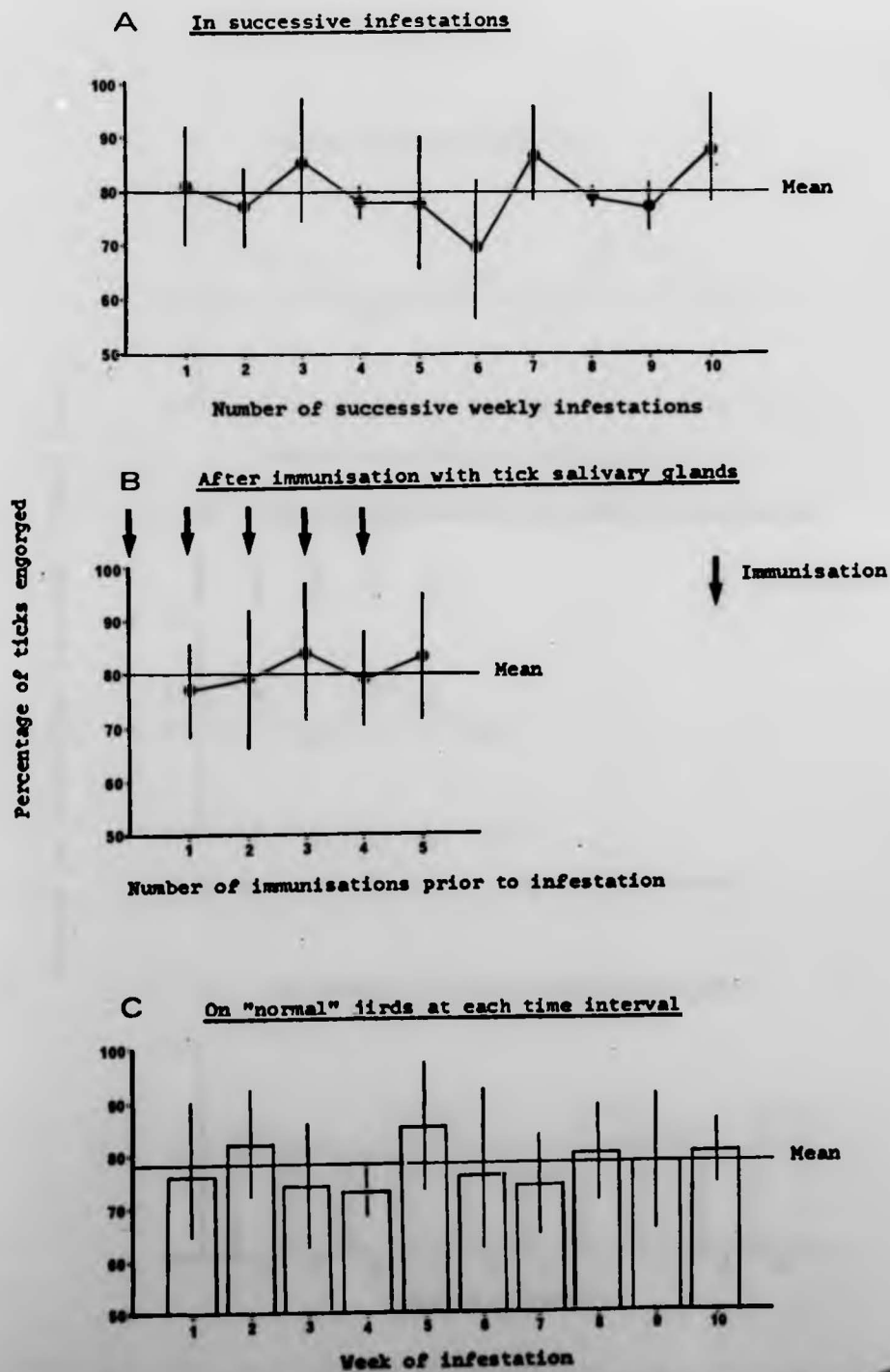
25 jirds of either sex were randomly divided into 2 groups. Jirds of the first group (5 animals) were used to raise 3 batches of ticks (A, B and C) from egg to adult. Initially, each jird received 3 consecutive infestations with 100 first-stage nymphs, fed on the host at fortnightly intervals. The succeeding nymphal stages were then refed on the same host and in the same order as before. Thus, each jird received 3 consecutive infestations with each nymphal stage using the 3 batches of ticks. Jirds of the second group (20 animals) were used for control feedings, and infested only once. Initially, 5 of these animals were each infested with 200 first-stage nymphs. The second stage nymphs arising from each batch were then transferred to 5 new hosts to be refed. This process was repeated for third and fourth stage nymphs. At the end of the experiment all the adult ticks raised on each group of jirds were pooled; 50 females were then taken at random from each pool to determine egg production.

RESULTSExperiment A (Figures 38-40)

Third-stage nymphs fed on all 3 groups of jirds (i.e. repeatedly infested, immunised, and normal control animals) showed a similar pattern of feeding and post-feeding behaviour throughout the course of this experiment, with respect to the percentage of ticks that engorged (Figure 38), size of blood meal ingested (Figure 39) and time interval between feeding and moulting (Figure 40). Whilst values for each of these parameters were subject to weekly fluctuations no trend appeared which suggested that the behaviour of ticks was in any way influenced by the development of a hypersensitivity reaction to tick-bite in jirds of the sensitised groups. Thus, ticks fed as readily on repeatedly infested animals after 10 infestations as after 1 (Figure 38A) and still ingested an equivalent amount of blood (Figure 39A) and moulted in a similar period of time (Figure 40A) to those fed in a primary infestation.

On immunised jirds, a similar picture emerged (see Figures 38, 39 and 40). The percentage of ticks that engorged (Figure 38B), the weight of blood ingested (Figure 39B) and time required to moult (Figure 40B) varied little between ticks fed on hosts sensitised with 5 doses of salivary gland antigen and those fed on hosts after only a single dose of antigen. Ticks that had fed on all 3 groups of jirds survived equally well and the mortality amongst pre-moulted nymphs never exceeded 4%.

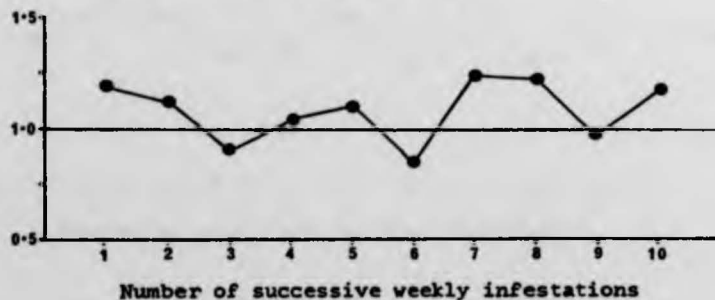
**FIGURE 38** Percentage of third-stage nymphs of *O. moubata* feeding on birds ( Mean values  $\pm$  S.D.).



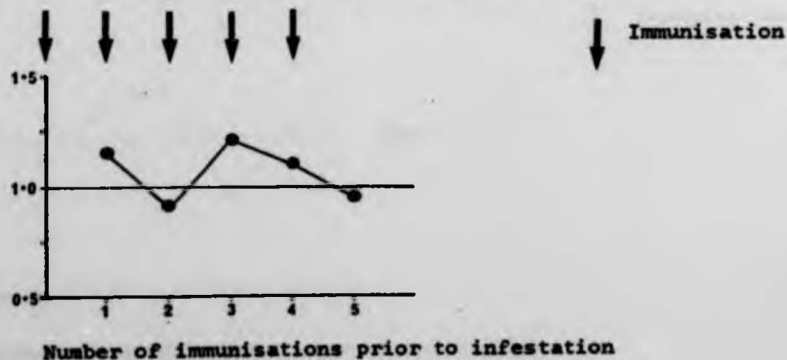
**FIGURE 39** Relative sizes of the blood meals ingested by various batches of third-stage nymphs of *O. moubata* on jirds

