STUDIES ON THE MIGRATION AND TRANSMISSION OF *BRUGIA PAHANGI* INFECTIVE LARVAE IN *AEDES AEGYPTI*.

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by

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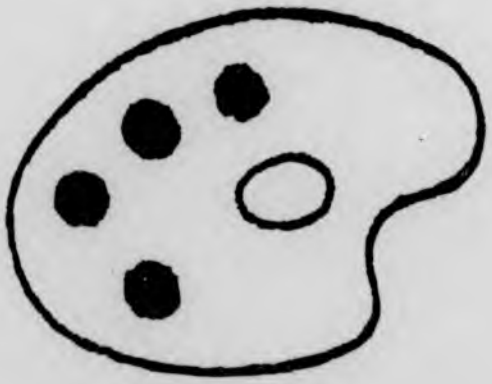

To the worm and the mosquito.

ABSTRACT

The filarial worm Brugia pahangi and the mosquito Aedes aegypti were used as a model system to study the inter-relationship between migrating infective larvae and the vector.

The distribution of parasites within the host population was shown to be over-dispersed and could be described by a negative binomial distribution. The degree of over-dispersion and mean parasite burden progressively declined during the course of infection. The decline in mean parasite burden was attributed to density-dependent parasite-induced host mortality as well as to the direct loss of infective larvae from the mosquitoes.

The course of larval migration within the mosquito was described and the evidence suggested that migration may occur solely due to "non-directional" translatory movement by the larvae. The effects of larval migration on the spontaneous flight activity and survival of infected mosquitoes were shown to be detrimental.

Transmission of larvae during blood-feeding often resulted in abnormal feeding behaviour and an increased mortality of infected mosquitoes. Humidity, temperature and the nature of the skin surface upon which the infective mosquitoes fed were shown to effect the efficiency of filarial

transmission.

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1 Central Services Unit

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CHAPTER 1

A HISTORY OF THE ROLE OF THE MOSQUITO IN FILARIAL TRANSMISSION

The relationship between disease and mosquitoes has been recognised for many centuries in various parts of the world. However, it was not until the late 1800's that the role of mosquitoes as vectors of parasitic diseases was elucidated.

Elephantiasis, a gross display of filariasis, was recognised as a distinct disease as early as 2000 B.C. by the Egyptians but may initially have been confused with leprosy (Foster, 1965; Hoeppli, 1969). From these early times elephantiasis was associated with still water, the breeding sites of mosquitoes. In India its manifestation was also regarded as a punishment by destiny for a breach of a vow of chastity and in early Indian medicine, as a derangement of the three dosas; wind, bile and phlegm (Hoeppli, 1959). The ancient indigenous population of Tahiti believed elephantiasis to be caused by eating certain foods, inheritance, exposure of wounds to salt water and the swallowing of urine from an infected patient (Hoeppli, 1959), but they too believed the principle cause was frequent contact with water.

The association of disease with mosquitoes was also

recognised by certain indigenous populations within the tropics, as recorded amongst the tribes of Tanzania, the Orinoco and Tahiti (Harrison, 1978).

Protection of the individual from the bite of the mosquito has been practised through many centuries and most probably developed as a defensive measure against the physical nuisance of the insect rather than a prophylactic device. The ancient Egyptians slept on high ground or towers to avoid contact with mosquitoes (Herodotus; Lyons and Petrucelli, 1978). Jeevas fishermen of the Punjab slept amongst mosquito infested swamps swathed in netting and it has been suggested that the habit of Europeans to have mosquito nets in India may have been copied from them (Ross, 1910).

Undoubtedly the single most important factor for the correlation of mosquitoes with disease was the acuteness of some of the diseases spread by those insects, malaria in particular, rather than a chronic disease, such as filariasis. The early scientific research into what is now recognised as vector transmission was often conjectural and with time many hypotheses floundered. Rasori, at the beginning of the eighteenth century, considered that mosquitoes might transmit malaria. The impetus for this idea came when he contracted the disease after being bothered by mosquitoes (Harrison, 1978). Later, Crawford in

1807 believed that mosquitoes were responsible for the transmission of yellow fever and "every other fever" by injecting their eggs into man. His work was greeted with ridicule (Harrison, 1978) and unfortunately his research proceeded no further. Beauperthuy in 1854 wrote that he believed that malaria was caused by mosquitoes sucking up poisons from their swampy breeding sites and injecting this "venom" into people (Harrison, 1978).

Metaxenie, the theory that parasites could be transferred between different species of animals was conceived at the end of the eighteenth century (Abilgaard, Harrison, 1978), but it was many decades later that strong supportive evidence emerged, principally through the work of Dr. Patrick Manson, whilst working on filariasis.

On the 4th January 1878, Professor Cobbold at the Royal Veterinary College, London, received an article from Manson describing the discovery of the embryo Filaria sanguinis hominis, now recognised as Wuchereria bancrofti (Cobbold, 1877) in the stomach of a mosquito, now thought to be Culex quinquefasciatus (=Culex fatigans; Edwards, 1922; Manson-Bahr and Alcock, 1959). Manson's work received acclaim when Cobbold communicated it to the Linnean Society later that year (Manson, 1878).

A year earlier, Bancroft had written to Cobbold from Brisbane, suggesting that "mosquitoes would be capable of

inbibing embryo filariae from the blood of the host" (Cobbold, 1880). However, he was unable to find any within the mosquitoes, believing them to be digested once within the insect (Manson-Bahr, 1959).

Manson continued with his studies and produced his historic paper (Manson, 1878) on "the mosquito considered as a nurse" in which he described the early development of the young larvae within the stomach of a mosquito. The uptake of the young embryos or microfilariae by mosquitoes was confirmed independently in 1878 by Lewis and da Silva Araujo in a correspondence to Cobbold and later by Sonsino (Cobbold, 1879).

By 1879 both Manson and Cobbold realised that filarial larvae had to mature within the mosquito before becoming infective (Manson-Bahr, 1959). Manson's earlier suggestion (Manson, 1878) that the larvae developed within the abdomen was shown to be incorrect when Lewis (1878) discovered the larvae within the thorax. However, Manson repeated much of his early work and went on to describe the morphological changes associated with the maturation of the larvae within the thorax; 6-7 days post infection. Cobbold then communicated this work to the Linnean Society in 1884 (Manson, 1884).

The mechanism of transmission at this time was still in

doubt, with early opinion being that the disease was water-bo rne. Manson (1878), noting the large number of empty mosquito carcasses found on water, proposed that the larvae fed directly on the internal tissues of the mosquito, precipitating the insect's death. He believed the papillae at one end of the worm were burrowing appendages (these are now believed to have a sensory function) and that the larvae used them for tunneling out of the drowning insect. Infection then occured with the larvae either burrowing into the skin of the host, or what was considered more probable, by ingestion of the larvae. Cobbold (1878) reiterated Manson's views when he wrote: "undoubtedly the larvae of Filaria bancrofti (=Wuchereria bancrofti) are swallowed with potable water" and went on to suggest that eradication of the disease could be achieved by filtering drinking water. Manson's belief in the theory of filariasis being water transmittable was influenced by books he possessed on natural history which stated that mosquitoes were ephemeral animals which died in water after laying their eggs (Sambon, 1922). He was unaware that the true reason for the early death of his mosquitoes was starvation and thought their demise was a natural occurrence. Manson was not alone with his views and his theory found major allies with Laveran and Blanchard (Chernin, 1983).

Although an unknown author, possibly Cobbold, writing in "the Veterinarian" in 1883, (Manson-Bahr, 1959), suggested that larval filariae were deposited in the act of biting,

this idea was not followed up.

A few years later controversy raged between Manson and Bignami about the transmission of malaria. Manson believed this disease was transmitted in a similar manner to his proposal for filariasis. Bignami observed that malaria could be transferred from an infected patient to a healthy person by blood transfusion. He reasoned that the mosquito proboscis acted like a needle, injecting the parasite into man. According to Bignami this work had been greatly influenced by the work of Smith and Kilbourne (1893) who showed that Texas Fever, a protozoal disease of cattle, was transmitted by the bite of a tick (Harrison, 1978).

Ronald Ross was in regular communication with Manson at this time and his immediate reaction was to defend his mentor from Bignami's "intrusion" (Harrison, 1978). However, Ross, whilst working in Calcutta discovered the mosquito vector of malaria (Anopheles spp.) and found Plasmodium protozoa concentrated in the salivary duct which led him to suggest that the disease is inoculated by the bite of the insect. He later confirmed this hypothesis by infecting healthy sparrows with an avian form of malaria transmitted by mosquitoes. Perhaps appropriately, it was left to Manson to deliver Ross's work to a meeting of the British Medical Association held in Edinburgh in 1898.

In Australia, Bancroft, encouraged by Manson, began his research on filariasis transmission. He found that he was able to keep his mosquitoes alive by feeding them on bananas and showed that full development of the filarial larvae in the thorax took between two and three weeks. Furthermore, he showed that W. bancrofti infected larvae could not survive for more than a few hours in water or saline (Bancroft, 1899); a fact later corroborated by Annett, Dutton and Elliot (1901), Lebredo (1905) and others. Bancroft initially suggested that transmission was by swallowing filariae within the insect. He considered that infection by this route was "possible, although somewhat improbable". In the addendum to this paper he suggested that infective larvae might gain access to the body through the bite of an infected mosquito. The biting theory of transmission was later supported simultaneously by James (1900) who noted the presence of a mature larva within the proboscis and Low (1900a and b) who demonstrated larvae in tissue sections of the mosquitoes head and proboscis. A few years later Bancroft tested his hypothesis and found that Dirofilaria immitis could be transmitted to healthy dogs from the bite of infected mosquitoes (Bancroft, 1904) and concluded by analogy, that mosquitoes directly transmit filariasis to man.

Today the biting theory of transmission is regarded as being the most common method of infection. However,

because the incidence of filariasis does not correspond closely with the distribution of a suitable vector, the role of mosquitoes has been questioned by some workers. Brug (1919) in Java even considered that direct mosquito transmission from man to man was "extremely doubtful". Yokowaga (1939) also questioned the role of the mosquito as a vector when he found, in an area with a high incidence of filariasis, low levels of infection amongst the mosquitoes. However, these facts do not negate the mosquito theory of transmission, they merely serve to illustrate the complex epidemiology of filariasis.

Kawakami (1922) and Katsudura and Mujagawa (1937) believed that another intermediate host besides mosquitoes might be responsible, possibly fleas. Infection was thought to occur when the flea was crushed onto the skin. However, it has since been shown that filarial larvae cannot pass through intact skin (Yokogawa, 1939; Gordon and Crewe, 1953; Ewert, 1967).

Similarly, bed bugs, were once considered to be possible vectors. However, they too fail to transmit filariasis (Wharton and Bin Omar, 1962; Nelson, 1963).

Yokogawa (1939) provided evidence to show that water-bo rne transmission was an insignificant source of infection. Confirming Bancroft's earlier observation that

larvae could only remain viable in water for a few hours he also showed that zooplankton, some of which are susceptible to certain species of nematode larvae proved refractory to filarial infection. Thus, the belief that the zooplankton might act as paratenic hosts was shown to be unlikely. He also indicated that oral infection of humans was unlikely as larvae died, within a few seconds, when placed into normal human gastric juice. His unsuccessful attempt to infect a monkey and a dog *per os* with *W. bancrofti* is not supportative of his previous claim because dogs are innately refractory to *W. bancrofti* infections and only a few species of monkey, such as *Macaca cyclopis*, are susceptible (Cross, Partono, Hsu, Ash and Oemyati, 1979).

Despite the seemingly strong evidence against the water-bourne theory of transmission, recent work has indicated that it might be a means of infection, albeit of minor importance. Although Lebredo (1905) had been unable to show the emergence of any *W. bancrofti* larvae from the corpses of infected mosquitoes, Yamada and Komori (1926) had shown that *W. bancrofti* larvae could emerge from an intact proboscis after only a few hours, and recent work (Gwadz and Chernin, 1973b; Bosworth, Sullivan and Chernin, 1976), has shown how readily *Brugia pahangi* infective larvae emerge from dead and dying mosquitoes, remaining viable in water for up to 72 hours. Furthermore oral transmission has been demonstrated with *B. pahangi* in the jird, *Meriones unguiculatus*, (Gwadz and Chernin, 1972 and 1973a; Sullivan

and Chernin, 1976; Bosworth, Sullivan and Chernin, 1976). Brugia malayi in the jird (Bosworth and Chernin, 1976a) and with B. pahangi in dogs (Bosworth and Chernin, 1976b). However, penetration of the stomach does not occur and the opportunity for infection, via the buccal cavity mucosa, is brief.

Today it is generally recognised that human filariasis is principally transmitted by the bite of an infected mosquito. The reason that this issue has produced such controversy in the past is a reflection of the complex epidemiology of the disease.

CHAPTER 2

A REVIEW OF THE LIFE-CYCLE OF FILARIAL NEMATODES TRANSMITTED BY MOSQUITOES (CULICIDAE).

The experimental studies described in this thesis are concerned with the migration and transmission of the infective larvae of B. pahangi within the mosquito Aedes aegypti. Although this work is treated largely from a "worm's-eye" view it is also an attempt to obtain a better understanding of this parasite-host relationship. In this chapter I will describe the typical life-cycle of lymphatic-dwelling filariae which are transmitted by mosquitoes (Figure 2.1), although other filarial worms will be considered where relevant. Because these studies are concerned principally with the infective larvae I will discuss in more detail those factors which govern the establishment of infective larvae within a population of infected mosquitoes. The detrimental consequences of parasitism on the arthropod host (vector) are not included in this review but are discussed in later chapters.

The filarial nematodes W. bancrofti (Cobbold, 1877), B. malayi (Brug, 1927) and Brugia timori (David and Edeson, 1964, 1965), are transmitted by the bites of mosquitoes. Together, they are responsible for much morbidity in man. The World Health Organisation (1984) estimated that 90.2 million people are infected with mosquito-transmitted

filariae throughout the world, with 905 million people living in areas of risk. Moreover, it is probable that these numbers are increased annually due to the rapid increase in urban development occurring in many areas of the tropics and the consequent increase in the C. quinquefasciatus population (Curtis and Feachem, 1981).

The distribution of the important human dwelling filariae is limited to the wet tropics. Although W. bancrofti is widely distributed throughout this range B. malayi and B. timori are confined to Asia (reviewed by Hawking and Denham, 1976; Hawking, 1976, 1977 and 1979).

Bancroftian and brugian filariasis are characterised by a wide spectrum of clinical manifestations, the signs and symptoms of which often differ from one endemic area to another. Although the most severe forms of the disease are now rarely seen (Nelson, 1978), recurrent fever, lymphangitis, lymphadenitis, lymphoedema, hydrocoeles, ulceration, lymphuria and chyluria are common. Typically filariasis is not found in very young children, but with continual exposure, over a number of years, an infection generally becomes established. The incidence of the disease increases with age, the chronic symptoms becoming more prevalent in later life, with a concomitant decrease in microfilarial densities in many of the most severely affected individuals. It is generally considered that the

more severe disease is a reflection of continuous and intense transmission.

THE ADULT WORM:-

Adult filaria may live within the lymphatics for many years, usually in the lower limbs and groin. W. bancrofti may also be found in the upper limbs and chest.