Ratio of the Observed/Expected weight of blood ingested

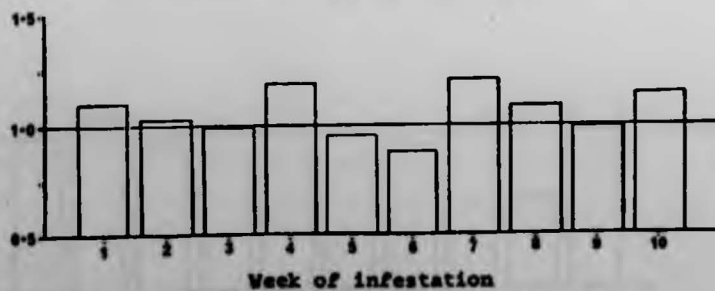
**A** In successive infestations

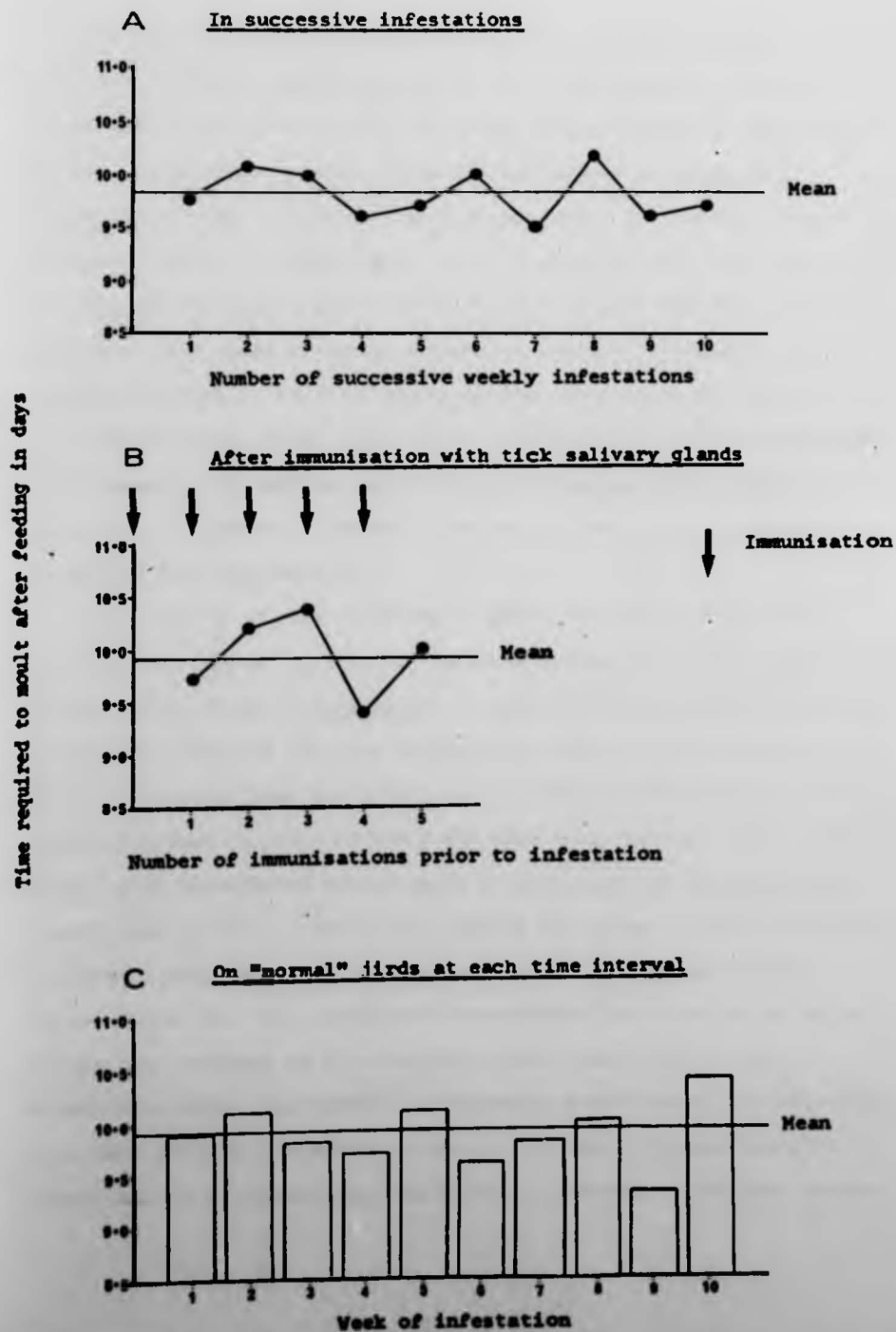


**B** After immunisation with tick salivary glands



**C** On "normal" jirds at each time interval







Experiment B (Table 25)

No differences were found in the feeding and post-feeding behaviour of ticks raised from egg to adult on repeatedly infested hosts and those transferred to new hosts for each stage in their life-cycle. Within each nymphal stage, the percentage of ticks that engorged and time required to moult were similar for batches raised on both groups of animals (Table 25). On repeatedly infested jirds it also emerged that ticks of batch C, that were always the last to be fed at each nymphal stage, engorged on the host and moulted as readily as ticks of batch A, that were the first to be fed (Table 25).

The mortality among ticks fed on both groups of jirds was extremely low. Late nymphal stages survived better than the early nymphs but there was no difference between ticks fed on the 2 groups and the mortality never exceeded 6%.

The results of this experiment suggest that tick-bite hypersensitivity acquired by jirds by repeated infestation had no appreciable effect on any stage of O. moubata. In addition, there appeared to be no cumulative effect of the bite reaction on ticks fed throughout their life-cycle on the same individual host. Female ticks raised in this way laid a mean ( $\pm$  S.D.) of 138 ( $\pm$  45) eggs while those raised on normal jirds (i.e. transferred to new hosts at each stage in the life-cycle) laid a mean of 131 ( $\pm$  43) eggs. During the course of this experiment a higher proportion of ticks engorged with each successive nymphal stage (Table 25). This could not be attributed to a cumulative effect of the bite reaction as it occurred on both sensitised and non-sensitised jirds. The probable explanation would seem to be that ticks reluctant to feed were gradually eliminated from the population, as those failing to engorge would also fail to proceed to the next nymphal

**TABLE 25** The feeding and moulting behaviour of *Ornithodoros moubata* raised from egg to adult on jirds by either successive refeeding on the same host ( Reinfested ) or transfer to new hosts for each blood meal ( Control ).

**TICK  
BATCH.**

**NYMPHAL STAGE**

	NYMPHAL STAGE			
	1	2	3	4
	Reinfested	Reinfested	Reinfested	Reinfested
	Controls	Controls	Controls	Controls
<b>A</b>	56 (8)	73 (19)	83 (9)	94 (7)
<b>B</b>	51 (11)	71 (13)	82 (9)	96 (6)
<b>C</b>	52 (23)	73 (21)	85 (9)	99 (2)
	9.8 (0.4)	8.3 (0.4)	9.3 (0.3)	12.6 (.7)
<b>A</b>	10 (0.6)	9.8 (0.5)	9.5 (0.3)	12.2 (.4)
<b>B</b>	9.8 (0.4)	8.2 (0.3)	9.4 (0.4)	12.5 (.5)
<b>C</b>	9.8 (0.4)	8.3 (0.3)	9.3 (0.3)	12.4 (.3)

Mean percentage  
of ticks fed  
( $\pm$  S.D.)

Mean number of  
days to moult  
( $\pm$  S.D.)

instar.

Conclusions from section II

- 1) Jirds that responded to tick bites with immediate and delayed hypersensitivity reactions remained fully susceptible to reinfestations with all stages of O. moubata.
- 2) The feeding and post-feeding behaviour of ticks did not vary between those fed on sensitised jirds and those fed on controls.

III. The effect of tick-bite hypersensitivity on the transmission of infective larvae and their establishment in the vertebrate host

It is clear from the results of the previous section that tick-bite hypersensitivity did not affect the feeding process of O. moubata. This suggests that the transmission of infective larvae to sensitised hosts will not be governed by the ability of ticks to engorge, but does not preclude that the bite-reaction may have a direct bearing on the escape of infective larvae from feeding ticks and their establishment in a sensitised animal.

In a number of parasitic relationships it has been shown, or suspected, that antigenic determinants are shared between the parasites and their intermediate hosts (Capron et al., 1968; Kemp et al., 1974; Lackie, 1976; Jackson, 1976). To determine whether the same is true in the case of Dipetalonema viteae, and if a host response to the bite of the vector might act directly on invading parasites, infective larvae were collected from ticks and incubated in the sera of guinea-pigs immunised with either whole tick or salivary gland antigen. Whole tick antigen was prepared from 10,000 unfed first-stage nymphs using the method described for salivary glands. The method of immunising guinea-pigs was similar to that used for birds (described in section I) only that each immunising dose of whole tick antigen contained 500  $\mu$ g of protein. In Cuchterlony tests, the serum of whole tick immunised guinea-pigs produced 6 lines of precipitation against whole tick extract and 3 against salivary gland extract while the serum of salivary gland immunised guinea-pigs produced 3 reaction bands against both of these antigens. No lines were obtained with the serum of control inoculated guinea-pigs.

Fifteen batches of 10 infective larvae were transferred by cat's

whisker into the wells of a Microtiter plate containing 100  $\mu$ l aliquots of neat serum from immunised or control guinea-pigs. The Microtiter plate was covered and placed in an incubator at 37°C. At half hour intervals the plate was examined under a binocular microscope and the number of motile larvae in each well recorded. In all of the sera infective larvae remained motile for up to 6 hours but were non-motile, presumably dead, within 24 hours. However, within the first 4 hours of incubation, 35 of 50 infective larvae in the serum of whole tick immunised guinea-pigs developed an adhering precipitate around the anus which was not observed with larvae maintained in the sera of salivary gland or control immunised animals.

In view of this result, an attempt was made to determine whether these precipitates were composed of immune complexes and whether other tick antigen (or shared antigenic determinants) were present on infective larvae. To do this, direct and indirect fluorescent antibody tests were performed on living worms; these were collected from ticks, washed 5 times in P.B.S. and incubated for a half, one, or four hours in serial dilutions of whole tick immunised guinea-pig serum. Other larvae were incubated in P.B.S. (negative control), serial dilutions of normal guinea-pig serum (negative control) or serial dilutions of serum from a guinea-pig immunised against *D. viteae* infective larvae (positive control for the antigen). This last serum produced 3 strong lines of precipitation against a homogenate of infective larvae in Ouchterlony tests and had been prepared by injecting 300 infective larvae subcutaneously into the guinea-pig on 5 occasions. As a positive control for the anti-whole tick serum, pieces of fresh salivary gland were also incubated in each of the guinea-pig sera.

After incubation, the infective larvae and salivary glands were

washed in 3 changes of P.B.S. and transferred into 25  $\mu$ l aliquots of fluorescein labelled rabbit anti-guinea pig serum (Wellcome) diluted 1 in 10 with P.B.S. These were left for 30 minutes at 37°C, washed again in P.B.S., mounted in buffered glycerine and examined under a Nikon fluorescence microscope.

In undiluted anti-whole tick serum precipitates formed around the anus of infective larvae which fluoresced in labelled anti-guinea pig serum. Cuticle fluorescence was not seen on these worms, or on those incubated in normal serum, although it was observed on larvae incubated in anti-larval serum at titres up to 256. Salivary glands fluoresced in anti-whole tick serum up to a titre of 4096, although non-specific fluorescence occurred in normal serum up to a titre of 32. Larvae and salivary glands incubated in P.B.S. did not fluoresce.

Results of these in vitro studies suggest that larvae excreted tick material soon after leaving the vector. In the presence of specific antibody, this became bound in immune complexes that formed a precipitate around the anus of living worms. It is not known whether such an event occurs when infective larvae enter a tick-bite sensitised host but the formation of these precipitates in vitro did not appear to reduce the viability of the parasites. No evidence could be found that the infective larvae of D. viteae share common antigenic determinants with their tick vector. Anti-whole tick serum, which was highly active against tick tissue, failed to react with infective larvae: Moreover, the cuticle of worms was readily coated with antibody directed against the larvae themselves.

The transmission of infective larvae and their establishment in tick-bite sensitised and normal jirds

The aim of this work was to determine whether infective larvae may be transmitted to, and establish in, jirds sensitised to the bite of the vector.

Experiment 1

Thirty-six male jirds were randomly divided into 2 equal groups and immunised with 5 weekly doses of salivary gland antigen (sensitised group) or P.B.S. (control group). One week after the final immunisation, each jird was infested with 50 second-stage nymphs confined to the back in a feeding capsule. These had been infected with D. viteae 30 days previously by feeding them on jirds with counts of 70-90 microfilariae per cu. mm. of tail blood. Infected ticks placed on jirds were allowed 3 hours to attach and engorge and those failing to do so were replaced so that each animal received 50 infective bites. Fifty ticks taken at random before the blood meal and dissected individually contained a mean ( $\pm$  S.D.) of 24 ( $\pm$  26) infective larvae.

Immediately after the ticks had fed, 6 jirds of each group were autopsied (as described in Chapter 3) and the larvae collected. Fifty days later, the remaining jirds were autopsied and the worms recovered were sexed, counted and measured. Female worms were examined under a compound microscope to determine whether mature microfilariae were present in their uteri.



### Results

Immediately after the ticks had fed, infective larvae were recovered from 4 of 6 sensitised jirds and 3 of 6 controls. All worms were found within the connective tissues and skin close to the site of infestation. The recovery of larvae from the sensitised group was 26 (4-52) per infected host while that from controls was 48 (19-79). As it was estimated that each tick contained a mean of 24 infective larvae before the blood meal, the 50 ticks fed on each host could potentially transmit 1,200 worms. Only 1.4 (0-4)% of the larvae in ticks were transmitted to sensitised animals and 2 (0-7)% to controls.

Mature adult worms were recovered from both groups of jirds autopsied 50 days after tick feeding (see Table 26). Half the animals of each group harboured adult worms with a mean ( $\pm$  S.D.) of 8.5 ( $\pm$  7.5) per infected host in the sensitised group and 9.8 ( $\pm$  6.6) in the controls. This difference was not statistically significant. The proportion of male to female worms in the 2 groups of jirds was similar (see Table 27). Worms recovered from sensitised hosts were slightly larger than those from controls (Table 27) but only the males were significantly longer ( $t = 2.3$   $0.0125 < P < 0.025$ ). The difference in length of the females was not statistically significant and all of these worms contained mature microfilariae.

From these results it is clear that infective larvae may escape from ticks feeding on both sensitised and normal jirds and establish themselves in these hosts. However, it was not possible to determine the proportion of worms that matured in each group as the original number of larvae received varied greatly from host to host (i.e. 0-79 larvae per jird in animals killed on the day of transmission). This was because the level of infection in the tick population and number

**TABLE 26** Transmission of *D.vitae* by *O.moubata* to jirds sensitised or non-sensitised to the bites of this tick

	<u>SENSITISED GROUP</u>	<u>NON-SENSITISED GROUP</u>
Number of animals in group	12	12
Number of animals harbouring worms, 50 days after tick feed	6	6
Mean number of worms per host ( $\bar{x}$ S.D.)	4.3( $\bar{x}$ 6.7)	4.9( $\bar{x}$ 6.8)
Mean number of worms per infected host ( $\bar{x}$ S.D.)	8.5( $\bar{x}$ 7.5)	9.8( $\bar{x}$ 6.6)
<b>TOTAL NUMBER OF WORMS PER GROUP</b>	<b>51</b>	<b>59</b>

**TABLE 27** Transmission of D.viteae by O.moubata to jirds sensitised or non-sensitised to the bites of this tick : Details of the sex and length of adult worms recovered 50 days after infection

	<u>SENSITISED GROUP</u>	<u>NON-SENSITISED GROUP</u>
Total of male worms recovered (%)	30 (59%)	34 (58%)
Mean length in mm. (+ S.E.)	24.8( $\bar{x}$ 0.7)	22.5( $\bar{x}$ 0.6)
Total of female worms recovered (%)	21 (41%)	25 (42%)
Mean length in mm. (+ S.E.)	44.6( $\bar{x}$ 1.1)	41.6( $\bar{x}$ 1.9)
<b>TOTAL WORMS PER GROUP</b>	<b>51</b>	<b>59</b>

of larvae transmitted by ticks were both highly variable. To determine the proportion of worms surviving in sensitised and non-sensitised jirds, an accurate estimate was required of the number of larvae transmitted. This could be done by either:-

- 1) Increasing the number of infected ticks fed on each host.
- 2) Directly controlling the number of parasites each jird received.

The first of these options proved impractical as jirds could not tolerate infestations of more than 50 second-stage nymphs. Therefore the second approach was taken in the following experiment.

#### Experiment 2

30 male jirds were randomly divided into 2 equal groups and immunised with either salivary gland antigen (sensitised group) or P.B.S. (control group). One week after the final immunisation, jirds were infected with D. viteae by the syringe inoculation of a counted number of infective larvae performed in such a way as to simulate the natural mode of transmission. To do this, each jird was infested with 30 uninfected third-stage nymphs confined to the back in a feeding capsule. As soon as these had fed, 100 infective larvae collected by dissecting ticks (see Chapter 8) were slowly inoculated intradermally over the site of the fresh tick bites. Larvae were suspended in 0.01 ml of tick salivary gland extract containing 25  $\mu$ g of protein and injected using a 0.1 ml microlitre syringe coupled to a 26 G x  $\frac{1}{4}$  inch needle. After injection, the number of larvae left in the syringe was determined (and generally found to be half that of the original inoculum).

To test the validity of the procedure used here to simulate the

natural mode of D. viteae transmission, a comparative histological examination was made of skin biopsies taken from naturally (Experiment 1) and artificially (Experiment 2) infected jirds. Two sensitised and 2 non-sensitised animals exposed to the bites of infected ticks, together with 2 jirds from each group of the present experiment, were killed immediately after infection and deep biopsies taken of the skin and underlying muscle at the site of infection. These were processed, sectioned and stained with haematoxylin and eosin.

50 days after infection the remaining animals of each group were autopsied. These consisted of 13 normal and 11 sensitised jirds as 2 of the sensitised animals died accidentally due to fighting. All worms recovered on autopsy were sexed and counted and forty of each sex were taken at random from both groups to be measured.

### Results

#### Comparative histology of the site of infection in "naturally" and "artificially" infected jirds

##### 1) Sensitised animals

The histological appearance of biopsies taken from jirds exposed to the bites of infected ticks revealed an acute inflammatory reaction (as described in section 1) containing the occasional infective larva. In artificially infected jirds the cellular infiltration was more intense and contained many more worms but was otherwise similar (Plate 41). Most larvae were present in the dermis although some had already reached subcutaneous tissues.

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50  $\mu$ m

Plate 41. Infective larva of D. vitreus in the skin of a tick-bite sensitised jird, immediately after infection. Note the marked infiltration of polymorphonuclear cells due to tick saliva inoculated along with the parasites.

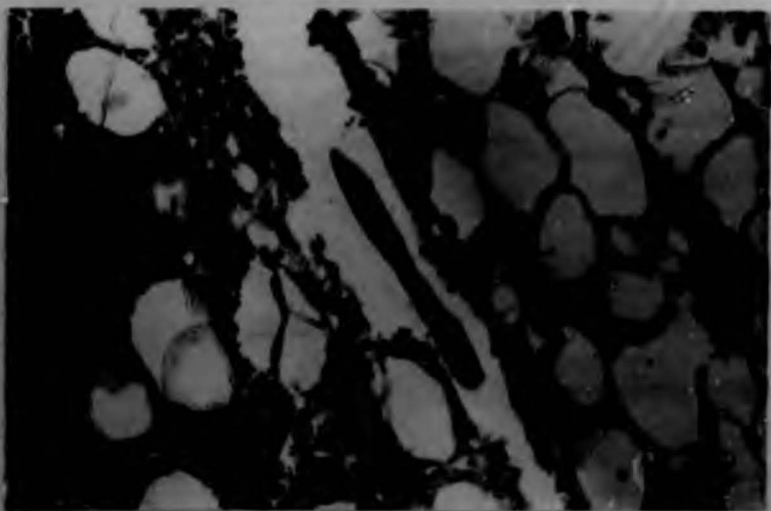
50  $\mu$ m

Plate 42. Infective larva of D. vitreus in the skin of a normal jird, immediately after infection. Note the absence of a marked tissue response due to tick saliva inoculated along with the parasites.



## 2) Non-sensitised animals

In naturally infected jirds, the sites of tick bites were infiltrated by few cells (as described in section 1) and infective larvae lay in the dermis surrounded by little host reaction. The histological appearance of biopsies from artificially infected jirds was essentially the same (Plate 42) but for the presence of many more larvae and some localised tissue damage at the point of injection.