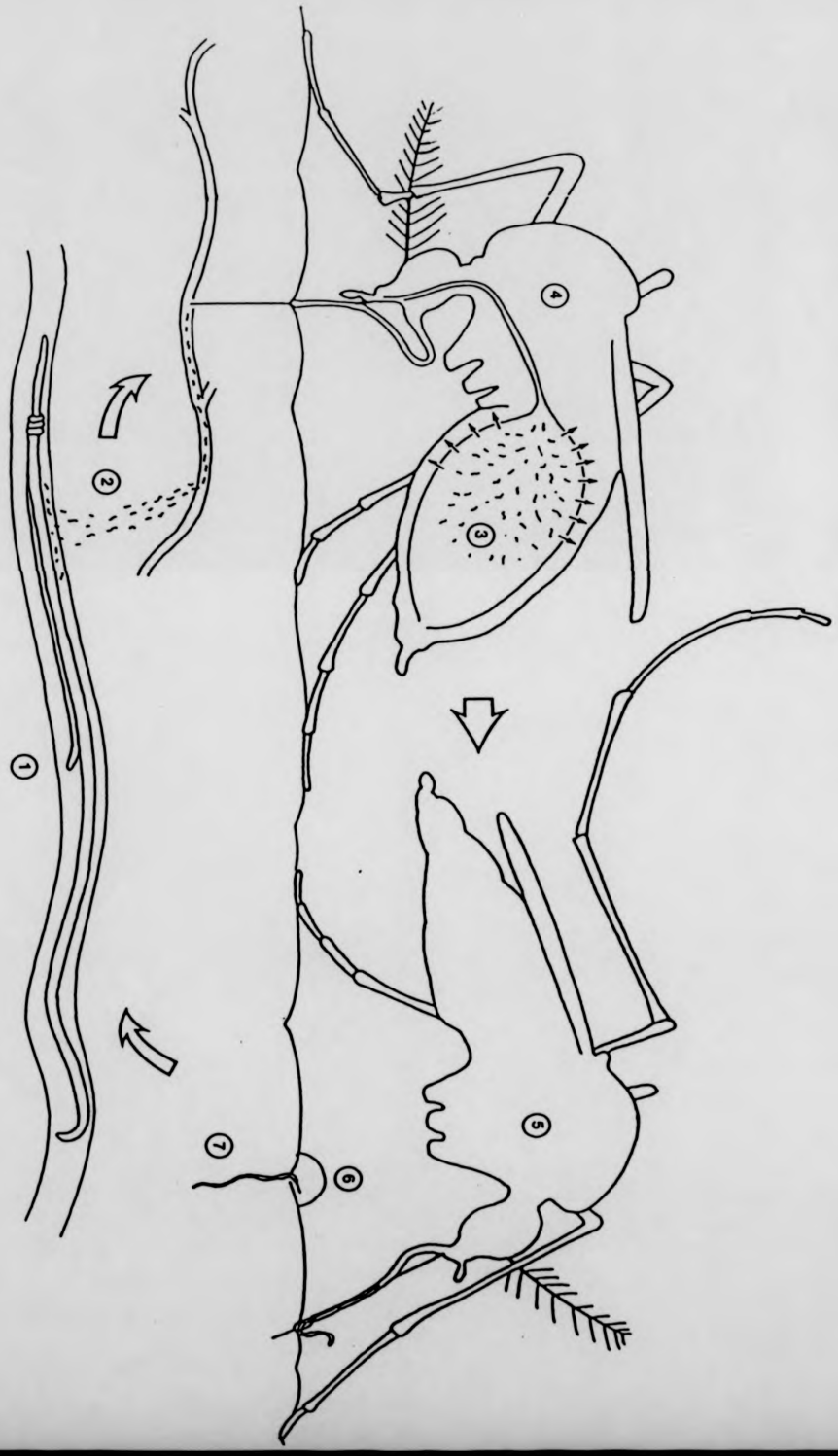
W. bancrofti adults are larger than those of Brugia species. Females measure between 80-100 mm in length compared with 60 mm, and males measure 40 mm in length compared with 25 mm (Buckley, 1960; Faust, Russel and Jung, 1970). A comprehensive desription of the adult morpholgy of B. malayi and B. pahangi is given by Buckley and Edeson (1956) and Schacher (1962). This work has recently been supplemented by scanning electron micrographs (SEMs) showing the surface morphology of adult B. malayi (Franz and Lenze, 1982; Lim, Loh and Singh, 1983).

THE MICROFILARIAE:-

After the adults have mated, the female produces hundreds of ovoviviparous young larvae (microfilariae, mff). The morphology of microfilariae has been extensively studied in the past by a number of workers including Fulleborn (1913), Brug (1928), Taylor (1960), Laurence and Simpson (1968a,

Figure 2.1 A schematic representation of the life-cycle of lymphatic-dwelling filarial worms transmitted by mosquitoes (not drawn to scale).

1. Mature adult filarial worms copulate in the lymphatics of the vertebrate host and produce microfilariae.
2. The microfilariae migrate into the peripheral blood circulation.
3. The microfilarae are imbibed with the blood-meal of a feeding mosquito. They then migrate from the mid-gut to the flight-muscles in the thorax.
4. Development to infective larvae takes one to two weeks.
5. Infective larvae migrate out of the thorax and eventually collect in the head and proboscis.
6. Infective larvae escape from the mosquito during blood feeding and remain on the skin surface covered in mosquito haemolymph.
7. These larvae penetrate the definitive host via the puncture wound after the mosquito has withdrawn it s mouthparts. The infective larvae then migrate to the lymphatics and mature into adults.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1968a, 1968b, 1969 and 1971) amongst others.

The microfilariae normally migrate from the lymphatics into the blood. The microfilariae of B. pahangi in cats may even cross the placenta and pass into the kittens' circulation (Kimmig, 1979). In man, they can also be found in hydrocele fluid or chylurous urine.

The density of microfilariae in the peripheral blood circulation amongst individuals in a vertebrate host population is extremely variable. Typically the distribution of microfilariae within this population describes a negative binomial (e.g. Grove, 1983). That is, the majority of the vertebrate host population have no or few microfilariae and the majority of the parasite population is carried by only a few individuals. In our laboratory, counts of more than 4000 microfilariae per 20 mm^3 of blood have been found in perfectly healthy cats infected with B. pahangi.

Many species of filariae exhibit a periodicity. Peak numbers of microfilariae appear in the peripheral circulation at those times of the day when the vector is most likely to feed. Moreover, within certain species some physiological races also exhibit different circadian rhythms. Extensive research on the mechanism of the periodicity has been carried out by Hawking (1965, 1967;

Hawking, Pattanayak and Sharma, 1966; Hawking, Jennings, Louis and Twira, 1981).

The distibution of microfilariae within the peripheral blood circulation, as assessed by finger-prick samples, is generally considered to be homogenous (Pichon, Riviere, Thirel, Chebret, Tetuanui and Toudic, 1981; Petit and Pichon, 1982). However, higher concentrations of Mansonella ozzardi microfilariae have been found in blood from the ear lobe compared with the finger (Nathan and Raccurt, 1979) as well as the scapular region compared with the buttocks (Moraes, Shelley, Dias, Mangabeira and Silva, 1983).

Although the microfilaraemia of infected individuals does not vary significantly from day to day (Hairston and Jachowski, 1968; Wilson and Ramachandran, 1971) there have been accounts of large seasonal variations. These peak periods of microfilarial density coincide with the peak biting densities of the vector (i.e. W. bancrofti; Ravindranathan, Sethumadhavan, Roychowdhury, Babu, Karioujia, Narasimham and Rao, 1980; Onchocerca volvulus; Hagiguchi, Kawabata, Tanaka, Okazawa, Flores and Recinos, 1981).

Geographical differences in the infectivity of microfilariae to their mosquito vectors occur. In general, urban strains of W. bancrofti are more infective to C. quinquefasciatus, whereas rural strains are more

infective to anophelines (Grove, 1983). Nelson, Heisch and Furlong (1962) have shown striking differences in infectivity between the non-periodic B. patei, a parasite of cats, from Pate Island and the nocturnally periodic mainland B. patei to Aedes pembaensis, the former being more infective.

THE VECTOR:-

Microfilariae cannot undergo further development unless imbibed by a susceptible mosquito. The advantage of this alternation is that it combines a large host with a longer life, constituting a lasting reservoir, with a small mobile vector, which facilitates transmission.

The main vectors of human filariasis are recorded from four main genera of mosquitoes (Culicidae), Anopheles, Aedes, Culex and Mansonia (reviewed by Ahmed, 1966; Sasa, 1976; White, 1979). Vectors of B. pahangi have been reviewed by Edeson, Wharton and Laing (1960). Only female mosquitoes transmit filariasis because it is only this sex which feeds on blood. However, B. pahangi microfilariae injected into males of both a susceptible and a refractory strain of Ae. aegypti underwent full development (Terwedow and Rodriguez, 1973; Townson, 1974).

The efficiency of mosquitoes as vectors, according to Sasa (1976), is not of an all-or-none type, as there exist

various grades of compatability between the vector and the parasite. The development of W. bancrofti and Brugia spp. in refractory and susceptible strains of Ae. aegypti has been shown to be controlled by sex-linked alleles, the f^m gene for susceptibility being double recessive (reviewed by Macdonald, 1976; Curtis and Graves, 1983). The susceptible strains of this insect are of particular importance as it is commonly used in laboratories for parasite production as well as studies on parasite-vector relationship.

Ae. aegypti is not an important vector of human filariasis as most strains are completely refractory. However, under laboratory conditions 53% of one wild strain from Uganda was susceptible to B. pahangi infections (Rodriguez and Craig, 1973). Ten other strains from Zimbabwe and Tanzania varied in their susceptibility from 1-37% and two strains from the Americas had very low susceptibilities of 2 and 6%. Later, Paige and Craig (1975) found that in different East African strains susceptibility varied from 0-59%, with sylvatic strains being more susceptible and domestic strains more resistant. However, it is unlikely that these susceptible strains would ever transmit filariasis in the field. This is because the microfilariae of W. bancrofti in Africa and South America exhibit nocturnal periodicity and Ae. aegypti is a diurnal feeder.

In the laboratory, the BLACK EYE strain of Ae. aegypti varies in it's susceptibilty to infection with a number of different species of filariae. It is more susceptible to B. pahangi infections than B. malayi (Beckett and MacDonald, 1971) and both species "take" better than W. bancrofti infections. The microfilariae of Brugia spp. are even more successful in developing within Mansonia spp. (Wharton, 1957; Laurence and Pester,1961). This latter, more equitable, relationship occurs naturally and may be a reflection of their longer shared evolutionary history.

For a feeding mosquito the only non-infective level of microfilariae in blood is zero. Fewer microfilariae in a patient simply results in a smaller percentage of mosquitoes being infected. Patients with ultra-low microfilaraemias (ULM) are considered to play a significant role in transmission (Jordan, 1959; Bryan and Southgate, 1976; Carme and Laigret, 1979) although ULM was thought not to play an important role in transmission in the Phillipines (Ishi, Cabrera, Suguri, Kobayashi, Go and Valeza, 1983). ULM may be of most importance when one is attempting to eradicate the disease, especially as adult females of W. bancrofti can produce microfilariae for 8 years (Webber, 1977) and, exceptionally, for up to 40 years (Carme et al., 1977). Although in areas where Anopheles gambiae is the vector of W. bancrofti mosquito infections seldom develop below 10 mff/ml (W.H.O., 1984).

Generally, an increase in the density of microfilariae within the peripheral blood results in an increase in the proportion of mosquitoes which become infected e.g. B. malayi in Mansonia longipalpis, (Wharton, 1957); an urban strain of W. bancrofti in C. quinquefasciatus, (Wharton, 1960); and W. bancrofti in An. gambiae, (Gelfand, 1955). In contrast, Wharton (1960) failed to demonstrate a similar relationship with a rural strain of W. bancrofti in C. quinquefasciatus. Other workers have shown that increasing the density of microfilariae in the blood meal will only raise the percentage of mosquitoes infected to a certain plateau level e.g. W. bancrofti in Ae. polynesiensis (Rosen, 1955) and B. pahangi in C. quinquefasciatus (Obiamiwe, 1977). The presence of mosquitoes refractory to filariae in these populations could explain why this plateau is reached. Indeed higher concentrations of microfilariae might well decrease the percentage of mosquitoes infected even in fully susceptible strains because imbibing large numbers of microfilariae will increase vector mortality.

The number of microfilariae a mosquito takes up during a blood-meal not only depends on the density of microfilariae circulating in the host's skin capillaries, but also the quantity of blood imbibed. The volume of blood ingested is roughly proportional to the weight of the mosquito (Christophers, 1960; Jordan and Goatly, 1962), with most

mosquitoes feeding until fully engorged. Younger mosquitoes have been shown to imbibe greater quantities of blood and this is reflected in their higher level of microfilarial intake (Maeda and Kurihara, 1980).

The uptake of microfilariae in relation to the size of the blood-meal has been a source of investigation for over a century. However, the conclusions of many studies have often been opposed. Manson (1883) and O'Connor and Beatty (1937) found that mosquitoes took up a greater number of microfilariae than one would expect from the number of microfilariae in a similar quantity of blood taken from a finger-prick sample. This has since been supported by many workers including Nicholas and Kershaw (1954), Wharton (1957), Ramachandran (1966), Bryan and Southgate (1976), Obiamiwe (1977b) and Trpis (1981). Other workers including Rosen (1955), Webber (1955), Wharton (1960), and Petit and Pichon (1982) found that microfilarial uptake was more or less as expected from the microfilarial counts in peripheral blood and the volume of blood ingested.

Turning full-circle, many authors have found that some species of mosquito take up fewer microfilariae than one would expect, e.g. Kershaw, Lavoipierre and Chalmers (1953), Duke (1956), Ramachandran and Zaini (1967) and Obiamiwe (1977b). Roychowdhury, Kanan and Das (1969) found this to be true only when mosquitoes were feeding on high concentrations of microfilariae. When mosquitoes fed

on low concentrations of microfilariae they found that a greater number had been ingested than one would expect.

Others reported that when microfilaraemia is high, vectors took in more than expected and when low, less than expected (Kershaw, Lavoipierre and Chalmers, 1955; Gubler, Innui, Black and Bhattachaya, 1973).

A classic paper on the feeding behaviour of mosquitoes by Gordon and Lumsden (1939) provides a series of possible explanations as to why the uptake of microfilariae by mosquitoes is so variable. They observed that mosquitoes could take up blood directly from capillaries in the skin, although occasionally, on perforating a capillary, the mosquito would feed on blood taken from a pool extravasated into the tissues. As erythrocytes escape more readily from ruptured capillaries than do microfilariae it follows that pool-feeding mosquitoes will take up fewer microfilariae than would be expected.

Microfilariae have also been seen clumped together in the capillaries (Augustine, Field and Drinker, 1936; Gordon and Lumsden, 1939). Consequently large numbers of microfilariae can collect in one capillary whilst in an adjacent one few, if any, may be found. These observations supported O'Connor and Beatty's hypothesis (1937) that greater numbers of microfilariae would be taken

up when a mosquito fed shallowly on a narrow, convoluted capillary, when clumping is more likely to occur, than if it were feeding more deeply on a wide and straight venule. This has also been suggested by Burton (1964), Nakamura (1964), Nathan and Raccurt (1979) and Pichon, Prod'hon and Riviere (1980). Indeed, this would explain why the density of microfilariae varies between different sites (Nathan and Raccurt, 1979; Moraes et al., 1983). The greater the density of narrow, convoluted capillaries, the greater the density of microfilariae.

The apparent concentration of microfilariae by certain species of mosquitoes was originally thought to occur because microfilariae were believed to become entangled in the mosquito's fascicle during feeding (Manson, 1883). However, Gordon and Lumsden (1939) failed to observe this phenomenon and thought it unlikely as microfilariae were freely and rapidly taken up from the capillaries.

Microfilariae may be concentrated in the blood-meal if they exhibit a positive chemotaxic response to mosquito saliva as has been suggested by Harley (1932) and Obiamiwe (1977b). However, Hiniman (1935) was unable to show this and Gordon and Lumsden (1939) considered chemotaxis impossible because of the increased blood-flow which occurs when a mosquito feeds.

The excretion of fluids from mosquitoes which occurs during

feeding (Reid, 1953, 1982; Boorman, 1960; Wharton, 1962), will also lead to an apparent concentration of microfilariae. Blood-meal volume will then be greatly underestimated if it is determined by weighing mosquitoes before and after feeding. This is because of the large quantities of urine (Mansonia annulifera, Ae. aegypti) or blood (Anopheles barbirostris) which is excreted during blood-feeding.

From these studies it is evident that the uptake of microfilariae by individual mosquitoes is extremely variable. Schmid and Robinson (1972) showed that the distribution of microfilariae within a population of midges, after an infective blood-meal followed a negative binomial distribution. This clumped distribution, which is typical of a great many parasite-host associations, may originate in a number of ways. Firstly, due to the periodicity of microfilariae in the peripheral blood stream. Secondly, due to the clumping of microfilariae in the blood capillaries and, lastly, due to the variation in microfilarial density at different sites in the same host. As a final comment on this subject, Omori (1966) demonstrated that the frequency distribution of W. bancrofti microfilariae ingested by C. quinquefasciatus was again aggregated except when the density of microfilariae in the peripheral blood was low (1-2 mff/20 mm^3 blood), the distribution then being random.

THE FATE OF INGESTED MICROFILARIAE:-

Even in susceptible vectors the survival of the parasite is not guaranteed. In many Anopheles spp. microfilariae can be damaged by the cibarial and pharyngeal armature lining the fore-gut (Coluzzi and Trabuchi, 1968; McGreevy, Bryan, Oothuman and Kolstrup, 1978; Buse and Kuhlow, 1979), with Brugia spp. being more vulnerable than W. bancrofti (W.H.O.,1984). However, when large numbers of microfilariae are imbibed many may be shielded from the armature by the presence of others.

Once within the insect's gut the microfilariae must avoid being digested, trapped within the coagulated blood-meal (Kartman, 1953; Ramachandran, Jimenez and Edeson, 1963, Ramachandran, 1966; Nayar and Sauerman, 1975; Obiamiwe, 1977b; Christensen, 1981) or being excreted in a bloody diarrhoea (Jordan and Goatly, 1962; Wharton, 1962). Nayar and Sauerman (1975) have suggested that symbiotic bacteria in the mid-gut of the mosquitoes can indirectly lead to the killing of microfilariae by the formation of oxyhaemaglobin crystals after the erythrocytes have lysed. However, not all physiological processes active within the gut of a mosquito are detrimental to the survival of filariae. The presence of anti-coagulant in the salivary secretions of some species of mosquito can facilitate migration out of the gut (Kartman, 1955; Nayar and Sauerman, 1975).