In view of these findings it was concluded that the procedure of infection used in Experiment 2 sufficed to simulate the natural process of transmission for the purpose of this study.

## Autopsy findings

All jirds autopsied 50 days after infection contained mature adult worms. A mean ( $\pm$  S.D.) of 33.5 ( $\pm$  16.4)% of inoculated larvae survived to maturity in the sensitised group while 32.6 ( $\pm$  16.8)% survived in controls. The 170 worms recovered from the former group and 202 from the latter were made up of a similar proportion of males and females (44% males and 56% females in each case). In contrast with the first experiment, male worms from sensitised and control animals were of similar size, with mean ( $\pm$  S.E.) lengths of 24.6 ( $\pm$  0.6) mm and 24.4 ( $\pm$  0.4) mm in the 2 groups respectively. However, female worms from the sensitised group were significantly longer than the ones from controls ( $t = 2.7$   $0.01 > P > 0.005$ ). These had a mean ( $\pm$  S.E.) length of 46.2 ( $\pm$  1.7) mm in sensitised animals while only 40.9 ( $\pm$  1.0) mm in controls although all contained mature microfilariae.

Conclusions from section III

- 1) The escape of infective larvae from the vector to the host is not influenced by the host response to the bite of the vector.
- 2) The infective larvae of D. viteae do not share common antigenic determinants with their tick vector and are not therefore subject to direct immunological attack in hosts that respond to tick antigens.
- 3) Nevertheless, infective larvae inoculated by ticks pass through an intense cellular reaction (provoked by tick saliva) in tick-bite sensitised animals.
- 4) These larvae survived and matured to establish normal filarial infections. The difference in size of adult worms from sensitised and non-sensitised jirds may have been anomalous as only males were affected in one experiment and only females in the other. Different rates in the growth of worms, rather than an absolute difference in the size attained, would seem the most likely explanation of this phenomenon.

IV. The effect of tick-bite hypersensitivity on the uptake of microfilariae by the feeding vector

In the previous section it was established that both sensitised and normal jirds may become infected with D. viteae but it had not been determined whether ticks can ingest microfilariae as readily from either group of hosts. The final section of this chapter therefore deals with a study made to compare quantitative aspects of microfilarial uptake by ticks fed on sensitised and non-sensitised jirds infected with D. viteae.

Sixteen male jirds were each inoculated with 50 infective larvae and their blood examined 60 days later for microfilariae. Twelve animals that developed patent infections were randomly divided into 2 equal groups and immunised with either salivary gland antigen (sensitised group) or P.B.S. (control group). Seven days after the final immunisation, each jird was infested with ticks and the following parameters measured.

- 1) The density of microfilariae in host blood.
- 2) The weight of blood ingested by each tick (i.e. fed - unfed weight).
- 3) The number of microfilariae ingested by each tick.

Two hours before infestation, each jird was bled from the tail and 60 cu.mm. of blood examined in a counting chamber to determine the density of microfilariae in the peripheral circulation. A feeding capsule was then fitted to each host and further samples of blood taken at half hour intervals and examined for microfilariae. This was done as it was found that the stress produced in jirds by the attachment of feeding capsules caused a marked, but transient rise in the peripheral microfilaraemia (cf. Hawking et al., 1964). Jirds were infested with

ticks as soon as microfilarial counts had resumed their original level. While the microfilariae of D. viteae are non-periodic in jirds (Hawking, 1967), precautions were necessary to avoid further changes in the microfilarial level during infestation. Jirds were maintained in a warm room (26°C) and at no time were they restrained or anaesthetised as it is known that the lowering of host body temperature, stress, or anaesthesia may each result in an elevated level of circulating microfilariae (Hawking et al., 1964; Hawking, 1967; Hawking et al., 1967).

Fifty second-stage nymphs of similar size were selected for each infestation. These were weighed before feeding, placed on the host, and the first 30 to complete engorgement reweighed individually as they detached. This was done to minimise the effects of excretion. Weighed ticks were dissected individually in counting chambers to determine the number of microfilariae ingested. The remaining ticks that fed were held in an insectary (at 26°C and 80% R.H.) and dissected one month later to check whether ingested microfilariae developed into infective stage larvae.

Immediately after the ticks had fed, 2 jirds of each group were killed and skin biopsies taken from the site of infestation. These were processed, sectioned and stained in haematoxylin and eosin for a histological examination of the feeding lesions.

### Results

The histological appearance of feeding lesions confirmed that sensitised animals reacted vigorously to tick-bite (as previously described) while normal hosts did not. In all jirds, microfilariae

were found in the pool of extravasated blood that collected beneath the bite-site and which in sensitised animals was heavily infiltrated with cells.

On both groups of jirds, ticks ingested microfilariae which successfully matured to infective stage larvae. Nevertheless, there was great variability in the uptake of microfilariae from tick to tick and from host to host. The quantitative data from this experiment are presented in Appendix 3 and a synopsis of the findings is given in Table 28.

#### Processing of the data

It was not possible to make a direct comparison of the numbers of microfilariae taken up from sensitised and non-sensitised jirds as the various batches of ticks imbibed different quantities of blood and were fed on hosts with different microfilaraemias. Instead, ratios of the observed/expected ( $O/E$ ) intake of microfilariae were calculated for each individual arthropod which served as a useful index to compare the relative capacity of ticks to acquire microfilariae from the various hosts. The expected intake of microfilariae was calculated by the number present in a unit volume of host blood multiplied by the volume of blood that the tick ingested. For the conversion of blood meal weights into volumes, the specific gravity of blood was assumed to be 1.

When the data had been processed it was found that nearly all ticks contained many more microfilariae than would be expected from the microfilarial density in the host and size of blood meal ingested (see Appendix 3). In part, this was undoubtedly due to underestimating the weight of blood meals, as O. moubata excretes coxal fluid before feeding

**TABLE 28** The relative capacity of O. moubata ticks to take up microfilariae of D. viteae from jirds sensitised or non-sensitised to tick bites : A synopsis of the data presented in Appendix 3.

ANIMAL CODE NUMBER	SENSITISED GROUP Mean O/E uptake of mff. ( $\bar{x}$ S.D.)	NON-SENSITISED GROUP Mean O/E uptake of mff. ( $\bar{x}$ S.D.)
1	4.98 ( $\bar{x}$ 3.49)	2.94 ( $\bar{x}$ 1.53)
2	6.82 ( $\bar{x}$ 5.14)	3.29 ( $\bar{x}$ 2.30)
3	5.80 ( $\bar{x}$ 3.33)	4.90 ( $\bar{x}$ 3.74)
4	6.25 ( $\bar{x}$ 4.36)	6.92 ( $\bar{x}$ 4.96)
5	2.62 ( $\bar{x}$ 2.30)	2.49 ( $\bar{x}$ 0.94)
6	6.33 ( $\bar{x}$ 5.78)	3.82 ( $\bar{x}$ 1.68)
GROUP MEAN	5.46 ( $\bar{x}$ 4.40)	4.06 ( $\bar{x}$ 3.21)

**Note :** Figures given in this table are mean values ( $\bar{x}$  S.D.) for ratios of the Observed/Expected (O/E) uptake of microfilariae by 30 third-stage nymphs fed on each individual host.

is complete (Lees, 1946; Lavoipierre and Riek, 1955, and personal observation). However, there was no loss of microfilariae in coxal fluid (personal observation) as reported by Londono (1976b). Another possible source of error might have arisen in the measurement of host microfilaraemia. This was determined in blood from the tail, while ticks were fed on the back (as it was impractical to infest unanaesthetised jirds on the tail). Presumably these inaccuracies applied to both groups of jirds as the experimental technique was never varied. It was therefore felt that these errors were acceptable as the sole objective of this study was to compare the relative capacity of ticks to acquire microfilariae from sensitised and non-sensitised hosts.

#### Analysis of the data

Means ( $\pm$  S.D.) of the O/E intake of microfilariae for all ticks fed on sensitised jirds (178 nymphs) and all ticks fed on controls (180 nymphs) were 5.46 ( $\pm$  4.4) and 4.06 ( $\pm$  3.2) for the 2 groups respectively. In a student's 't' test, the difference in these values was highly significant ( $t = 3.46$ ,  $P < 0.0005$  with 356 degrees of freedom). To analyse the data in this way it must be assumed that all jirds within each group were uniformly infective to ticks. However, it can be seen from Table 28 that this was clearly not the case.

In view of this finding, the data were reanalysed by the Mann-Whitney rank test using means of the O/E uptake of microfilariae from each individual host (see Table 28). This enabled the 2 groups of jirds to be compared while allowing for the variability between hosts in their infectivity to ticks. It was also important to establish that



variations in the O/E uptake of microfilariae were not due to differences in the level of host microfilaraemia. To explore this possibility, the significance of the relationship (if any) between these parameters was tested by the coefficient of correlation.

Reanalysis of the data confirmed that there was a greater intake of microfilariae by ticks fed on sensitised animals than by those fed on controls. However, the difference between the groups was far less significant ( $P = 0.09$  with 10 degrees of freedom). This result emphasises the great variability between hosts in their infectivity to ticks and indicates that the host response to tick bite may not have been the only factor influencing the uptake of parasites. Nevertheless, there was no statistical significance in the relationship between host microfilarial levels and the O/E uptake of microfilariae (coefficient of correlation  $r = -0.085$  with 4 degrees of freedom on normal, infected jirds).

#### Conclusions from section IV

- 1) There was great variability in the uptake of microfilariae from tick to tick and from host to host.
- 2) Ticks fed on sensitised animals ingested relatively more microfilariae than those fed on controls.
- 3) Nevertheless, further work is necessary to verify that the host response to tick bite significantly alters the capacity of ticks to ingest microfilariae because other host factors, that were not identified, appeared also to affect the uptake of parasites.

DISCUSSION

Humphrey and White (1970) defined hypersensitivity or allergy as "a specifically induced altered reactivity in which there is evidence of an underlying immunological mechanism". There seems little doubt that jirds developed such an altered capacity to react to the bites of O. moubata after repeated exposure to ticks, or immunisation with salivary gland extract: Animals sensitised by either procedure showed the rapid evolution of a hypersensitivity response, as judged by the development of immediate and delayed footpad responses, the histopathology of the bite reaction and the appearance of circulating serum antibodies (see Section I).

Nevertheless, jirds that had been immunised or had been repeatedly infested remained fully susceptible to all stages of O. moubata. In addition, the survival and fecundity of ticks raised on sensitised animals was similar to that for ticks raised on non-sensitised hosts (see Section II).

From previous studies on hypersensitivity to arthropods it has become increasingly clear that only those species which feed for several days are adversely affected by the host response to biting (Trager, 1939a and b, 1940; Mellanby, 1943; Brumpt and Chabaud, 1947; Chabaud, 1950; Risk, 1956; Nelson and Bainborough, 1963; Arthur and Snow, 1967; Roberts, 1968a and b; Nelson *et al.*, 1972; Balashov, 1972; Allen, 1973; Bagnall and Rothwell, 1974; Bennett, 1974). This suggests that components of the host response effective against arthropods are ones that appear relatively late, or are slow to develop, after the initial introduction of salivary secretions. This view is supported by observations on the rejection process of ixodid ticks on resistant

animals in which it appears that the mouthparts are walled off by an inward growth of the epidermis (Trager, 1939a), or are isolated from the blood source by the appearance of vesicles packed with inflammatory cells at the site of tick attachments (Allen, 1973).

In the present study, all stages of O. moubata completed engorgement within 90 minutes. It therefore seems likely that these ticks detached too early to incur potentially adverse effects of the host response. Interestingly, Trager (1940), also working with argasid ticks, found that the rapidly feeding nymphs and adults of Argas persicus could readily engorge on bite-sensitised chickens while larvae, which require up to 4 days to feed, were partially inhibited from normal engorgement.

There have been few reports on the fate of arthropod-borne parasites in vertebrates which have been sensitised to the arthropod vector. Alger and Harant (1976a and b), working with Plasmodium berghei, claimed to obtain a 30% protection of mice against sporozoites from infected mosquitoes if the animals were firstly immunised with the salivary glands of normal mosquitoes. In contrast, McGreevy et al. (1975) working with filarial worms, found no difference in the survival of Brugia pahangi infective larvae in cats that had been immunised with mosquito thoraces compared with others that were injected into a normal animal. These authors also reported that by means of an indirect fluorescent antibody technique they were unable to detect significant quantities of mosquito antigen on the cuticle of B. pahangi third-stage larvae (McGreevy et al., 1975).

A similar picture emerged in the present study as it was found that D. viteae third-stage worms do not share common antigens on their surface with O. moubata ticks (see Section III). Moreover, the escape

of infective larvae from the vector to the host and their subsequent establishment in the vertebrate tissues did not appear to be influenced by allergic reactions to tick feeding.

Gordon (1958) proposed that for a number of arthropod-borne diseases a host reaction to the vector bite may markedly enhance transmission of infection. To illustrate this point he chose examples of Trypanosoma cruzi and louse-borne typhus (a rickettsial infection) which are both transmitted to man in the arthropod faeces if these contaminate the wound which the vectors leave after feeding. Gordon (1958) suggested that individuals who respond strongly to the vector bites are at greatest risk of contracting infection, as they are more likely to contaminate the feeding lesion by rubbing and scratching than others in which the bite is relatively benign. Host behavioural responses to the bites of haematophagous arthropods are indeed an interesting aspect of the host-vector relationship that could have an important bearing on parasite transmission. However, in the present study dealing with transmission of filarial worms these were not considered, as the behaviour of rodents during infestation with an argasid tick vector can have little relevance to man and his actions against vectors of human filariae.

In the present work on infection of the arthropod vector it was found that the uptake of microfilariae by ticks fed on bite-reactive animals was relatively greater than on non-sensitised controls. However, the difference between these groups was not statistically significant and appeared to be complicated by host factors other than hypersensitivity to the tick bites (see Section IV).

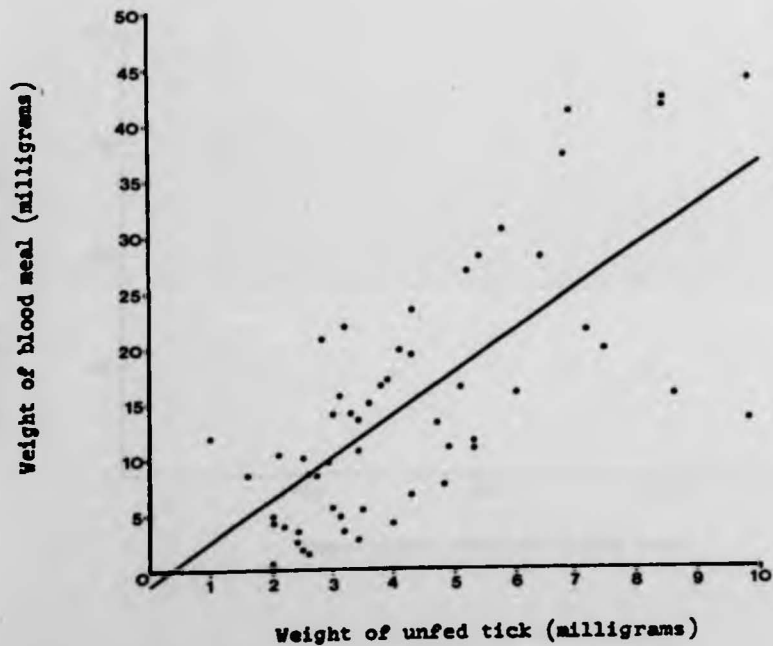
From the results of this study it seems highly improbable that host allergy to tick bites influences the transmission of D. viteae. If the findings of this work have relevance to human filarial infections,

then the reactions of man to the bites of mosquito vectors are also likely to have a negligible effect on the transmission of infection. However, an appreciation of the relationship (or lack of it) between bite-hypersensitivity and the transmission of infection can only serve to add to our understanding of the epidemiology of filariasis. Further work will be necessary using other model infections to evaluate the possible role of arthropod-bite hypersensitivity in the transmission of filarial parasites which have skin-dwelling microfilariae.



Figure 1. Relationship between the percentage of bites with hypersensitivity and the percentage of bites with infection. The line represents the theoretical relationship if hypersensitivity is a result of infection.

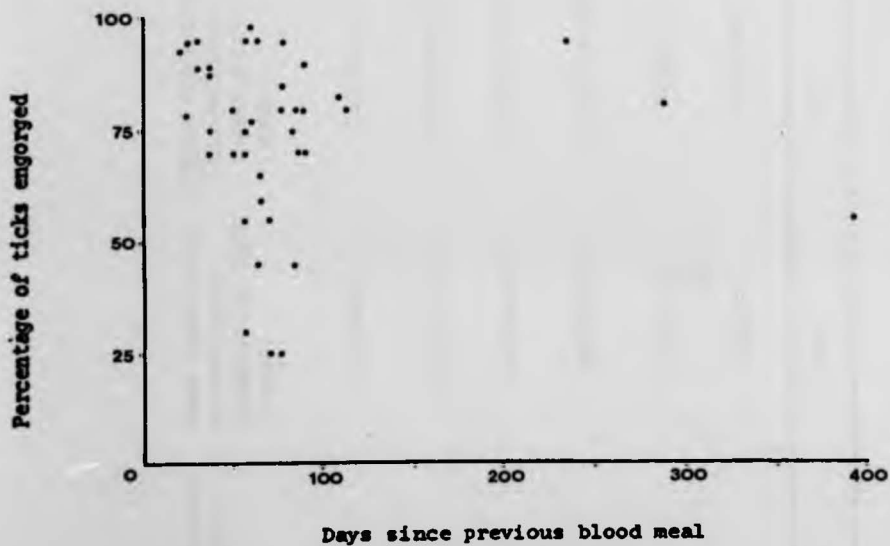
APPENDIX 1 Relationship between the unfed weight of O.moubata ticks and the weight of blood ingested ; based on examination of 55 individual third-stage nymphs fed on "normal" jirds under identical conditions.



Coefficient of correlation  $r = + 0.72$   
Degrees of freedom (  $n-2$  ) = 53  
 $P < 0.001$  Highly significant

It is concluded that there is a positive, proportional relationship between the unfed weight of third-stage nymphs and the weight of blood they ingest while feeding

APPENDIX 2 Success rate of feeding of *O.moubata* ticks after various periods of fasting ; based on examination of 40 batches of third-stage nymphs (each containing 20-40 ticks) fed on "normal" jirds under identical conditions.



Coefficient of correlation  $r$  ( between success rate of feeding and period of prior fasting ) =  $-0.07$

Degrees of freedom  $(n-2)$  = 38

Not significant

It is concluded that there is no proportional relationship between the percentage of ticks that engorge and the period of fasting prior to infestation.



APPENDIX 3 Uptake of microfilariae of Dipetalonema viteae by Ornithodoros moubata fed on jirds sensitised or non-sensitised to tick bites ; based on individual examinations of 30 third-stage nymphs fed on each of 12 infected hosts.