Agglutination of erythrocytes occurs within a minute of a blood meal. The blood-mass contracts and becomes surrounded by a peritrophic membrane within 30 minutes. The microfilariae usually exsheath immediately in the blood mass and, assisted by the action of the cephalic hook, pass readily through the surrounding peritrophic membrane (Esslinger, 1962).

The mid-gut wall, in the abdomen of a mosquito, constitutes further mechanical and physiological barriers to larval egress. Microfilariae pass through swollen digestive cells in the stomach wall (Bain, 1971; Bain and Brengues, 1972) and into the haemocoel. The migration out of the gut can take 2-3 hours (Feng, 1930; Iyengar, 1936; O'Connor and Beatty, 1938; Lewis, 1953) though sometimes it is as little as a few minutes (Wharton, 1957a; Ramachandran, 1966; Laurence and Pester, 1961; Esslinger, 1962; Hockmeyer, Schiefer, Redington and Eldridge, 1975). Owen (1978) found that the length of time taken for microfilariae to leave the gut was related to the susceptibility of the vector. In refractory strains of the Aedes scutellaris species complex, migration of B. pahangi microfilariae ceased after 1.5 hours and only 50% of the microfilariae reached the thorax. In susceptible strains over 95% of microfilariae migrated to the flight muscles over several hours. It has also been shown that microfilariae of B. malayi collected from the

peritoneal cavity of a jird are less able to penetrate the mosquito mid-gut (30% microfilariae penetrate) than those from jird blood (70% microfilariae penetrate; Schrater, Rossignol, Hamill, Piessens and Spielman, 1982). However, many microfilariae found in the peritoneum are deformed. It is likely that adult females in the lymphatics also produce such deformed microfilariae but in this case they either fail to migrate into the bloodstream or if they do are filtered out by the liver.

The proportion of microfilariae which are successful in reaching the haemocoel relative to the number of microfilariae in the mid-gut follows one of two relationships (Bain, 1971; Pichon, Perrault and Laigret, 1974). Firstly, "facilitation" may occur. In these instances the proportion of microfilariae passing into the haemocoel increases as the number of microfilariae ingested increases, e.g. W. bancrofti in An. gambiae A. Excrescences formed on the gut epithelium by the penetrating microfilariae facilitate the passage of other microfilariae (Bain and Brengues, 1972). Secondly, "limitation" may occur. In this case the proportion of microfilariae crossing the mid-gut wall is proportional to the number of microfilariae imbibed when the number of microfilariae ingested is small, but when a large number of microfilariae is ingested, a limiting figure is reached, above which the proportion reaching the haemocoel is

reduced, e.g. W. bancrofti in Ae. aegypti, O. volvulus in Simulium damnosum, Dipetalonema dessetae in Ae. aegypti. This limiting value which occurs in heavily infected Ae. aegypti is believed to be due to an intense lysis of the mid-gut epithelium and the secretion of a substance(s) which hampers the egress of microfilariae (Bain and Chaubaud, 1975; Bain, Philippon, Sechan and Cassone, 1976).

Migration of microfilariae proceeds through the fat body and haemocoel forward into the thoracic muscles (W. bancrofti; Manson, 1878; Bahr, 1912; O'Connor and Beaty, 1938; Koboyaski, 1940; B. malayi; Brug and de Rook, 1930; Feng, 1936; Wharton, 1957a and b; B. pahangi; Edeson and Wharton, 1957). In some instances this can take up to three days to complete e.g. B. patei in M. uniformis (Laurence and Pester, 1961).

The developing larvae of W. bancrofti have been reported in the head muscles of C. quinquefasciatus (Gigliolo, 1948) but this observation has never been repeated and must, therefore, be recognized as an unusual occurrence.

LARVAL DEVELOPMENT WITHIN THE THORAX:-

The microfilariae on reaching the thorax, pass into the spaces between the muscle fibres before penetrating the sarcolemma, usually where the fibres insert on the cuticle. Here the worms become quiescent, developing intracellularly

along the axis of the muscle fibres of the indirect flight muscles. The larvae lie in "tunnels" between myofibrils and cause little damage to the mosquitoes muscle (Esslinger, 1962; Beckett and Boothroyd, 1970; Beckett, 1971). The development of Brugia spp. in Ae. aegypti and M. uniformis occurs principally within the bottom and middle bundles of the dorsal longitudinal muscles (Beckett and Macdonald, 1970a, b; Beckett, 1971a) although Esslinger (1962) found no such predilection in An. quadrimaculatus.

The metamorphosis of filarial larvae within mosquitoes has been studied in detail. e.g. Iyengar (1956) for W. bancrofti, by Feng (1936) for B. malayi and by Schacher (1962) for B. pahangi. Additional, recent information on the external morphology of the infective larvae of Brugia spp. (Aoki, 1980; Aoki, Vincent, Ash, and Katamine, 1980) and W. bancrofti (Franz and Zielke, 1980) has been obtained using SEM.

THE DEVELOPMENT OF MICROFILARIAE:-

Essentially the microfilariae metamorphose into short, thick larvae, growth occurring on the third day after the blood meal when the mosquitoes eggs have developed. There then follows a period of differentiation, followed by a moult. The second-stage larva, so formed, is of a similar shape to the previous stage, but is larger and has a well differentiated alimentary canal. There then follows a

second moult with the emergence of the highly active male or female third-stage larva. This infective larva is ideally suited for conditions both within the mosquito vector and the mammalian definitive host.

The highest parasite mortality occurs in the first few days after the infective blood meal (Pratt and Newton, 1946; Beckett and MacDonald, 1971). Indeed, one week post infection 75-80% of B. pahangi larvae and only 22% of B. malayi larvae develop normally in a susceptible strain of Ae. aegypti (Beckett and MacDonald, 1971). The cause of death is uncertain and is not density-dependent, within the range of 40-206 mff/20 mm^3 blood.

Intuitively, one would expect that there is a large variability in the fitness of individuals within a population of microfilariae. This is, to an extent, age dependent. De Hollanda, Denham and Suswillo (1982) found that microfilariae have to be greater than two days old before they are capable of undergoing development within the mosquito. It has been suggested that microfilariae which fail to develop do so because they are unable to exsheath after penetrating the gut (Laurence and Pester, 1961). But it is also likely that a proportion of microfilariae are simply inately incapable of development within the mosquito.

Recent in vitro work suggests that a rise in osmotic

pressure which occurs when microfilariae pass from the mid-gut to the flight-muscles of the mosquito may be important for the development of microfilariae to second-stage larvae (Ando and Kitamura, 1982).

In resistant strains of mosquitoes worms may become encapsulated by haematocytes and often undergo melanisation (Noe, 1901; Brunhes and Brunhes, 1972; Oothuman, Simpson and Laurence, 1974; Lehane and Laurence, 1977; Chen, 1982) but this rarely occurs in most of the normal vector species (W.H.O., 1984). The abnormal development of B. pahangi in refractory strains of Ae. aegypti is not caused by melanisation in the flight muscles (Lehane and Laurence, 1977) but is due to the inability of the first stage larvae to carry out hypodermal and cuticular reorganisation (Lehane, 1978).

A mathematical model for the relationship between the host microfilaraemia and the number of developed infective larvae mosquitoes has been described by Sasa (1976). However, because of the large variability in microfilarial uptake by individual mosquitoes, it has not been possible, except by statistical estimation, to evaluate the number of infective larvae produced from mosquitoes feeding on a patient with a known concentration of microfilariae. Recently, Klowden (1981) attempted to overcome this problem by infecting Ae. aegypti with known numbers of B. pahangi

microfilariae in an enema. He produced a log linear relationship between the number of microfilariae administered and the number of infective larvae recovered. Of course the number of infective larvae capable of being supported by a mosquito is not infinite and an increase in the number of microfilariae imbibed will not necessarily result in an increase in the number of infective larvae.

In high density infections filarial larvae develop at an unequal rate (Rosen, 1955; Webber, 1955; Wharton, 1957). In the wild even mosquitoes with low worm burdens have been found with larvae at different stages of development (Feng, 1931; Hu and Chang, 1933; Byrd, St. Amant and Bromberg, 1945), although this has been interpreted as showing that microfilariae, originating from separate feeds, were developing simultaneously.

The ability of filarial worms to develop within mosquitoes is independent of prior exposure (Bahr, 1912; Duxbury, Moon and Sadun, 1961; Bosworth and Ewert, 1967; de Meillon, Hayashi and Sebastian, 1967). This implies that there is no immunological memory in mosquitoes which operates against filariae. Beir's recent attempt to reduce the "vector competence" of Aedes triseriatus by infecting mosquitoes concurrently with D. immitis and different species of gregarine protozoans also me t with failure (Beir, 1983).

Sterilization of mosquitoes with chemicals or radiation in

an attempt to increase susceptibility to infection has largely been unsuccessful (Ahmed, 1969; Sharma, Das, Bendle and Razdan, 1981). However, A 12K Rad radiation dose did significantly increase the infection rate of Ae. togoi with B. malayi (Ahmed, 1969).

Duhrkopf and Trpis (1981) treated pupae of the Ae. scutellaris complex with tetracycline and found that the adult mosquitoes had reduced levels of infection. They found that tetracycline removed symbiotic rickettsiae from the insects and suggested that these symbionts might provide some necessary component for filarial larval development. However, Curtis, Ellis, Doyle, Hill, Ramji, Irungu and Townson (1983) were unable to demonstrate similar findings with C. quinquefasciatus.

Gaaboub and Busvine (1975, 1976) showed that sub-lethal doses of DDT and PH 60:40, an insect development inhibitor, "broke down" the resistance to infection of B. pahangi in a refractory strain of Ae. aegypti. This is undoubtedly of some importance in filariasis endemic areas where one may encounter DDT and PH 60:40 resistant vectors. Clearly the effect of other pesticides on the vectorial capacity of different insecticide resistant and susceptible strains would be of great interest.

Recent work has demonstrated that meal Ae. togoi reared and

stressed as larvae in high salt concentrations developed fewer B. malayi infective larvae compared with those raised as larvae in a low salt concentration (Sucharit, Vutikes, Leemingwasdi, Kerdpidul and Chomcharn, 1982).

There is also some evidence to suggest that uninfected blood meals either before or after an infective blood meal, increases the number of larvae surviving within the mosquito (Desowitz and Chellapah, 1965; Petit, Bain and Kaveh, 1977) and the rate of their development (Le Corroler, 1957).

The age of the mosquito can also affect the survival and development of filarial larvae. Duxbury et al., (1960) found that more and larger larvae of D. uniformis developed in An. quadrimaculatus fed when 3 days old compared with those fed 9 days after emergence. Similarly, more B. malayi larvae were recovered from Ae. togoi when fed 5 days old than from those fed when 12 days old; although proportionately fewer of the young mosquitoes were infected (Maeda and Kurihara, 1980). This is not altogether surprising since mosquitoes imbibe the greatest quantity of blood around five days after emergence. Larger blood-feeds will, of course, result in a larger intake of microfilariae, sufficient in some cases to kill the mosquito and thus reduce the proportion of mosquitoes infected.

The development of filarial larvae has also been shown to be independent of mosquito hormones (Yoeli, Upmanis and Most, 1962; Gwadz and Spielman, 1974).

THE RATE OF INFECTIVE LARVAL DEVELOPMENT:-

Development of Brugia spp. infective larvae usually takes between one and two weeks, with W. bancrofti taking slightly longer to mature, depending on environmental conditions. The importance of temperature on larval development was first recognised by Acton and Rao (1930) and Rao and Iyengar (1930). In general, at temperatures below 22 °C few microfilariae are able to penetrate the gut of the mosquito (Hu, 1962) and little or no development occurs (Rao and Iyengar, 1930; Omori, 1958; Nakamura, 1964). As the temperature rises the rate of development increases, becoming optimal approaching 30 °C (Rao and Iyengar, 1930; Rodriguez and Thompson, 1974). However, at higher temperatures the yield of infective larvae decreases because of increased filarial larva mortality (Singh, Mammen and Das, 1967; Brunhes, 1969; Rodriquez and Thompson, 1974). Such elevated temperatures also decrease the longevity of infected mosquitoes (Nakamura, 1965).

Rao and Iyengar (1930) believed that humidity had a greater bearing on development than did temperature. At a low humidity both the percentage of mosquitoes infected and the worm burden of those insects were reduced.

Another environmental parameter, atmospheric pressure has been shown to have no affect on the development of D. immitis in Ae. aegypti (Williams, 1959). Therefore, altitude alone is unlikely to alter the development of filarial worms.

Infective larvae can remain viable within the mosquito for as long as the mosquito survives. For instance, Lavoipierre and Ho (1966) found live B. pahangi larvae in Ae. togoi after 29 day whilst Hu (1934) found W. bancrofti larvae alive in C. quinquefascitus after 79 days. In dead mosquitoes larvae can remain viable for up to 72 hours providing the corpse does not dehydrate (Gwadz and Chernin, 1973).

The many factors which operate against the successful development of microfilariae to infective larvae leads to relatively few infective larvae being found in wild mosquito populations. This is despite the presence of infected humans with high levels of circulating microfilariae. Thus mean parasite loads in infected mosquitoes have been recorded of 1.8 (W. bancrofti in C. quinquefasciatus in Rangoon), 2.2 (W. bancrofti in C. quinquefasciatus in Sri Lanka), 2.8 (B. malayi in Mansonoides spp. in Malaysia; Sasa, 1976) and 2.6 (W. bancrofti in C. quinquefasciatus in Colombo;

Samarawickrema and Laurence, 1978) infective larvae per mosquito. Even in areas of high filarial endemicity only 1% of the mosquito population may be infected. However, under controlled laboratory conditions, using a highly susceptible mosquito vector 99% of the insect population may be infected (Macdonald and Ramachandran, 1965; Rodriguez, 1973). Similarly, much higher levels of infective larvae are found, mean loads of 10.9 (*W. bancrofti* in *Ae. aegypti*; Paige and Craig, 1975) and 6.5 - 15.5 (*B. pahangi* in *Ae. togoi*; Lavoipierre and Ho, 1966) occuring.

THE INTENSITY OF TRANSMISSION:-

Estimations of the number of infective bites received annually vary a great deal. Whether this is a true reflection of the levels of transmission in these areas or due to some factors which bias the calculations remain unclear (see Kuhlow and Zielke, 1978). Estimates of 14 infective bites received annually in Trinidad (Nathan, 1981) and 1,850 in Calcutta (Gubler and Battacharya, 1974) illustrate this disparity.

The transmission of filarial worms is an inefficient process. Hairston and de Meillon (1968) first drew attention to this by showing that bites by 15,500 mosquitoes carrying infective larvae were necessary for the production of each new case of microfilaraemia in Rangoon.

MIGRATION AND DEVELOPMENT WITHIN THE HOST:-

Once within the host penetration of the local lymphatics occurs within a few hours and the larvae migrate with the lymph flow to the perinodal lymphatic sinus of the nearest lymph node (Ewert and Ho, 1967; Ewert, 1971; Ewert and El Bihari, 1971; Suswillo, Denham and McGreevy, 1982). Later they migrate against the flow and return to the afferent lymphatics where they remain for the rest of their life. The migration of the infective larvae of Brugia spp. in cats is completed after 1-12 days (Edeson and Buckley, 1959; Schacher, 1962; Ewert and El Bihari, 1971; Suswillo, Denham & McGreevy, 1982).

Bey (1938) believed that migration was dependent on a thermopositive response from the larvae; the worms being attracted to warmer areas of the body. He argued that infective larvae penetrate from the subcutaneous lymphatics and migrate to the lower limbs for this reason. A small percentage of larvae, he believed, would remain in the superficial inguinal nodes during periods of hot weather and in persons whose limbs are exposed to the sun. During warm weather the larvae would travel toward the spermatic cord, as he thought (wrongly) that this region was one of the warmest areas of the body.

Until recently, the infectivity of third-stage larvae was

thought to be independent of age (Bosworth and Ewert, 1971). However, Eberhard and Lowrie (in press) have found that young, 11 day old, infective larvae are not as infective as 14 day old larvae.

The infectivity of infective larvae is though, independent of the vertebrate host species donating the microfilariae. Vincent, McCall, Courgill, Ash and Sodeman (1982) found no difference in the infectivity of B. pahangi larvae to jirds from jird-passaged or dog-passaged strains.

The infective larvae of B. pahangi moult after 8-9 days to form fourth-stage larvae (Schacher, 1962). The final moult occurs around 25 days. Mating of the two sexes can occur with microfilariae being detected in the blood as early as 54 days post infection.

CHAPTER 3

THE MIGRATION OF THE INFECTIVE LARVAE OF *B. PAHANGI* WITHIN THE MOSQUITO *AE. AEGYPTI*

SUMMARY

The distribution of *B. pahangi* infective larvae within a population of *Ae. aegypti* mosquitoes is over-dispersed, although the degree of over-dispersion declines during the course of infection. The decreasing mean worm burden which occurs with time is probably due to density-dependent, parasite-induced host mortality as well as the direct loss of larvae from these insects. The course of larval migration within the mosquito is described and evidence is provided to suggest that this takes place due to the "random" movement of the larvae. In heavy infections the migration of larvae into the proboscis is delayed.

INTRODUCTION

Since the earliest description of the development of *W. bancrofti* within the *C. quinquefasciatus* (Manson, 1878), much work has been carried out to elucidate the relationship between parasite and vector (reviews by Lavoipierre, 1958; Nelson, 1964; Piessens and Partono, 1980). Certain aspects of this relationship remain poorly investigated, particularly in respect to the behaviour of the third-stage, infective larvae during its migration

within the vector and its transmission to the vertebrate host. A more complete understanding is not only of zoological interest, but may also provide useful information for epidemiological studies and for improving the efficiency of the production of infective larvae in the laboratory.

The primary aim of this study was to investigate the behaviour of the infective larvae of B. pahangi during their migration in Ae. aegypti. A qualitative account of the migration of infective larvae would provide useful information on the intrinsic nature of larval migration and serve as a valuable "template" on which subsequent studies could be based. The methodology was similar to that used by Lavoipierre and Ho (1966) who investigated the migration of B. pahangi in a larger mosquito, Ae. togoi. However, comparatively few mosquitoes were used in their study and it was considered important to use large numbers in order to be able to attach greater statistical weight to any conclusions reached.

MATERIALS AND METHODS

The strain of B. pahangi, originally from East Malay sia, was obtained by the London School of Hygiene and Tropical Medicine in 1968 from the Liverpool School of Tropical Medicine. It has since been maintained by passage in domestic cats (Felis catus).

The BLACK EYE strain of Ae. aegypti, homozygous for the f^m gene, which is susceptible to infection with many filariae (Macdonald. 1962a and b), was used as the vector throughout the study. The procedure for producing infected mosquitoes was as follows.

Mature Ae. aegypti eggs were hatched each week on Wednesday morning. Two to three egg papers were placed on the water surface of an enamel or fibreglass domestic bath, half full of water and kept at 30 °C ± 1. The water was allowed to stand for 24 hrs and liver powder (Armour Pharmaceutical Company) added until the water appeared cloudy. The following morning air was bubbled through the water to facilitate the dispersal of the liver powder and to prevent the formation of fungal mats on the water surface which would kill the mosquito larvae. As soon as pupae appeared in the baths (usually Monday) the baths were drained and the pupae and larvae collected in a sieve before transfer to a plastic bucket. The larvae, male pupae and female pupae were then separated according to their relative sizes using a mechanical separator (Plate 3.1). The larvae were returned to the baths and the separation procedure repeated the following day.

The pupae were collected by filtration using a glass funnel (Plate 3.2) and mixed in a ratio of 1 male to 3 females.

Plate 3.1 Device for the mass separation of mosquito larvae, male and female pupae.

Plate 3.2 Glass pupae collecting funnel used for standardising the number of male and female pupae added to each cage. One volume for females and one-third of a volume for males.

Approximately one thousand pupae were then divided between two water-filled, plastic tubs (9 cm diameter, 5.5 cm high) in each mosquito cage. The cages were 30 X 30 X 30 cm in volume, constructed from plastic-coated, wire-frames and covered with sandfly netting (Dauris and Co., London). In addition to the two pupal tubs each cage also contained a plastic pot (4.5 cm bottom diameter, 6.5 cm top diameter, 7.5 cm high) holding tap water and a folded filter paper (12.5 cm diameter, Whatman No. 1) to allow the adult mosquitoes to drink and lay their eggs. The adults were maintained on dried sultanas placed on top of the cage and maintained at 27 $\pm$ 1 ^{o}C, 80% relative humidity with a 12 hour light : 12 hour dark regime (i.e. light 08.00 - 20.00 hrs).

On Thursday, one cage of stock mosquitoes was fed on a guinea-pig. By Monday the egg-paper was covered with eggs. These were used for rearing all mosquitoes. This procedure was adopted to prevent the selection of filarial resistant genes in the population. The egg-paper was replaced and kept for three to five days in a glass dessicator at 95% relative humidity (using a 7.5% solution of potassium hydroxide in water in the base of the dessicator) and 30 $\pm$ 1 ^{o}C. After this time each paper was stored until needed in another glass dessicator, kept at 70% relative humidity (33% potassium hydroxide) and 30% $\pm$ 1 ^{o}C. The potassium

hydroxide solutions were changed monthly.

The mosquito cages were cleaned after use by immersing the cages in a weak solution of bleach overnight and then rinsing them in clean tap water the following day.

The dried fruit was removed from the experimental cages on Thursday, 2 days post emergence, and the mosquitoes allowed to feed on an infected cat the next morning. A cat with approximately 80-200 microfilariae per 20 mm^3 of peripheral blood (counted by the technique of Denham, Dennis, Ponnudurai, Nelson and Guy, 1971) was anaesthetised with 1 ml/kg body weight sodium pentobarbitone (60 mg/ml solution, Sagatal, May and Baker Ltd) given intraperitoneally. The animal was then shaved on one flank with a pair of electric clippers and placed on top of a pair of cages for approximately 15 minutes. After feeding the pupal tubs were removed and fresh dried fruit placed on the cage.

Mosquito autopsies to assess the parasite burdens were performed as follows. Adult mosquitoes were taken at random from a cage with a pooter and blown into a boiling tube which had a cotton wool bung wetted with a small quantity of pyrethroid insecticide (Cooper "Super Strength Fly Killer", Wellcome Foundation Ltd). Each female mosquito was divided into four parts (proboscis, head, thorax and abdomen) under a dissecting microscope at a magnification

of X 8. Each part was teased apart in a separate drop of PBS on a glass slide using two pairs of fine-tipped forceps. The slide was left for several minutes, to allow the larvae to move out of the tissues, before being examined under a dissecting microscope at a magnification of X 20. The number of larvae found in each section of mosquito was recorded.

In this thesis experiments on transmission were invariably performed 12 days post infection (p.i.). It was, therefore, of interest to produce a reliable assessment of the statistical distribution of the parasites within infected mosquitoes at this time. This was achieved by pooling the worm burdens from the first ten mosquitoes autopsied from 85 consecutive trials conducted over a two-year period.

In order to demonstrate that the observed over-dispersed distibution was not the product of a combination of random distributions, the frequency distribution of infective larvae from 200 female mosquitoes taken at random from a single cage was obtained. The variance-to-mean ratio was again used to assess whether or not this population was over-dispersed. When this value is greater than unity the distibution is over-dispersed.

Autopsies was performed on days 7, 8, 9, 10, 12 and 15 days p.i. for six trials. Two trials were extended, with autopsies performed additionaly on days 20, 25 and 30 post

infection.

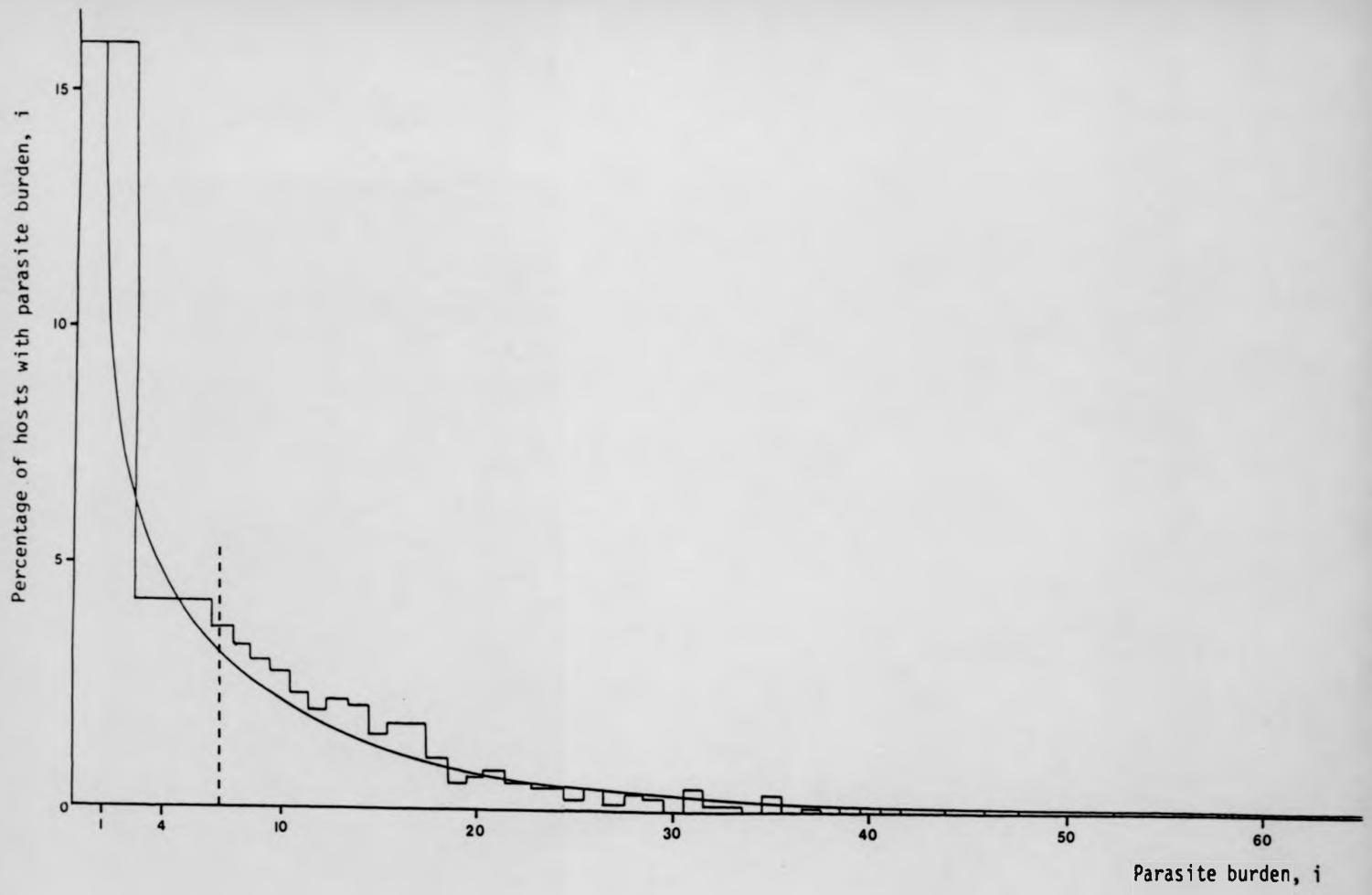
The mean number of larvae recovered from the proboscis and head together, the thorax and the abdomen of mosquitoes autopsied between 7-30 days p.i. were determined for each day and expressed as a percentage of the total number of larvae recovered. Furthermore, in order to determine whether the migration of larvae was influenced by the density of infection the data were segregated into three groups. Mosquitoes with 1-3 larvae, as is commonly found in the field, constituted the group with a low worm burden. Insects with worm burdens greater than 9 represented a high worm burden class and those mosquitoes with intermediary loads represented the group with a medium worm burden.

In addition the mean worm burden and the variance-to-mean ratio of the pooled data from the six trials were calculated for each day (p.i.) that autopsies were performed. Comparisons between the worm burdens on different days during the infection were made using the Mann-Whitney U-test. When p was less than 0.05 the difference between the two samples was considered significant.

RESULTS

The frequency distribution of infective larvae per mosquito, 12 days p.i., is shown in Figure 3.1. This

Figure 3.1 The frequency distribution of the infective larvae of B. pahangi within 850 Ae. aegypti mosquitoes autopsied 12 days post infection. An estimate of the value of the negative binomial parameter k (= 0.44) was obtained by a maximum likelihood method (see Southwood, 1978). The frequency distribution of infective larvae within mosquitoes is presented as the percentage of the total sample containing i larvae. The curve represents the prediction of the negative binomial distribution and the broken vertical line the mean worm burden per mosquito (= 6.3). The variance to mean ratio ($s^2 \div x$) of larval numbers per mosquito was used to assess the degree of over-dispersion (see Anderson and Gordon, 1982).


Percentage of hosts with parasite burden, i
15
10
5
0
1
4
10
20
30
40
50
60
Parasite burden, i

distribution is highly over-dispersed and fits the negative binomial probability model (Chi-squared = 51.7, d.f. 43). That is, the majority of mosquitoes had few larvae while a few mosquitoes harboured the major proportion of the larval population. The variance-to-mean ratio from the 200 mosquitoes autopsied on one occasion was 8.9 again indicating that the distribution is clearly aggregated.

The relationship between the mean worm burden, the variance-to- mean ratio of the number of larvae per mosquito, and the duration of infection are shown in Figures 3.2A and B. Both the mean worm burden and the variance-to-mean ratio decreased with the duration of infection. However, it was not until the day 10 p.i. that the recorded worm burden differed significantly from the day 7 p.i. worm burden ($z = 1.99$, $0.05 < P > 0.01$)

The percentage distribution of larvae within the mosquito during the course of infection is shown in Figure 3.3. The supporting data is provided in Appendix 3.1.

The migration of infective larvae from the thorax commences one week post infection. This is a dramatic exodus with 75% of the larvae leaving the thorax during the following few days. Subsequently a more gradual decline takes place. The movement of larvae from the thorax is accompanied by an increase in the percentage of larvae moving into the

Figure 3.2A The relationship between the mean worm burden and the duration of infection for larvae recovered from mosquitoes autopsied 7-30 days post infection. The points are observed data (± 95% confidence limits). For values of n see appendix 3.1.

Figure 3.2B The relationship between the variance-to-mean ratio and the duration of infection for larvae recovered from mosquitoes autopsied 7-30 days post infection. For values of n see appendix 3.1.