SENSITISED GROUP

Animal code number	Mean wt. blood (mg.) ingested per tick (range)	No. microfilariae per cu.mm. host blood	Mean expected no. microfilariae ingested per tick (range)	Mean observed no. microfilariae ingested per tick (range)	Mean ratio of the observed/expected uptake of mff. per tick (range)
1	4.1(1.9-7.6)	12.9	53(26-99)	281(25-707)	4.98(0.49-12.4)
2	4.7(2.3-8.1)	58.1	274(139-470)	1760(224-4830)	6.82(0.93-19.9)
3	4.2(1.7-8.8)	52.8	219(90-465)	1163(377-3608)	5.80(1.68-14.5)
4	3.8(1.1-6.2)	22.1	84(23-136)	478(113-1252)	6.25(1.08-20.5)
5	5.6(2.9-8.9)	3.7	20(10-33)	50(2-220)	2.62(0.13-11.0)
6	4.7(2.6-8.4)	2.6	12(6-21)	68(8-324)	6.33(0.67-21.2)
GROUP MEAN	4.52	25.4	110.3	633	5.46

APPENDIX 3 - continued

NON-SENSITISED GROUP

Animal code number	Mean wt. blood (mg.) ingested per tick (range)	No. microfilariae per cu. mm. host blood	Mean expected no. microfilariae ingested per tick (range)	Mean observed no. microfilariae ingested per tick (range)	Mean ratio of observed/expected uptake of mff. per tick (range)
1	4.8(2.5-8.4)	2.3	11(6-19)	29(10-80)	2.94(0.84-8.0)
2	4.6(2.1-7.2)	5.9	27(12-42)	90(9-279)	3.29(0.36-10.0)
3	5.0(3.2-8.0)	5.8	29(19-47)	146(26-406)	4.90(1.08-15.1)
4	5.0(2.6-8.1)	1.2	6(3-10)	38(13-92)	6.92(2.71-22.3)
5	5.2(3.1-8.0)	7.1	37(22-57)	90(27-183)	2.49(0.70-4.36)
6	4.7(2.2-8.4)	39.8	198(88-334)	786(91-2642)	3.82(0.91-6.8)
<b>GROUP MEAN</b>	<b>4.80</b>	<b>10.4</b>	<b>51.3</b>	<b>197</b>	<b>4.06</b>

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## Intrauterine development of the microfilariae of *Dipetalonema viteae*

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### ABSTRACT

The egg shell of *Dipetalonema viteae* separated from the oolemma and became highly convoluted at an early stage of development. No second oolemma or trilaminar membrane was seen. Channels containing electron dense material (thought to be nutrient material from the uterine wall) were formed between adjacent embryos. Many developing embryos died.

Microvilli were formed by the uterine wall and developing embryos were closely apposed to these (again, presumably to obtain nutriment).

Embryos emerged from the egg in the uterus and were born as unsheathed microfilariae.

Terry *et al.* (1961) studied the embryogenesis of *Dipetalonema viteae* by light microscopy and showed that it was ovoviviparous. The ultrastructure of the intrauterine development of several non-sheathed microfilariae has been reported. McLaren (1972, 1973) studied *D. viteae*, Harada *et al.* (1970) and Lee (1975) studied *Dirofilaria immitis*, and Kagei (1960) studied *Setaria cervi*. Rogers *et al.* (1976) described the development of sheathed *Brugia pahangi* embryos from after fertilization to birth, with particular emphasis on the origin and development of the sheath of the microfilaria and its possible role in the nutrition of the developing embryo. They showed that:

1. The secreted egg shell separates from the oolemma and becomes convoluted,
2. "Nutrient channels" containing the uterine secretions are formed between adjacent embryos,
3. Many embryos die in the central areas of the uterine lumen,
4. Nearly mature embryos become buried in the uterine wall suggesting additional direct contact with the uterine wall to increase nutrient supplies to the rapidly differentiating embryos,
5. Fully differentiated microfilariae detach from the uterine wall and straighten out, the convoluted egg shell unfolding to form the microfilarial sheath.

It is the purpose of this paper to study the development of *D. viteae* *in utero* and compare the development of this parasite with that of *B. pahangi*.

### MATERIALS AND METHODS

Fully mature female *D. viteae*, obtained from the subcutaneous connective tissue of infected *Meriones unguiculatus* were examined by light and electron microscopy using the methods described by Rogers *et al.* (1976).

### OBSERVATIONS AND DISCUSSION

#### Light microscope studies

The development of *D. viteae* within the egg shell, from morula, through C-shaped form, to the coiled fully developed microfilaria, was similar to that described by Rogers *et al.* (1976) for the sheathed *B. pahangi* and the unsheathed *Onchocerca gutturosa* microfilariae, except that, once differentiated, the microfilariae of *D. viteae* formed a tight spiral with up to 4 coils instead of a double ring (Fig. 9b). The microfilaria hatched out of the egg shell in the same way as did the embryo of *O. gutturosa*. Terry *et al.* (1961) also observed the hatching of *D. viteae* microfilariae *in utero* and reported the presence of degenerating embryos.

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#### Ultrastructural studies

Sectioning of the *D. viteae* uterus began at the vulval end of the fertilization chamber. It was assumed that the eggs had already been fertilized as uninucleate egg cells were surrounded by spermatozoa, but no sperm were seen inside ova. The undivided, fertilized eggs were similar to those of *B. pahangi*, having as an oolemma a distinct unit membrane, covered by an egg shell, presumably secreted during fertilization, as described by McLaren (1973). The egg shell membrane formed initially as an uneven secretion (Fig. 1) and did not become uniform in thickness until cell division started. McLaren (1973) also observed this feature of *D. viteae*. The egg shell of *B. pahangi* becomes defined earlier. Even at this early stage of *D. viteae*, there were channels between the future egg shells containing material which we suggest is, as with *B. pahangi* (Rogers *et al.*, 1976), a nutrient secretion of the uterine wall (Fig. 2), which may also have a lubricating function.

Even before cell division commenced, the egg shell sometimes separated from the oolemma (Fig. 3), although usually separation did not start until cell division had begun. This latter timing is comparable with the situation found in *B. pahangi* where separation of the oolemma and egg shell occurred at the 3-4 cell stage (Rogers *et al.*, 1976). We could find no trace of a trilaminate membrane formed from the original oolemma as was reported by McLaren (1973). The egg shell seems to be formed by oval secretion only, while the underlying oolemma is a clear unit membrane. We found abundant new membranous elements beneath the oolemma, as did McLaren (1973), but in our view these new membranes were being laid down ready for the rapid division stages of the very large cells found at this time (Fig. 4) rather than for the formation of a new oolemma to replace one discarded as part of the egg shell. Once cell division has started and the egg shell separates from the oolemma and enlarges, the additional membranous material can still be seen beneath the oolemma. It would, therefore, seem that these structures are not part of the egg shell, but are available for the membrane requirements of early embryonic division. McLaren (1973) stated that by the end of meiosis in *D. viteae*, both the surface coat and original oolemma had separated from the ovum surface and a new oolemma was formed under the stimulus of fertilization. Her most recent work (in press) has shown that, using Uranyl acetate as a fixative (as opposed to its later use as a stain), additional plasmalemma-like membrane structures can be visualised in schistosomulae. A similar fixing technique was used in obtaining her observations on *D. viteae* quoted above, and may possibly account for the apparent differences in our two findings with this particular unshathed microfilaria. We also found that the egg shell often became folded before complete separation from the oolemma (Fig. 5).

Development of *D. viteae* was not synchronous, like that of *B. pahangi*, so that development did not occur uniformly down the uterus. Figure 4 shows an early fully developed unshathed worm amongst embryos at the third cell division stage. The difference in nuclear and cell sizes of these stages of development is dramatic and shows the enormous amount of new membrane required for each new cell created at this early stage.

Channels between adjacent egg shells were filled with electron dense material (Figs. 6 and 7) which appeared identical with that formed by *B. pahangi*. We again presumed this material, similarly produced as an apocrine secretion from the uterine wall, to be nutrient. Close apposition of the egg shells of embryos to the uterine surface was also similar to that seen in *B. pahangi*. Embryos at quite different developmental stages could be adjacent and share the same nutrient channels, as shown by an embryo with a fully formed cuticle adjacent to an early form (Fig. 6), again demonstrating the asynchronous development of the embryos in any zone.

As the embryos differentiated, they curled up as spirals with up to 4 coils (Figs. 9a and b). *B. pahangi* embryos did not spiral in this fashion,  $1\frac{1}{2}$  coils being their usual maximum.