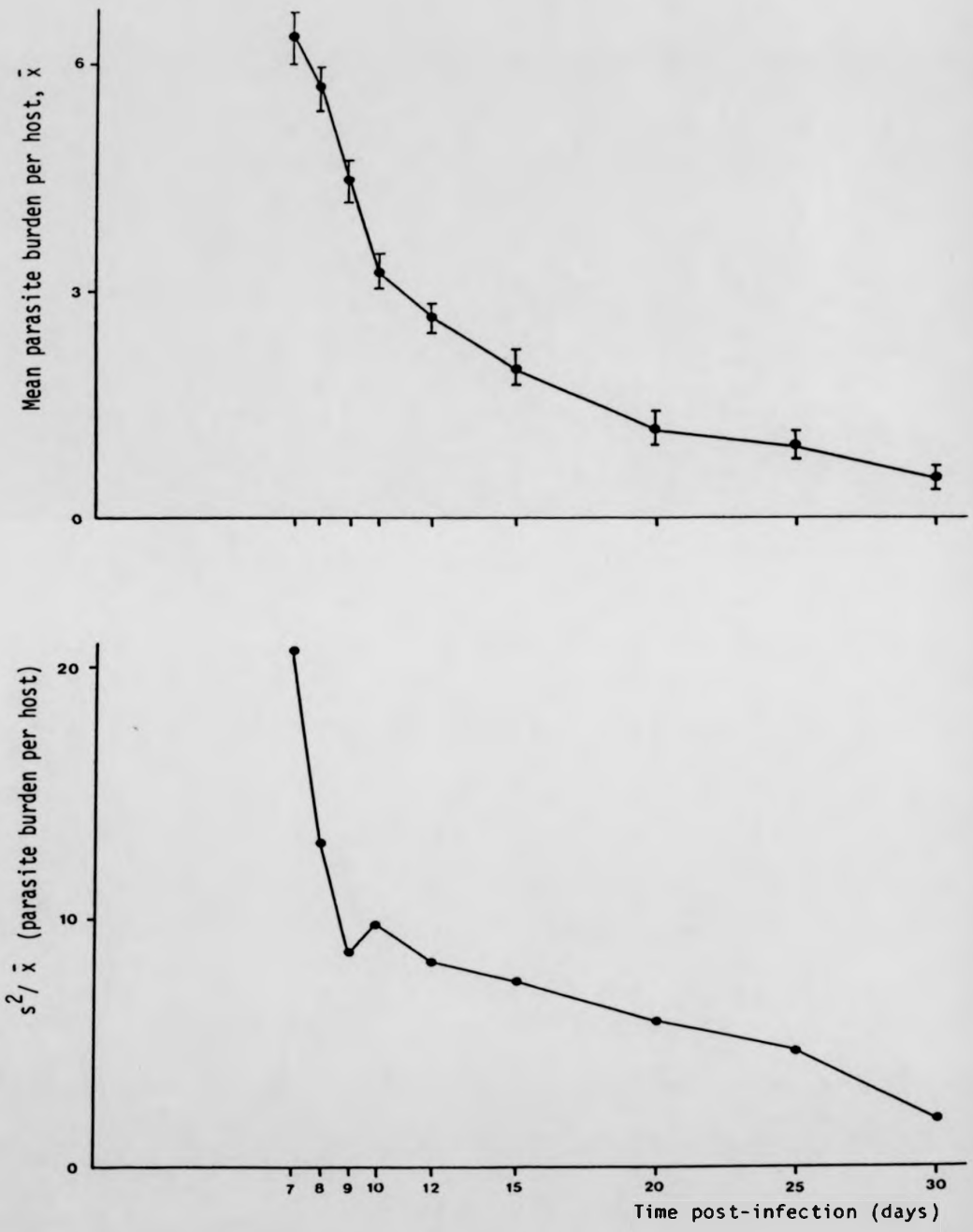
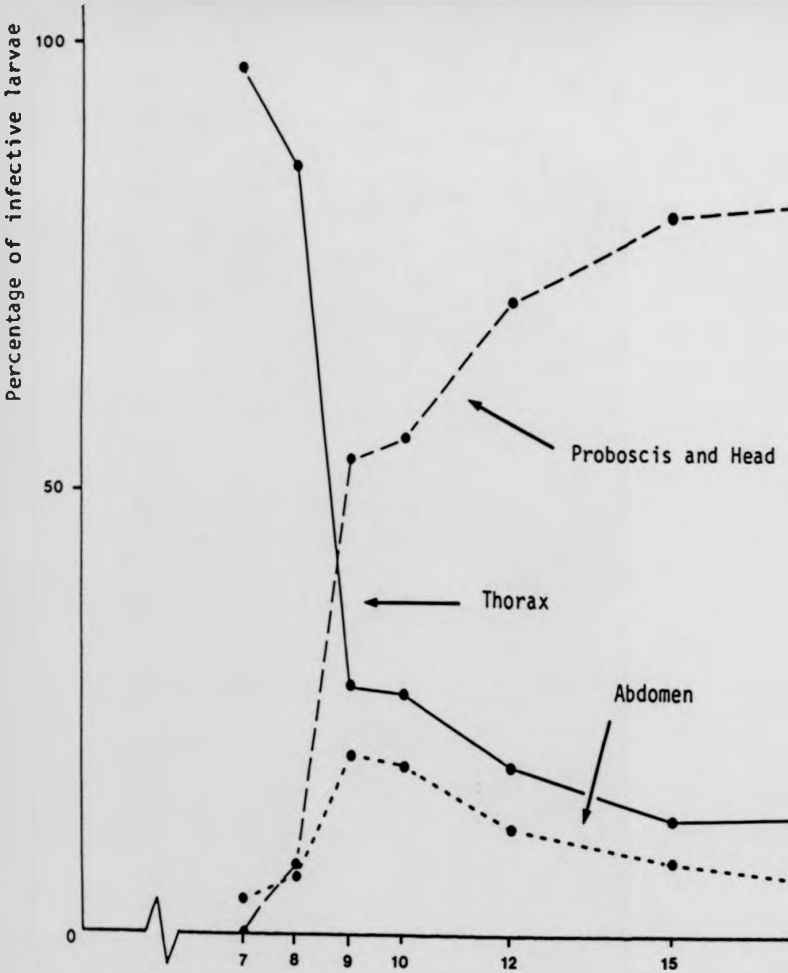
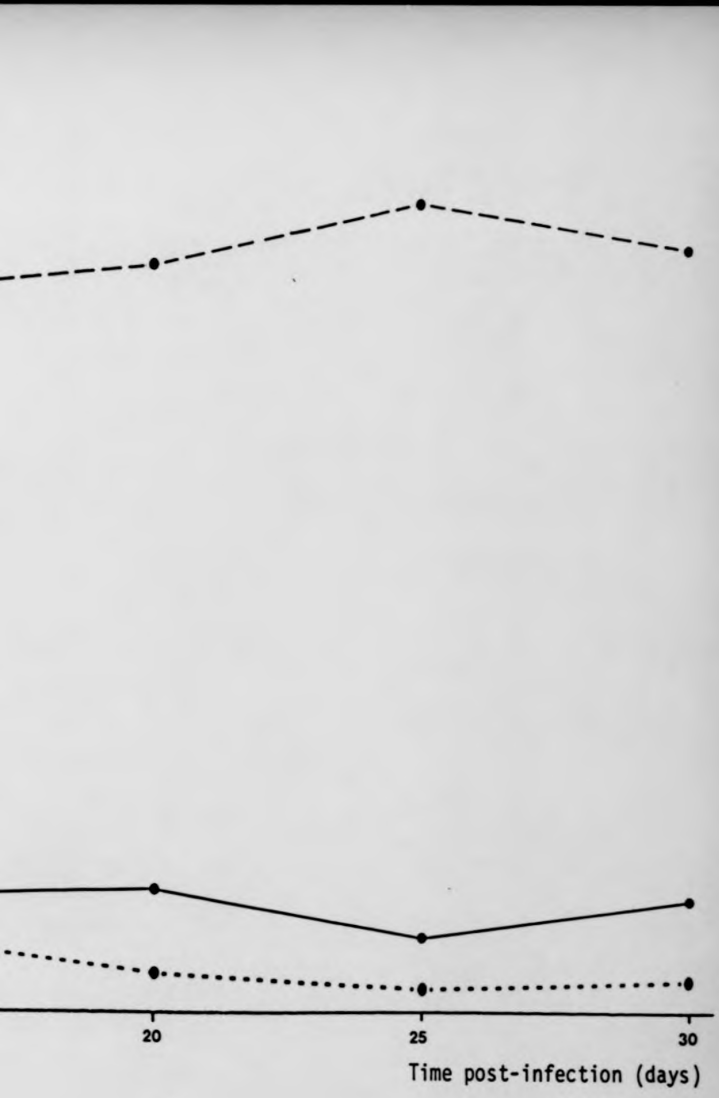

Mean parasite burden per host, $\bar{x}$
6
3
0
$s^2/\bar{x}$ (parasite burden per host)
20
10
0
7
8
9
10
12
15
20
25
30
Time post-infection (days)

Figure 3.3 Percentage distribution of larvae within mosquitoes autopsied 7-30 days post infection.
For values of n see appendix 3.1.


Percentage of infective larvae
100
50
0
7
8
9
10
12
15
Proboscis and Head
Thorax
Abdomen


20
25
30
Time post-infection (days)

proboscis and head, the two curves being symmetrically opposed. When the combined curve of the percentage of larvae in the proboscis and head is separated into its component parts both curves follow a similar pattern (Figure 3.4). However, a higher percentage of larvae is always found in the proboscis than in the head. The initial increase of larvae in the proboscis is also greater than that found in the head indicating that larvae fill-up the proboscis of the mosquito first.

Concomitant with the percentage increase of larvae in the proboscis and head there is a less dramatic rise in the percentage of larvae in the abdomen. This occurs rapidly, being at a maximum 10 days p.i., thereafter the percentage of larvae resident in the abdomen slowly declines.

The percentages of larvae in the thorax, proboscis and head together and the abdomens of mosquitoes with low, medium and high worm burdens, autopsied between 7-25 days p.i., are shown in Figures 3.5, 3.6 and 3.7, respectively. Supportative data is shown in in Appendix 3.2.

Irrespective of the parasite load, the rate at which infective larvae leave the thorax (Figure 3.5) is similar during the first 2 days. After this time, at low parasite levels, this exodus is more complete. This is similarly reflected in the accumulation of larvae in the proboscis

Figure 3.4 The percentage distribution of larvae in the head and proboscis of mosquitoes autopsied 7-30 days post infection. For values of n see appendix 3.1.

Percentage of infective larvae
60
30
0
7
8
9
10
12
15

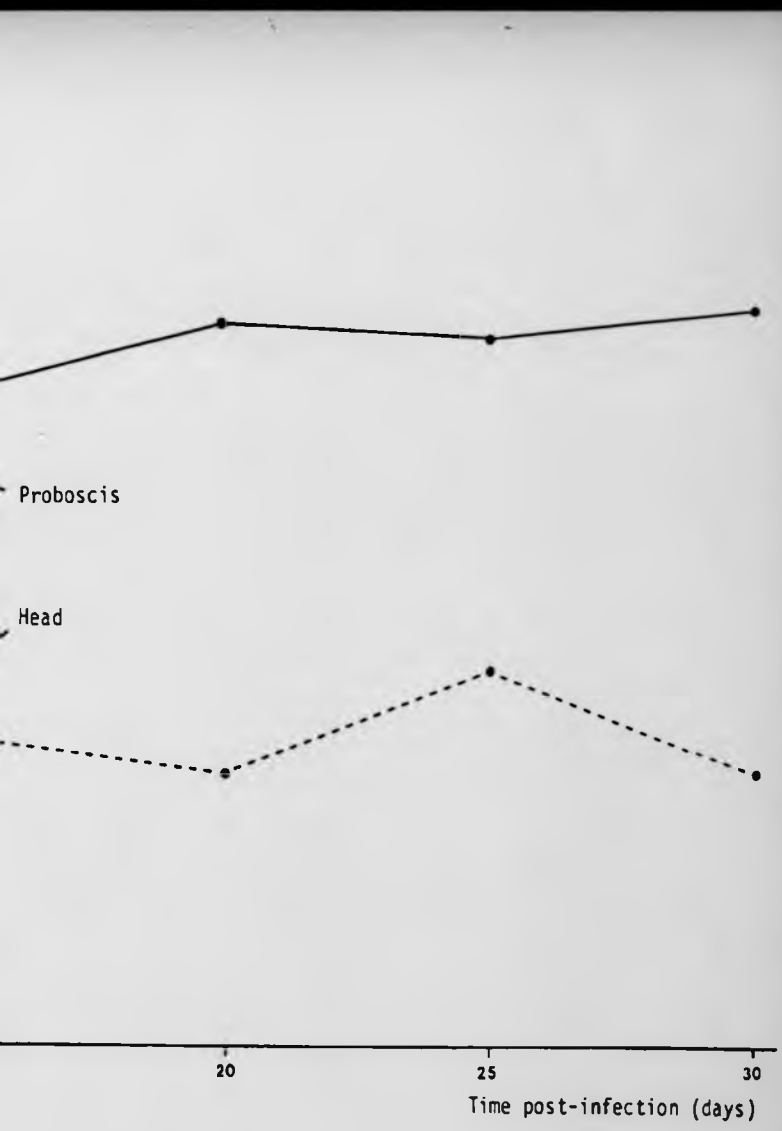

Proboscis
Head
20
25
30
Time post-infection (days)

Figure 3.5 The percentage distribution of larvae in the thorax of mosquitoes with a low, medium and high parasite burden 7-25 days post infection.

For values of n see appendix 3.2.

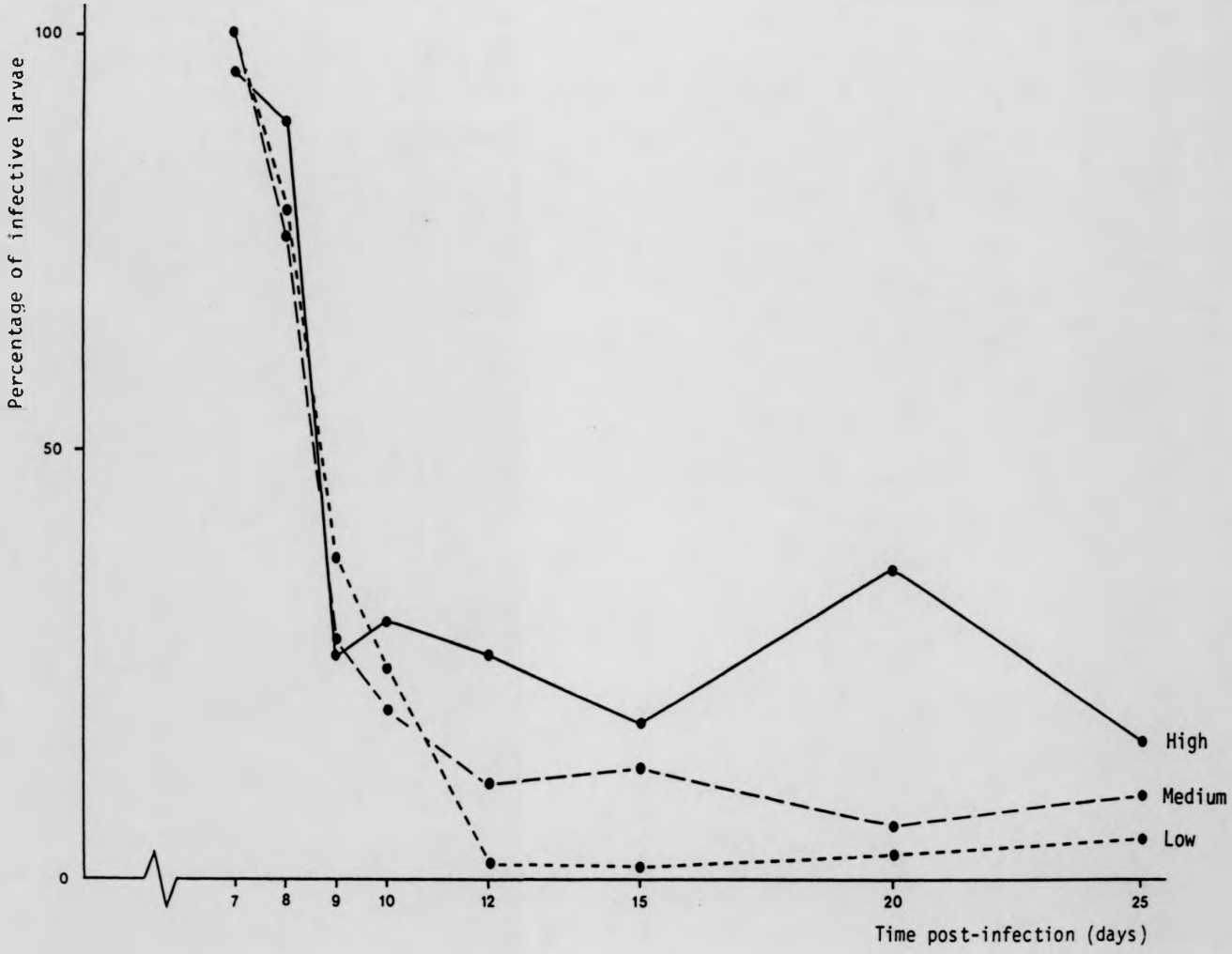
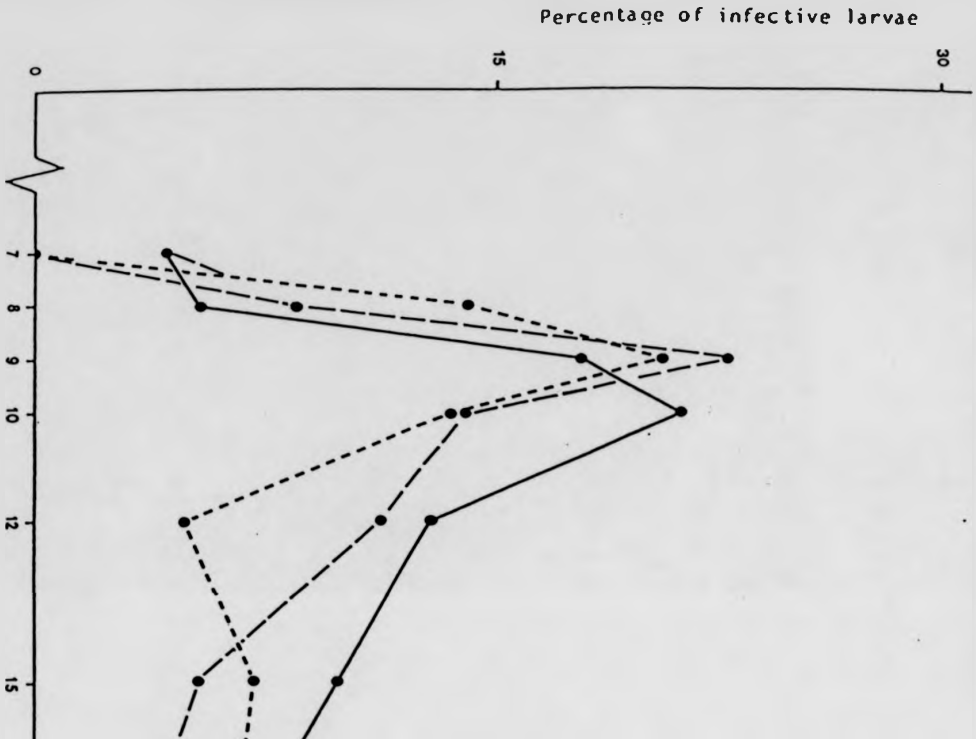

Percentage of infective larvae
100
50
0
7
8
9
10
12
15
20
25
Time post-infection (days)
High
Medium
Low

Figure 3.6 The percentage distribution of larvae in the abdomen of mosquitoes with a low, medium and high parasite burden 7-25 days post infection.

For values of n see appendix 3.2.


Percentage of infective larvae
0
15
30
7
8
9
10
12
15

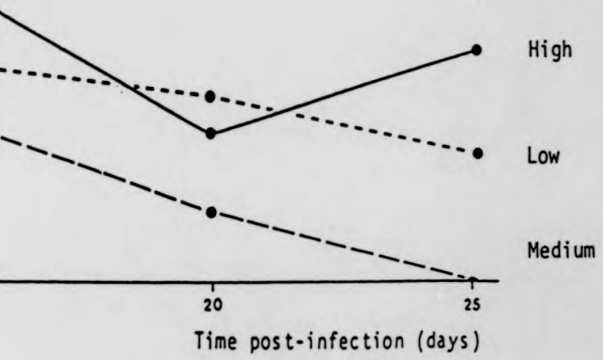
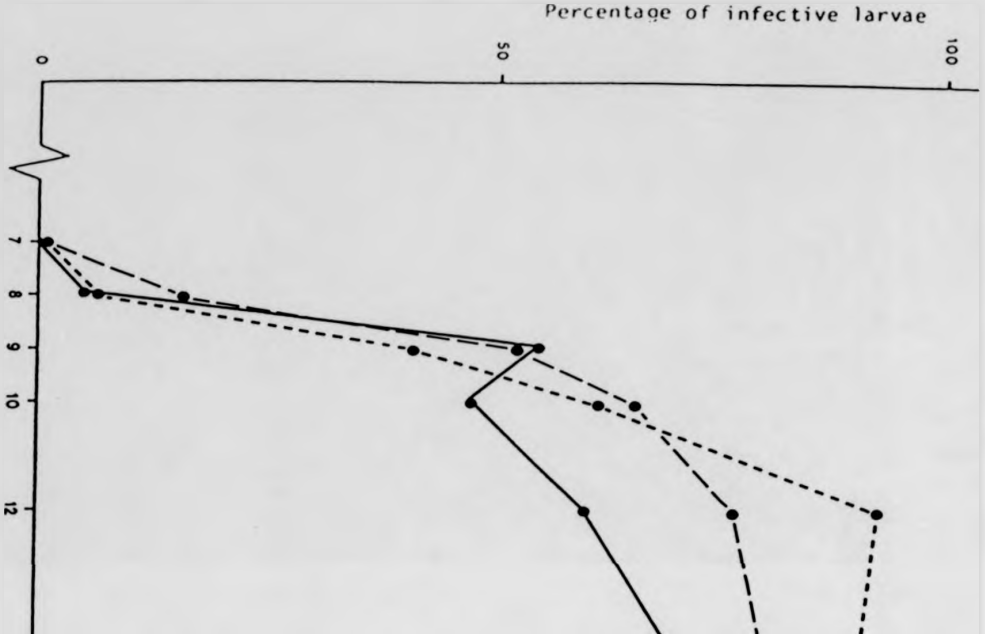
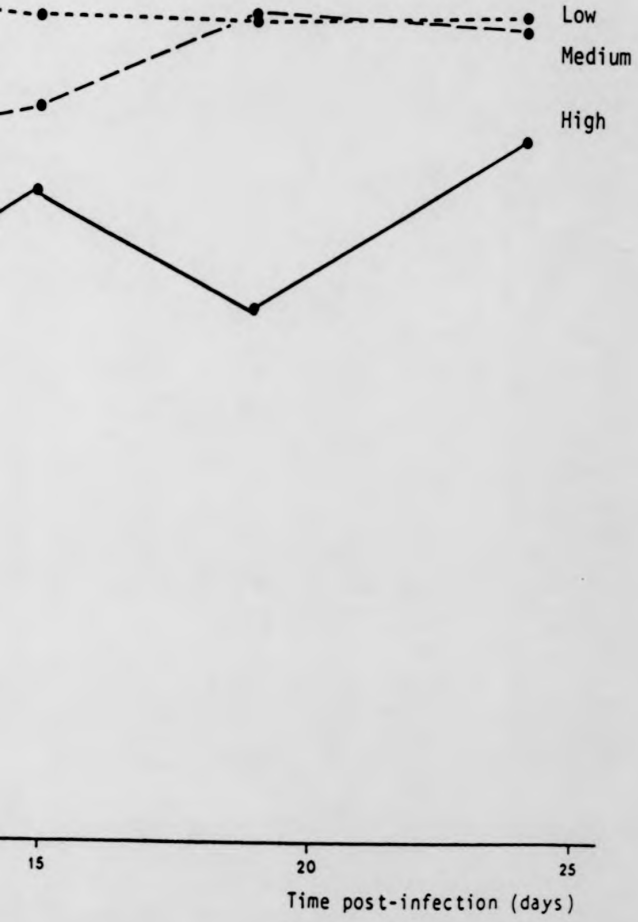

High
Low
Medium
20
25
Time post-infection (days)

Figure 3.7 The percentage distribution of larvae in the head and proboscis of mosquitoes with a low, medium and high parasite burden 7-25 days post infection.

For values of n see appendix 3.2.


Percentage of infective larvae
0
50
100
7
8
9
10
12


Low
Medium
High
15
20
25
Time post-infection (days)

and head (Figure 3.6). The retardation of larval migration in heavy infections was also demonstrated by the peak in the percentage of larvae accumulating in the abdomen (Figure 3.7) which occurred a day later in mosquitoes with a high worm burden. In Figures 3.5, 3.6 and 3.7 the curves formed by mosquitoes with a medium worm burden tend to be intermediary between the other two curves.

DISCUSSION

There is strong evidence to show that infective larvae within a population of infected mosquitoes are clearly not normally distributed. Their distribution is highly over-dispersed and may be described by a negative binomial model (Figure 3.1). This model is likely to be representative of the distribution of filarial worms in field populations of mosquitoes. Krafsur and Garrett-Jones (1977) found that the distribution of W. bancrofti in Anopheles funestus was aggregated. The biological significance of a negative binomial distribution is that it enhances the density-dependent regulation of parasite abundance (Crofton, 1971; Anderson, 1978, 1979, 1981). It is in the few hosts with large numbers of parasites that this regulatory effect is manifest.

The observed decline in the degree of over-dispersion

through time (Figure 3.2 B) indicates that the tail of the parasite frequency distribution (i.e. that portion where a few hosts harbour many parasites) is being lost. There are two possible causes of this relationship. Firstly, there may be density-dependent parasite-induced host mortality, where hosts with high parasite burdens suffer increased mortality due to the action of the parasites (Klowden, 1981), which would lead to a decline in both the mean parasite burden per host and the level of over-dispersion through time. It would be misleading to consider that there exists a clearly defined lethal level above which death occurs and below which the mosquito survives. More simply one should assume that the probabilty of a host dying is some function of its parasite burden (Anderson, 1978, 1979, Anderson and Gordon, 1982).

Secondly, there is evidence that larvae may be "lost" from the probosces of mosquitoes either when they feed on sugar or spontaneously as a result of the proboscis being too tightly packed with larvae. The former is probably the more important of the two mechanisms since the greatest decline in mean worm burden (Figure 3.2A) occurs when the infective larvae begin moving out of the thorax 7 days p.i. (Figure 3.4) and there are few larvae in the head and proboscis.

The initial degree of over-dispersion is most probably generated by the heterogenous uptake of microfilariae in

the blood meal (Kershaw et al., 1953; Schmid and Robinson, 1972; Pichon et al., 1980) as well as the heterogeneity in both mosquito feeding behaviour and susceptibility to infection.

Numerous authors have, in the past, used conventional parametric statistics when comparing the worm burdens from different groups of infected mosquitoes. Such an approach is inappropriate since the few mosquitoes with heavy infections will exert too great a weight on the comparison. Thus when a small sample is used, the inclusion of one or more mosquitoes with an exceptionally large worm burden would represent a serious source of error in the estimation of the mean parasite burden per host. A far better method is to use non-parametric statistical tests. For instance, the Mann-Whitney U-test may be used for comparing the worm burdens of different groups of mosquitoes. Essentially the comparison is made by ranking the individual worm burdens. In this way those mosquitoes with heavy infections are not over-emphasised in the comparison. Nonetheless mean values are included in this thesis as they represent a convenient description of parasite populations for comparisons between worm burdens from different groups of hosts. Any inherent errors will be minimized because of the large sample sizes.

It is difficult to prove conclusively that larvae are lost

from mosquitoes whilst feeding on sugar media since no investigator has shown infective larvae in these media. However, Pratt and Newton (1946) showed that the infective larvae of W. bancrofti emerge from the proboscises of infected mosquitoes immersed in saline and found evidence of some proboscises having ruptured prior to dissection. They also found that the mean number of larvae in the proboscis did not increase during the infection whilst the mean number of worms in the thorax and abdomen decreased, implying that larvae were lost from the proboscis. However, this relationship could also be explained in terms of the density-dependent parasite-induced host mortality previously discussed. In addition, a proboscis can only contain a limited number of larvae and so the number of larvae contained within it cannot increase indefinitely. However, Ho and Ewert (1967) demonstrated that probing alone is often enough to elicit the emergence of larvae from the proboscis.

In mosquitoes with a low worm burden (1-3 worms per host) there is little difference in mortality compared with that of an uninfected group (Laurence, 1963; Samarawickrema, 1967; Krafsur and Garett-Jones, 1977). Intuitively, if larvae are being lost from the proboscis one would predict that larvae would continue to accumulate in the head and proboscis until all the larvae were located there. Moreover, because there are so few larvae in the mosquito, larvae would not be physically prevented from moving into

the head and proboscis. The line in Figure 3.6 representing the group with a low worm burden does reach a plateau level, indicating that larvae are being lost from the mosquitoes.

However, there remains a further possible explanation for this plateau. A proportion of the population of infective larvae may be less suited to migration, possibly as a result of their lower activity. Hence the less active larvae of W. bancrofti (Laurence, 1979) are found more evenly distributed throughout the body of the mosquito (Newton and Pratt, 1946; Laurence and Pester, 1961) compared with the more active larvae of Brugia spp. (Laurence and Pester, 1961; Lavoipierre and Ho, 1966).

One might expect that there is a strong selective pressure against highly active larvae, since they would either escape from the mosquito too readily or so damage the mosquito by their vigorous peregrinations that it would result in it's death. In contrast, infective larvae with low activity would not present such a lethal threat to the host and as a consequence genes for low activity would be selected for. This would explain why a large proportion of larvae fail to move into the proboscis. Moreover, for transmission to occur it requires only one larva in the proboscis to trigger emergence, with larval recruitment occuring rapidly from other areas of the insect's body.

This aspect of transmission is discussed more fully in Chapter 6.

This present work confirms the work of Laurence and Pester (1961) and Lavoipierre and Ho (1966) who found that B. pahangi infective larvae concentrate within the proboscis and head 10-12 days post infection. Moreover, the accumulation of larvae in this region continues for as long as the mosquito survives. This is even more dramatic if one assumes that larvae are slowly being lost from the proboscis. The length of time taken for the larvae to accumulate in the proboscis must not only be a function of temperature but must be partly determined by the intrinsic activity of the larvae.

Lavoipierre and Ho (1966) demonstrated that in Ae. togoi, B. pahangi larvae oscillated between the head and proboscis in an 8 day cycle, commencing 9 days post infection. However, in my experiments with the same parasite species, clearly no oscillation occurred (Figure 3.4). This pattern of migration may indeed be peculiar to the larger insect but may equally be explained in terms of the inevitable fluctuations in recorded parasite levels associated with their small sample size, as each point is based on the dissection of only 8-10 infected mosquitoes. Lavoipierre and Ho (1966) also indicated that larvae moved back into the thorax from the proboscis and head in a similar 8 day cycle. Indeed Kan and Ho (1973) proposed

that this meandering had some biological significance and may be related to the feeding requirements of the larvae. They added that this would also allow time for the repair of damaged flight muscles. If this is so the failure to show such an effect in this study which is based on a considerably larger population sample is suprising. If B. pahangi does actually oscillate within Ae. togoi it would be interesting to know why this does not occur in Ae. aegypti. As a final comment it is worth pointing out that a great many worms straddle the head and proboscis junction so a clear distinction between worms in the proboscis and head alone is not possible.

Increasing worm burdens leads to a retardation in the migration of larvae from the thorax (Figure 3.5). This is most clearly demonstrated by the delay which occurs in heavy infections when larvae move into the abdomen, after 9 days p.i. (Figure 3.7). This is probably due to the unequal rate of larval development found in heavy infections (Rosen, 1955; Webber, 1955; Wharton, 1957) and the congestion of larvae which occurs in the head and proboscis.

It seems likely that the movement of infective larvae within the mosquito is essentially non-directional (i.e. random), but that the path of migration is dictated by the internal anatomy of the mosquito. "Non-directional" or

"random" are both over-simplifications and are used as short-hand to describe a more complex behaviour. Although, the overall pattern of behaviour in a population of nematode larvae is random many features of the tracks of individual larvae are idiosyncratic and measurable, occuring with a predictable frequency (Croll, 1971; Croll, 1972; Croll and Blair, 1973).

Larvae moving out of the thorax will follow the path of least resistance, usually through the loose connective tissue surrounding the gut. Larvae rarely accumulate in the legs and wings as these appendages are well packed with muscle at their junction with the thorax. Moreover the larvae preferentialy develop along the axis of the dorso-longitudinal flight muscles (Beckett and MacDonald, 1970; Beckett, 1971) and so when moving out of the thorax will tend to move anteriorly or posteriorly and not dorsally or ventrally.

Initially, larvae may accumulate in the abdomen simply because the junction between the thorax and abdomen is larger than the neck. But larvae accumulate in the head and proboscis because they have moved into a confined space. Randomly moving larvae on entering a long narrow structure would tend to remain there. Nelson (1963) found that B. patei microfilariae concentrated in the legs and tarsi of bed-bugs, an abnormal host. It may be too great a jump to compare the behaviour of one life stage with

another but even in normal vector species infective larvae are found in unusual sites. These include the legs, wings, halteres, antenae, intersegmental membranes and palps (Pandit, Pandit and Iyer, 1929; Brug and de Rook, 1930; Feng, 1936; Summers, 1943; Wharton, 1957; amongst others).

The infective larvae of Dipetalonema viteae are distributed randomly throughout the body cavity of the tick, Ornithodorus moubata (Bain, 1967). Perhaps no structure exists in the tick's body cavity within which the infective larvae can congregate or the worms may just be too large. In an evolutionary context the size of infective larvae in relation to the mosquito vector must be limited in order that the worms can enter the labium. Yet the infective larvae must be sufficiently large (see Chapter 5) and active to escape from the mosquito and infect the host.

If there is a stimulus which causes larvae to accumulate in the labium, other than by random movement it must be a weak one as larvae take several days to accumulate there. However, some larvae experimentaly implanted into the abdomen can migrate into the head and proboscis within a few hours (Newton and Pratt, 1945; Desowitz and Chellapah, 1965). Larvae are also able to move out of the labium and this posterior migration has been noted by Highby (1943) with Dipetalonema abuta larvae in Ae. aegypti and

Anopheles spp. and by Gwadz and Chernin (1973) with B. pahangi in Ae. aegypti.

Furthermore, if the migration of infective larvae occurs by a process of 'random-walk' it could partly explain why so many different genera of mosquitoes can act as filarial vectors, in contrast to most other mosquito-transmitted diseases where there are relatively few vector species. Indeed Mansonella ozzardi is transmitted by vectors from two different families, Simuliidae in Colombia and Culicidae in Haiti (Kozek, Eberhard and Raccurt, 1983). A migratory mechanism which only requires the parasite to be capable of moving forwards more often than moving backwards (as nematodes do; Croll, 1973) is far more likely to succeed in a wide range of vectors. This is, of course, providing that the physiological environment of the mosquito is suitable for larval development. Internally one species of mosquito may be much like the rest to an infective larva.

One can speculate that such a basic form of behaviour is likely to be a primitive one, similar perhaps to that of it's free-living ancestors. Darwin (1859) held the view that " although an organ [read behaviour] may not have been originally formed for some special purpose if it now serves for this end we are justified in saying that it is specially contrived for it".