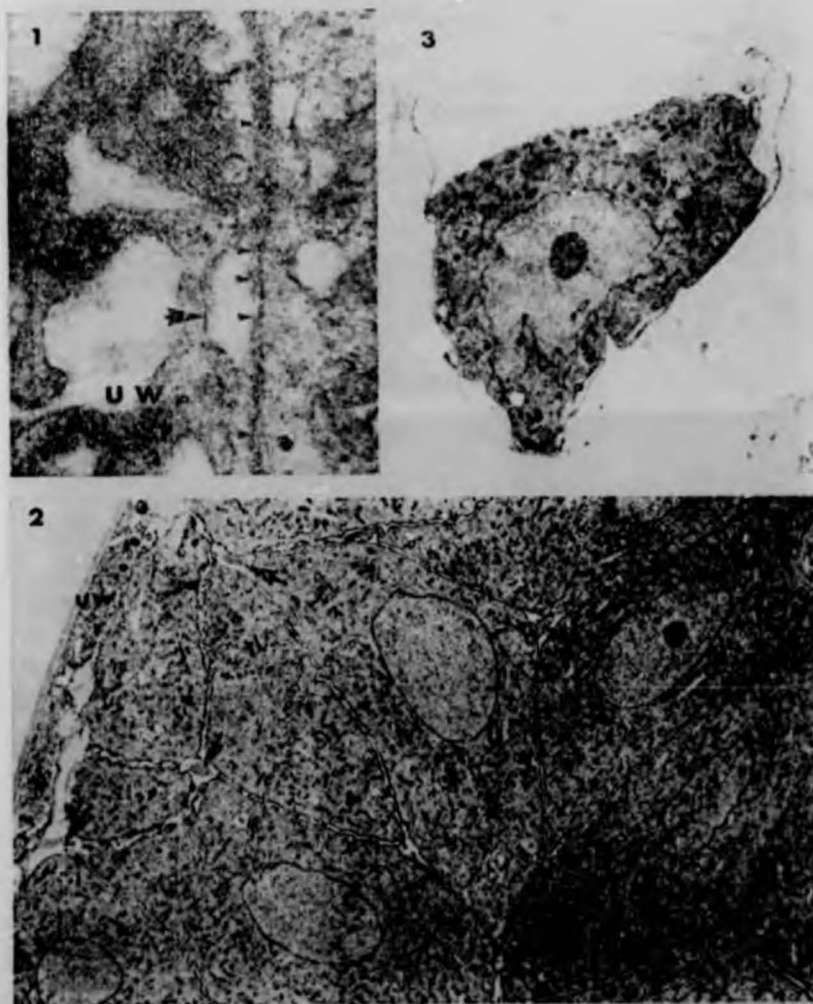


FIG. 1. "Egg shell" being secreted on the microfilarial embryo plasmalemma (small arrows). The membrane of the adjacent uterine wall (u.w.) is indicated by a large arrow ( $\times 70000$ ).

FIG. 2. Uninnucleate embryos packed tightly within the uterus, whose 'nutrient' secretions (arrowed) are seen passing between them ( $\times 2300$ ).

FIG. 3. Early 'egg shell' separation from the embryo ( $\times 6300$ ).

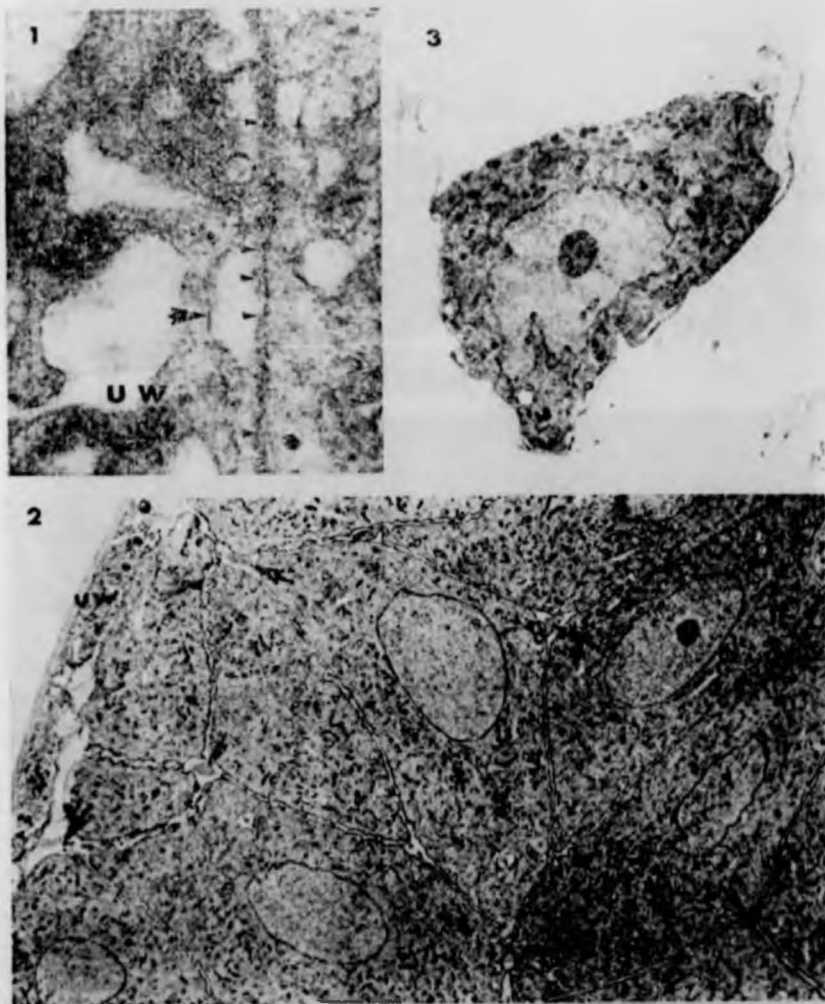


FIG. 1. "Egg shell" being secreted on the microfilarial embryo plasmalemma (small arrows). The membrane of the adjacent uterine wall (u.w.) is indicated by a large arrow ( $\times 70,000$ ).

FIG. 2. Uninucleate embryos packed tightly within the uterus, whose 'nutrient' secretions (arrowed) are seen passing between them ( $\times 2300$ ).

FIG. 3. Early 'egg shell' separation from the embryo ( $\times 6300$ ).

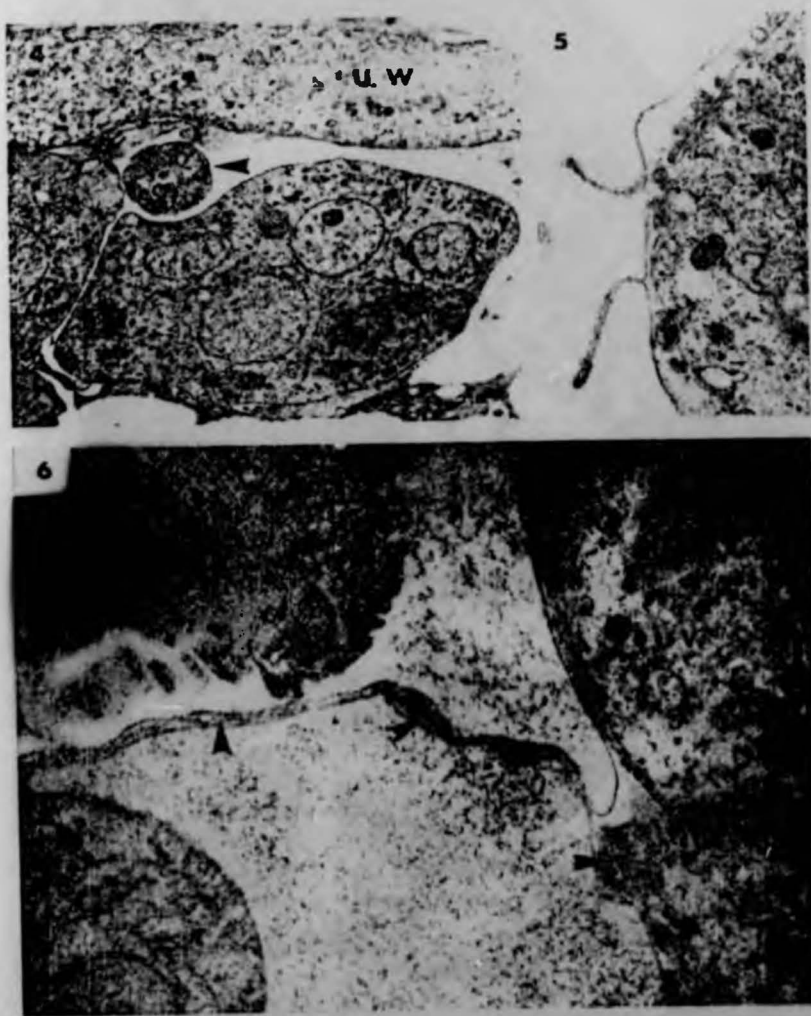


FIG. 4. Embryos at 4th and 5th division stage separated from the uterine wall by an apparently unsheathed microfilaria (arrowed). This lack of synchrony is seen again in Fig. 6. ( $\times 6300$ )

FIG. 5. Convoluted 'egg shell' seen at about the 3rd division stage ( $\times 23000$ ).

FIG. 6. Two embryos, the upper one having already developed its cuticle, the lower still at 4th or 5th division stage, sharing a 'nutrient' channel, whose contents are arrowed ( $\times 18000$ ).





FIG 4. Embryos at 4th and 5th division stage separated from the uterine wall by an apparently unsheathed microfilaria (arrowed). This lack of synchrony is seen again in Fig. 6 ( $\times 6,000$ ).

FIG 5. Convoluted 'egg shell' seen at about the 3rd division stage ( $\times 23,000$ ).

FIG 6. Two embryos, the upper one having already developed its cuticle, the lower still at 4th or 5th division stage, sharing a 'nutrient' channel, whose contents are arrowed ( $\times 18,000$ ).





FIG. 7. Section similar to 6, showing uterine secretion passing into the channel (arrowed) between two embryos ( $\times 10000$ ).

FIG. 8. Montage of a cross section of a uterus containing early embryos, nearly half of which are already dead (those starred) ( $\times 320$ ).



FIG. 7. Section similar to 6, showing uterine secretion passing into the channel (arrowed) between two embryos ( $\times 10,000$ ).

FIG. 8. Montage of a cross section of a uterus containing early embryos, nearly half of which are already dead (those starred) ( $\times 320$ ).