CHAPTER 4

THE EFFECT OF BLOOD FEEDING ON THE MIGRATION AND LOSS OF THE INFECTIVE LARVAE OF B. PAHANGI FROM AE. AEGYPTI

SUMMARY

The presence of blood or eggs in the abdomens of mosquitoes at a time when the infective larvae are beginning their migration out of the thorax leads to a significant increase in the proportion of larvae accumulating in the abdomen. The mortality of infected mosquitoes increased following an infective blood-meal, when the infective larvae moved out of the thorax and again after a blood-meal, when larvae were lost from the mosquito. The loss of larvae from infective mosquitoes when blood-feeding 12 days p.i. was marked (85.1% loss), but was reduced when mosquitoes were fed through a thin-layer of cotton (48.3% loss). The loss of infective larvae and their distribution within the mosquito before and after a secondary non-infective blood-meal is also described.

INTRODUCTION

In the preceeding chapter it was suggested that infective larvae accumulated in the proboscis of a mosquito as a consequence of the insect's internal anatomy. i.e. infective larvae moving into a confined area would tend to remain there. It was, therefore, of interest to determine

whether, at a time when many larvae move into the abdomen, this area of the body could be made more "attractive". The area available for the movement of infective larvae within the haemocoelomic cavity of the abdomen could be reduced by the presence of either a blood-meal or eggs.

Secondly, the loss of larvae and their distribution within infective mosquitoes following successive blood-meals was investigated. "Infective" mosquitoes were those insects containing third-stage, infective larvae in their proboscises.

It was also of interest to monitor the survival of infected mosquitoes during the migration of the infective larvae and following a blood-meal.

MATERIALS AND METHODS

In the first series of experiments, three cages, each containing approximately 750 female and 250 male, 4 day-old, adult mosquitoes were allowed to feed on an infected cat (i.e. one infected control cage, and two test cages). Mosquitoes which failed to feed at any time during these experiments were removed, autopsied and their worm burdens determined. The test cages of mosquitoes were fed either 5 days p.i. or 8 days post infection. The mosquitoes were allowed to feed between 11.00-12.00 hours on the shaved belly of russet-coloured, female jirds (*Meriones*

unguiculatus; obtained from Intersimian Ltd, Abingdon, Oxon). The jirds were anaesthetised with 0.06 ml of sodium pentabarbitone (60 mg/ml solution) given intraperitoneally. In each experimental replicate only litter-mates were used.

Mosquitoes were autopsied 9 days p.i., at the time when the greatest accumulation of infective larvae in the abdomen occurs (Figure 3.3). Mosquitoes were grouped according to the condition of their abdomen at this time. i.e.

1. Normal (mosquitoes from the infected control cage).
2. Blood-filled (mosquitoes from a test cage fed 8 days p.i.).
3. Egg-filled (mosquitoes from a test cage fed 5 days p.i.).
4. Egg-laid (mosquitoes from a test cage fed 5 days p.i. which had laid eggs 8 days p.i.).

The number and distribution of infective larvae within the mosquitoes was recorded. The entire procedure was performed on seven separate occasions.

The data was analysed using the Friedman two-way analysis of variance by ranks and the Wilcoxon matched-pairs signed-ranks test. In these and all later statistical tests, significance was assumed at the 5% level (i.e. $p < 0.05$).

In the second series of experiments, four cages each containing approximately 300 female and 100 male adult mosquitoes were reared. The bottom of each cage was covered with white paper to assist in the identification and daily removal of mosquito cadavers.

Three of the four cages of 4 day old mosquitoes were fed on an infected cat (one infected control cage and two infected test cages) whilst the fourth cage was fed on an uninfected cat (uninfected control cage).

The two test cages of mosquitoes were offered a blood meal 12 days p.i., when a large number of infective larvae would have accumulated in the proboscis (Figure 3.3). One test cage of infected mosquitoes was fed on a jird through a thin-layer of cotton as it had been found that this reduced the number of larvae which were lost from the mosquitoes. The larger residual worm population would allow greater statistical weight to be attached to any conclusions reached, although the maintained high parasite burden may have affected the behaviour of both parasite and host.

Approximately thirty mosquitoes were autopsied from the infected control cage and from each test cage following blood-feeding. The number and distribution of infective

larvae within each mosquito was recorded. Mosquitoes which failed to feed were removed and their worm burdens determined. Thirty mosquitoes were also removed from the uninfected control cage to simulate the conditions experienced by the other three cages.

The two test cages of infected mosquitoes were given a further blood-meal four days later (i.e. 16 days p.i.). On this occasion both cages fed directly on a jird. Autopsies were performed on approximately 30 mosquitoes from the test cages both before and after feeding. Thirty mosquitoes were also autopsied from the control cage of infected mosquitoes. The entire experiment was repeated on six separate occasions.

After the initial blood-meal the mortality data for each group of mosquitoes was pooled and recorded as daily percentage survival. When the number of dead mosquitoes was recorded over a two-day period (instead of daily) this number was divided by 2 to obtain a mean value for the number of deaths per day. The logrank test (Peto, Pike, Armitage, Breslow, Cox, Howard, Mantel, McPherson, Peto and Smith, 1977) was used for comparing the mortality of one group of mosquitoes at different periods following the initial blood-meal. This test was also used for comparing the mortality between different groups of mosquitoes for the same period of time. In addition, the per capita instantaneous death rate was determined for each of the

four groups of mosquitoes.

The mean worm burden of the pooled data for the six trials was determined. The worm burdens of the different groups of mosquitoes were compared using the Wilcoxon matched-pairs signed-rank test.

Mosquitoes with high density filarial infections are less likely to feed (Wharton, 1957; Lavoipierre, 1958; Nelson, 1964 and Zielke, 1976). This means that fewer larvae are lost from the feeding mosquitoes than one would expect and consequently the estimated values for larval loss would be underestimated. The Mann-Whitney U-test was used to compare the worm burdens of mosquitoes from the control cage with those from either test cages.

The pooled mean number of larvae recovered from the proboscis and head together, the thorax and abdomen, from each group of mosquitoes autopsied at various intervals after infection, was expressed as a percentage of the total number of larvae recovered.

RESULTS

The effect of different conditions in the abdomens of mosquitoes on the percentage of infective larvae accumulating in the abdomen is shown in Table 4.1.

Table 4.1 The mean parasite burden per host (and the percentage of infective larvae) in the abdomens of mosquitoes at different stages of assimilating a blood-meal, 9 days post infection.

Expt.	No. mosq. dissected	Mean no. of larvae per mosquito (percentage of larvae found in the abdomen			
		Control	Blood-filled	Egg-filled	Egg-laid
1	79	10.3 (20.4)	18.2 (41.0)	18.8 (41.3)	17.1 (23.4)
2	105	6.1 (29.0)	-	5.9 (41.3)	5.8 (24.1)
3	153	3.8 (10.1)	3.4 (38.7)	3.7 (12.0)	5.4 (7.5)
4	108	3.9 (8.6)	5.0 (30.4)	5.4 (17.9)	5.3 (5.1)
5	92	4.1 (17.6)	10.0 (59.8)	7.7 (27.1)	12.7 (22.5)
6	90	9.7 (26.1)	7.0 (45.5)	5.2 (28.6)	15.4 (18.8)
7	81	9.3 (23.3)	2.5 (37.5)	7.4 (32.7)	-
$\bar{x}$		6.1 (16.0)	7.2 (42.0)	6.7 (20.0)	8.9 (17.0)

The mean worm burdens of all four groups of mosquitoes were similar (chi-squared = 4.2, $0.95 < p > 0.05$), indicating that few or no larvae were lost from the test mosquitoes during their second blood-meal. This is important since one test group was fed 8 days p.i. when a few infective larvae may be found in the proboscis (Figure 3.4).

The fuller the abdomen the greater was the percentage of infective larvae found there (i.e. Blood-filled > Egg-filled > Control= Egg-laid). The differences were all significant.

The mortality data for the six replicates was combined for each of the four groups of mosquitoes. The survival curves for each of the four groups is shown in Figure 4.1. All three test groups upto day 12 received the same treatment. Thus, the data for these three groups was combined for the statistical analysis upto day 12. The survival of mosquitoes following a uninfected blood-meal was significantly greater than that of mosquitoes feeding on a microfilaraemic cat (chi-squared = 21.8, $p < 0.001$). The instantaneous death rate of uninfected mosquitoes was shown to be constant throughout the duration of the experiment (Figures 4.2A and 4.2B).

Comparisons of survival within the pooled group of infected mosquitoes at 5 day intervals during the course of

Figure 4.1 Survival curves for four groups of mosquitoes receiving different treatments. i.e. A, uninfected mosquitoes; B, infected mosquitoes; C, infected mosquitoes given a blood-meal 12 days p.i.; D, infected mosquitoes given a blood-meal through a thin-layer of cotton 12 days post infection. (chi-squared = 100.9, $p << 0.01$) and 4-8 days p.i. (chi-squared = 81.4, $p << 0.01$).

Approximately 1800 mosquitoes per group

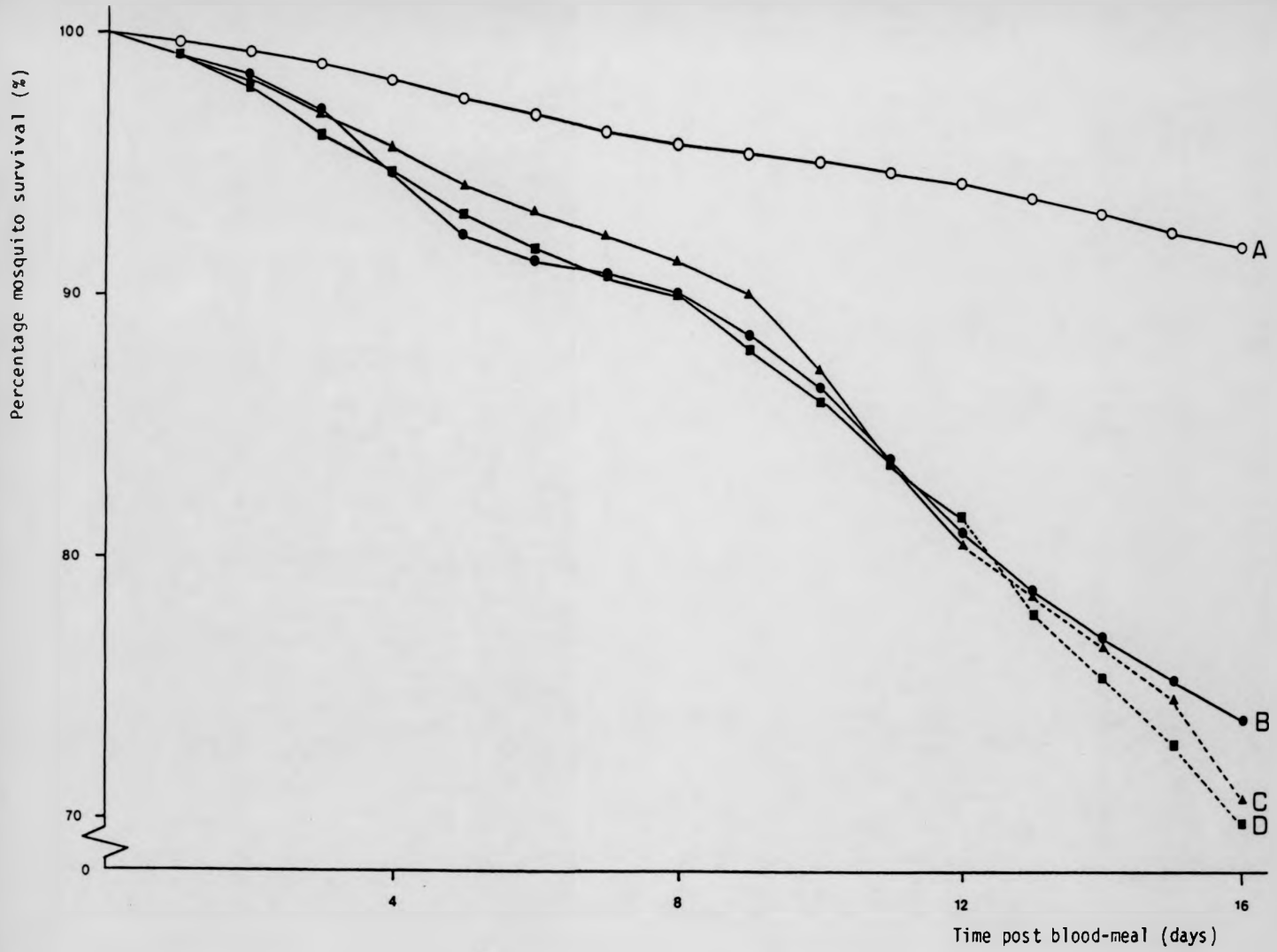
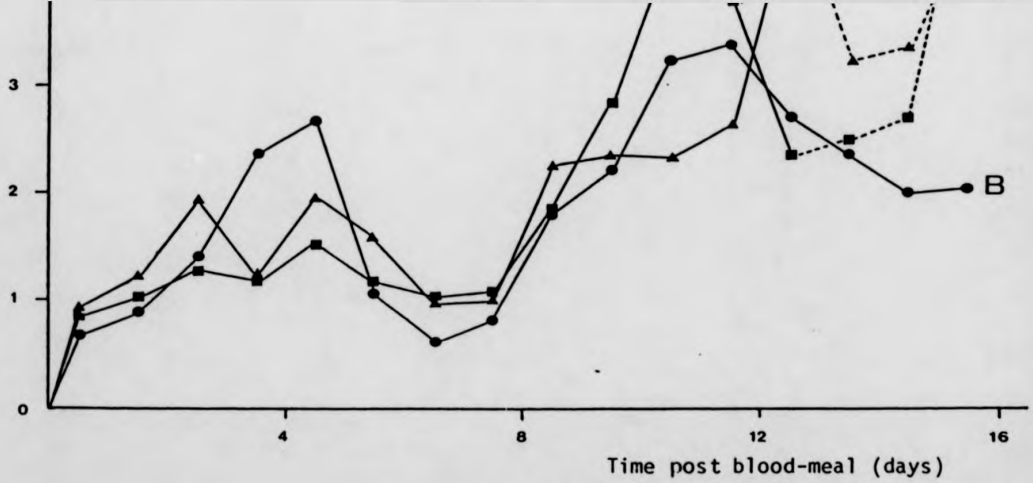

Percentage mosquito survival (%)
100
90
80
70
0
4
8
12
16
Time post blood-meal (days)
A
B
C
D

Figure 4.2A The per capita instantaneous death rate of uninfected mosquitoes (group A).

Figure 4.2B The per capita instantaneous death rate of infected mosquitoes (group B), infected mosquitoes given a blood-meal 12 days p.i. (group C) and infected mosquitoes given a blood-meal through a thin-layer of cotton 12 days post infection (group D).
Approximately 1800 mosquitoes per group


3
2
1
0
4
8
12
16
B
Time post blood-meal (days)

Per capita instantaneous death rate, $\mu_{(t)}$

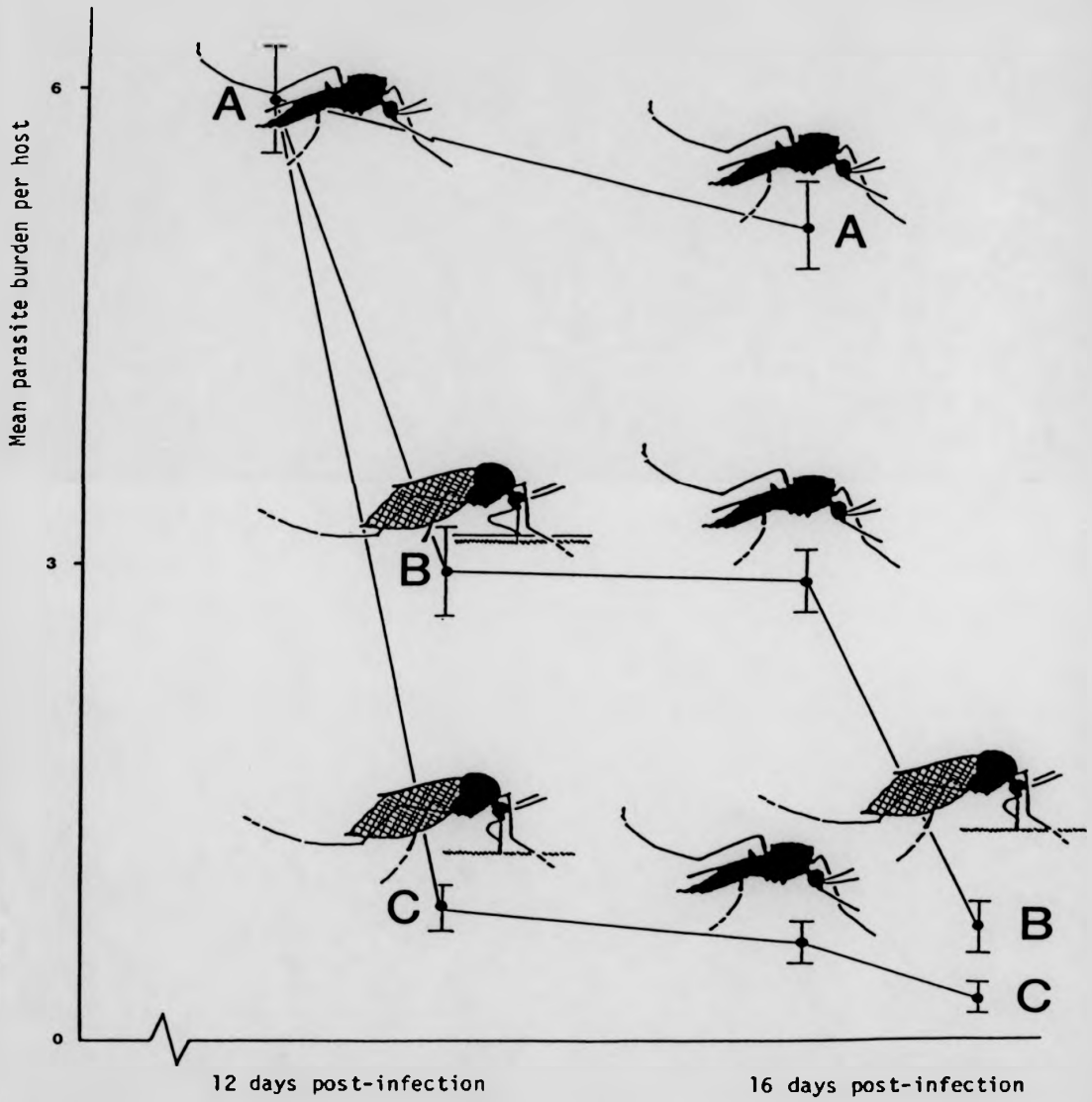
infection showed that there was no difference in mortality between 0-4 days p.i. and 4-8 days p.i. (chi-squared = 0.04, $0.95 < p > 0.05$). However, the instantaneous death rate did rise in at least one of the groups of infected mosquitoes (B) following a blood-meal. (Figure 4.2B).