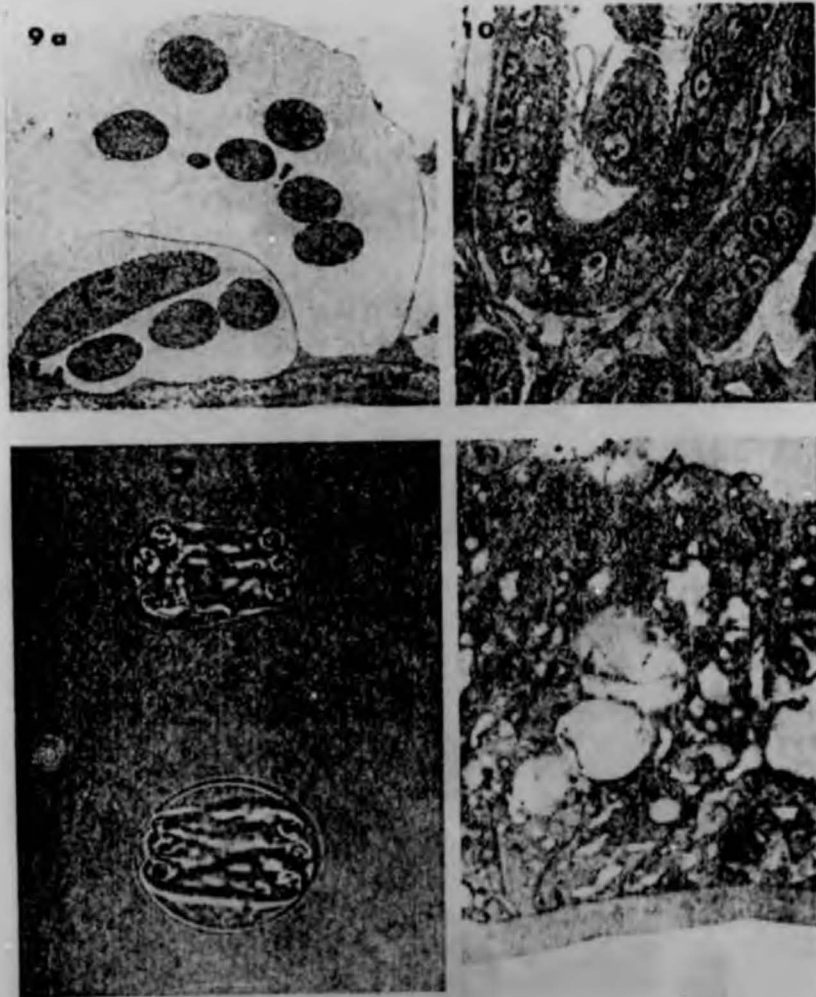


FIG. 9a. Embryos within their sheaths and coiled up at least 3 or 4 times ( $\times 1100$ ).

FIG. 9b. Phase contrast light micrographs of two embryos coiled up 3 or 4 times ( $\times 800$ ).

FIG. 10. Highly differentiated embryos, within their sheaths, whose 'nutrient' channels are still full of contents (arrowed) ( $\times 3000$ ).

FIG. 11. The villous surface (arrowed) of the uterine wall found during the final maturation stages of the embryos ( $\times 400$ ).

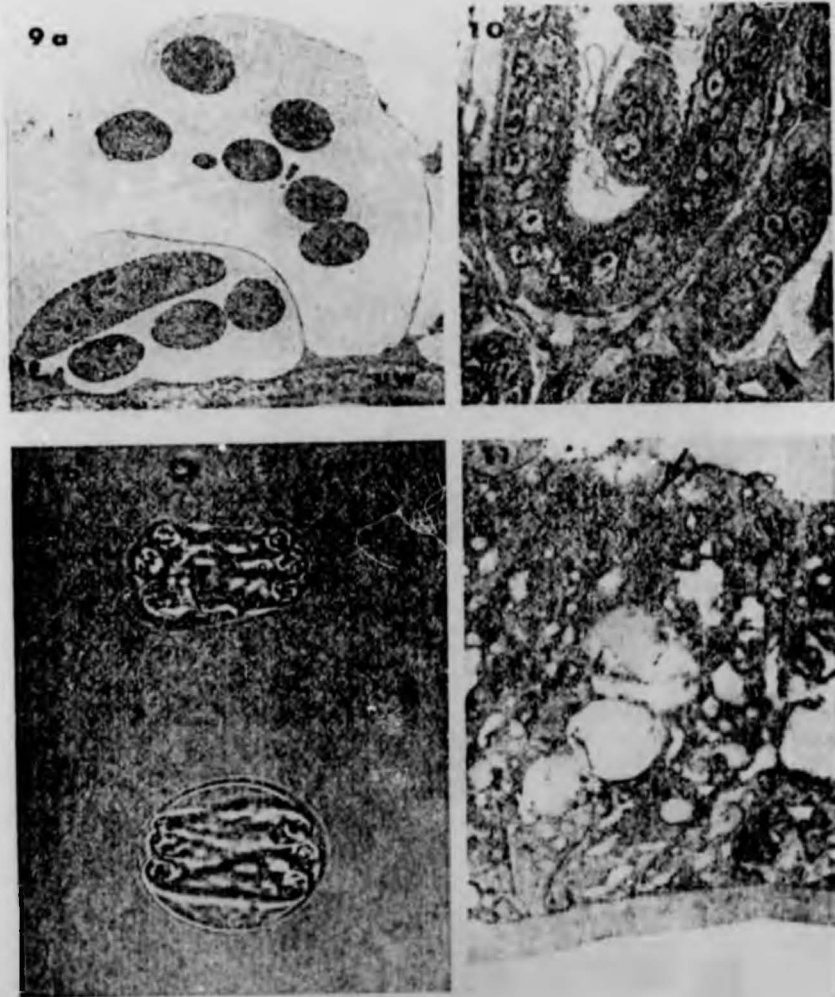


FIG. 9a. Embryos within their sheaths and coiled up at least 3 or 4 times ( $\times 1100$ ).

FIG. 9b. Phase contrast light micrographs of two embryos coiled up 3 or 4 times ( $\times 800$ ).

FIG. 10. Highly differentiated embryos, within their sheaths, whose 'nutrient' channels are still full of contents (arrowed) ( $\times 3000$ ).

FIG. 11. The villous surface (arrowed) of the uterine wall found during the final maturation stages of the embryos ( $\times 400$ ).



FIG. 12. A single unshathed mature microfilaria surrounded by many dead embryos (marked) and discarded sheaths ( $\times 3600$ ).

FIG. 13. Mature microfilariae among 2 still sheathed (small arrows) and a discarded sheath (large arrow), possibly from the adjacent worm ( $\times 3600$ ).





FIG. 12. A single unsheathed mature microfilaria surrounded by many dead embryos (marked) and discarded sheaths ( $\times 3600$ ).

FIG. 13. Mature microfilariae among 2 still sheathed (small arrows) and a discarded sheath (large arrow), possibly from the adjacent worm ( $\times 3,000$ ).

Early development of microfilariae of *D. viteae*

A comparison of the death rates of embryos in the centre of the uterine lumen at this stage of differentiation showed that up to two thirds of *D. viteae* were dying while *B. pahangi* uteri lost only about half this number. The montage (Fig. 8) shows nearly half the embryos already dead at an early stage in their development. Owing to the non-synchronous development of the embryos (see above) some of those seen in Fig. 8 appear to be very advanced at the time of their death. We noted that the majority of those embryos whose development was more advanced than their neighbours were found adjacent to the uterine wall; never in the centre of the lumen (Figs. 4, 6 and 8). We suggest that this position may be adopted to obtain the maximum nutrition at this stage, since the specialised uterine mechanisms (see below) are not yet available at these upper uterine levels. Embryos with amphids and other features of the fully differentiated microfilaria, were still within their egg shells with nutrient channels between these (Fig. 10).

The differentiated *D. viteae* embryos did not burrow into the uterine wall as did those of *B. pahangi*. Instead, at a similar level, the luminal cell surfaces of the uterine wall developed an extensive system of microvilli (Fig. 11), presumably to aid nutrition of the embryos. Other workers studying the development of microfilariae destined to be un-sheathed, have noted similar features. Kagei (1960) reported that the fertilization membrane of *Setaria cervi* developed papillary projections and that there was a dense, amorphous substance between these and microvilli-like structures on the uterine wall and that inclusion bodies in the uterine wall showed a decrease in polysaccharide content as the embryos developed, suggesting that the inclusion bodies and the electron dense substance are a nutritional source in this worm. Harada *et al.* (1970) also reported contact between the fertilization membrane around the *D. immitis* ovum and microvilli on the uterine wall by means of papillary projections on the membrane. The presence of vacuoles and inclusion bodies in the uterine wall and an amorphous substance between the cleaving ova and uterine wall again suggested a nutritional role.

The final stage in the development of *D. viteae* microfilariae was the mass casting of egg shells to produce fully differentiated, un-sheathed microfilariae (Fig. 13), which are then ready to pass out through the vulva of the female worm. The newly un-sheathed worms in the uterine lumen were surrounded by empty sheaths and dead worms (Fig. 12).

## CONCLUSIONS

At the light microscope level, the only differences noted between the development of the un-sheathed *D. viteae* microfilaria and that of sheathed *B. pahangi* (Rogers *et al.*, 1976) were the spiral coiling of the differentiating *D. viteae* and its hatching from the egg shell when fully formed.

At the ultrastructural level, the *D. viteae* embryo showed the following features:

1. The egg shell separated from the oolemma and became convoluted, as in *B. pahangi*; and we could not detect a second oolemma or trilaminar membrane beneath the egg shell as described by McLaren (1972, 1973).
2. "Nutrient channels" formed between adjacent embryos in the same way as in *B. pahangi*.
3. Embryonic death in the central uterine lumen was even more noticeable than in *B. pahangi* (about two thirds of the embryos died, compared with one third of *B. pahangi*).
4. Additional nutrient supplies to the rapidly differentiating microfilariae were obtained, not by the microfilariae burrowing into the uterine wall as in *B. pahangi*, but by the formation of microvilli on the uterine wall and the close apposition of the egg shells of the worms to these microvilli.
5. The mature microfilariae hatched out of the egg shells to produce un-sheathed microfilariae.



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