The difference in mortality of infected mosquitoes between 8-12 days p.i. was significantly greater than the mortality between 0-4 days post infection. This difference is most striking in Figure 4.2B where there is an increased instantaneous death rate in all groups of infected mosquitoes, commencing 7 days post infection.

Following a blood-meal 12 days p.i. the survival of the two test cages of infected mosquitoes was significantly less than that of the control group of infected mosquitoes which did not have a secondary blood-meal (chi-squared = 21.8, $p << 0.01$). This may be seen as the marked increase in the instantaneous death rate after the secondary blood-meal in groups C and D (Figure 4.2B).

The decline in mean worm burdens in infective mosquitoes following successive blood-meals is shown in Figure 4.3. Significantly greater numbers of infective larvae were lost from mosquitoes feeding directly onto a jird than from mosquitoes fed on a jird through a thin-layer of cotton (85.1% c.f. 48.3%).

Figure 4.3 The effect of blood-feeding on the loss of larvae from infective mosquitoes. Where, A are infected mosquitoes; B, are infected mosquitoes which fed 12 days p.i. and C are infected mosquitoes which fed 12 days p.i. through a thin-layer of cotton. The bars represent the 95% confidence limits about the points.


Mean parasite burden per host
6
3
0
A
A
B
B
C
C
12 days post-infection
16 days post-infection

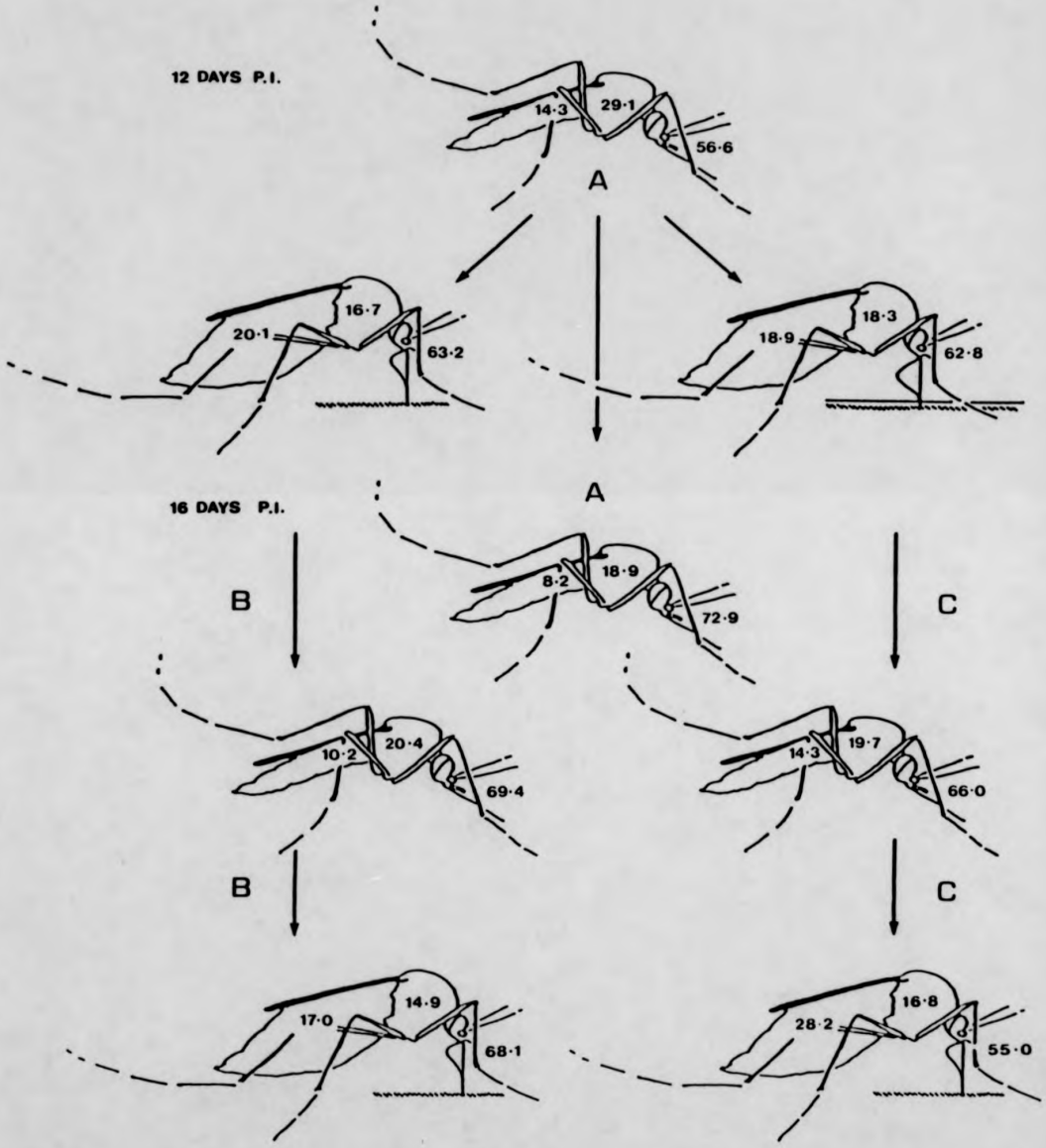
The decrease in mean worm burdens which occurs in all groups of infected mosquitoes between 12-16 days p.i. is not significant.

Following a second, non-infective blood meal (day 16 p.i.) both test groups of infective mosquitoes showed a significant decrease in mean worm burdens (Figure 4.3). This represented a loss of worms of 75.6% in the more heavily infected group of test mosquitoes and 56.2% in the less heavily infected group of mosquitoes.

Mosquitoes which failed to feed 12 days p.i. had significantly greater worm burdens than the control group of infected mosquitoes. i.e. $\bar{x}$ = 8.0 (jird only, n = 39, z = 2.4) and $\bar{x}$ = 9.0 (jird through cloth, n = 41, z = 2.3) c.f. $\bar{x}$ = 5.9 (control, n = 181).

The spatial distribution of infective larvae within the different groups of mosquitoes is shown in Figure 4.4. Following a blood-meal 12 days p.i. larvae from all parts of the mosquito move into the head and proboscis. Worms in the abdomen, however, were less likely to escape and formed a larger proportion of the residual, post feeding, infective larval population. Between the second and third blood-meal (i.e. 12-16 days p.i.) the tendency for infective larvae to accumulate in the head and proboscis continued. A blood-meal given to infective mosquitoes 16

Figure 4.4 Showing the percentage spatial distribution of infective larvae within mosquitoes 12 and 16 days post infection.


12 DAYS P.I.
14·3
29·1
56·6
A
16·7
20·1
63·2
18·3
18·9
62·8
16 DAYS P.I.
A
B
C
8·2
18·9
72·9
10·2
20·4
69·4
14·3
19·7
66·0
B
C
17·0
14·9
68·1
28·2
16·8
55·0

days p.i. resulted in a greater proportion of larvae being retained in the abdomen although larvae did not increase proportionately in the head and proboscis.

Mosquitoes with low numbers of larvae are not as efficient at transmitting infective larvae (56.2% loss) as mosquitoes with high worm burdens (75.6% loss, Figure 4.3). This may be offset by lower rates of mortality in those mosquitoes with low parasite burdens and the reduced feeding avidity of heavily infected individuals.

DISCUSSION

Francis (1919) first noticed that a large proportion of infective larvae can be found in the abdomens of mosquitoes. Since then many workers have suggested that infective larvae always migrate into the abdomen after moving out of the thorax (Yamada, 1927; O'Connor and Beatty, 1938; Newton and Pratt, 1945; Trpis, 1981). Gigliolo (1948) and Rosen (1955) even suggested that the passage of larvae into the abdomen was necessary for the larvae to reach full maturity. However, this has never been proven and more simply one should consider that the peregrinnations of the infective larvae do not immediately take them to the head and proboscis.

Limiting the space available for larval movement in the abdomen when the worms commence migration clearly leads to

a greater number of larvae accumulating there. This evidence, therefore, supports the hypothesis that larvae tend to remain in the proboscis because of the restricted space for movement in this appendage.

The ability to remain in the abdomen of a mosquito could be advantageous to the worm. For an infective larvae, the proboscis of a mosquito is a vulnerable place, since larvae may be lost from the proboscis due to any slight disturbance or may occur spontaneously. It would, therefore, be advantageous to the nematode to arrive only at the proboscis when the mosquito has laid her eggs and is seeking another blood-meal. Such a mechanism appears to operate for larvae which are delayed in the abdomen due to the presence of a blood-meal. Not until the blood-meal is assimilated and the eggs laid does the percentage of larvae in the abdomen drop to a level similar to that found in infected mosquitoes which have not had a recent blood-meal.

Low (1900) and Noë (1901) first recognised that filarial larvae could be harmful to mosquitoes. Since then it has been shown that during the course of an infection there are two periods at which the mortality can be elevated. The first may occur within a few days of an infective blood-meal and is associated with the migration of microfilariae out of the gut (Roubaud, 1936; Kershaw et al., 1953; Lavoipierre, 1958; Nelson, 1964; Ramachandran, 1966;

Townson, 1970; amongst others). This effect was demonstrated in the present study by the marked increase in the per capita instantaneous death rate of mortality of the mosquitoes (Figure 4.2). The second rise in mosquito mortality occurs when the infective larvae commence migration (Kershaw et al., 1953; Rosen, 1955; Wharton, 1957; Nelson, 1964; Townson, 1970; Beckett, 1971) This effect was clearly demonstrated in this study beginning 7 days post infection. It is likely that more active filariae, such as B. pahangi, cannot be tolerated at the same levels as less active species, such as W. bancrofti.

Jordan (1959) suggested that the continual loss of infective larvae through regular blood-feeding, as occurs in nature, would prevent the accumulation of large numbers of larvae. He argued that this would, therefore, reduce the lethal effects of filarial parasitism associated with high density infections. Whilst it is possible that the meanderings of the larvae within the mosquito may result in some neurological disturbances, according to Beckett (1971, 1973), the major damage, which is irreversible, occurs in the flight muscles and is caused by the emerging larvae. Therefore, a reduction in parasite burden after the flight muscles have been damaged would be of little consequence.

A further rise in mortality was demonstrated after infective mosquitoes had fed. This may be directly due to damage caused by the emerging larvae or, indirectly, due to

the loss of haemolymph from the mosquito (Chapter 6) or the increased chance of bacterial infection.

Townson (1971) considered that the poor survival of Ae. aegypti infected with B. pahangi reflected a host-parasite relationship in which the parasite was poorly adapted to its vector. Although the BLACK EYE strain has had one gene selected for susceptibility, the f^{m} gene, the remainder of the mosquitoes gene complement may not be so suitably adapted. In other words this parasite-host relationship carries the hallmark of a recent evolutionary marriage, poorly adapted participants. However, it should be appreciated that massive infections, as are found under laboratory conditions, are rarely seen in field populations of infected mosquitoes, perhaps as a result of parasite-induced host mortality.

It is an ecological truism that in the wild few organisms die of old age. Most die due to predation, disease and accidents (Krebs, 1972). For example, enormous daily mortalities of 24% for C. quinquefasciatus and Anopheles peditaeniatus have been recorded (Laurence, 1963). Because of this both age-dependent effects and parasite-induced mortality went undetected in the field. However, mosquito mortality has recently been shown to be age-dependent in the field (Clements and Paterson, 1981) and workers in holoendemic areas have found that host mortality is

parasite-dependent (Samarawickrema and Laurence, 1978, Bryan pers. comm.).

Parasitism clearly has a direct effect on host survival but indirect effects, such as a greater susceptibility to predation and a reduced competitive fitness may also be important. Such pressures, irrespective of their intensity, will inevitably lead to a more equitable relationship developing between parasite and vector. That is, provided it is not to the parasites advantage to damage it's host (e.g. in terms of increased transmission potential) or that the genetic control of pathenogenicity is not linked to transmission factor(s) in the parasite.

Many authors have shown that during a blood-meal infective larvae which escape from the proboscis are recruited from all parts of a mosquitoes body (Wharton, 1957; Jordan, 1959; Lavoipierre and Ho, 1966; Ho and Lavoipierre, 1975), although worms in the proboscis are more likely to infect the host (de Meillon et al., 1965). However, contrary to their previous findings, Kadri and Lavoipierre (1978), stated that it is only those larvae in the proboscis which are lost during feeding. This was based on their inability to detect movement of larvae in the head, thorax and abdomen during feeding. They also deduced from histological sections that the labium becomes virtually closed off from the cephalic haemocoele as the labium is bent back. In my study only 36% of the larvae were found

in the labium before feeding, yet 85% of the larvae were lost from the mosquito during feeding. This is unequivocal evidence that larvae from all areas of the mosquitoes body are capable of infecting a host. However, it will be argued in Chapter 6 that at least one larvae must be in the proboscis when the mosquito blood-feeds before larval emergence and recruitment occurs.

It is generally accepted that more infective larvae of Brugia spp. are lost during feeding than are infective larvae of W. bancrofti because of their greater activity (Laurence, 1979). Thus losses of 50-96% of B. pahangi infective larvae from Aedes spp. occur (Ho and Lavoipierre, 1975; Kadri and Lavoipierre, 1978); although Zielke found lower losses of 36-46% for Brugia spp. infective larvae in Ae. aegypti. Losses of W. bancrofti infective larvae are generally lower, estimates varying between 15-41% (de Meillon et al., 1965, Zielke, 1976, 1979), although Jordan (1959) found that in C. quinquefasciatus the majority of W. bancrofti larvae were lost during feeding.

The tendency of mosquitoes with high density infections not to feed demonstrates a further debilitating effect of filariasis on mosquitoes. Other manifestations include a reduction in fecundity (Javadian and Macdonald, 1974; Gaaboub, 1976; Christensen, 1981; Cheke, Garms and Kerner, 1983), impaired flight activity (Townson, 1970; Hussain and

Kershaw, 1971; Kan and Ho, 1973; Paige and Craig, 1975; Hockmeyers et al., 1975; Wijers and Kulu, 1977; amongst others) and an increase in mortality.

De Meillon et al. (1965) found that immediately after a blood-meal the proportion of W. bancrofti in the abdomen of C. quinquefasciatus drops prior to rising back to pre-blood meal levels. This was interpreted as being due to pressure from the blood-meal forcing the larvae forwards. In this study it was not possible to autopsy all mosquitoes immediately after a blood meal so it was not known whether the proportion of larvae in the abdomen fell before rising higher than pre-feeding levels. However, if a similar situation exists within this parasite-host system it suggests that after feeding a proportion of the larval population are attracted to the blood-meal, possibly due to a thermopositive response. However, this is not a strong effect as the mean number of larvae found in the abdomen following a blood-meal does not increase. This effect, more simply, could be due to larvae furthest from the proboscis standing the least chance of escaping from a feeding mosquito.

The failure of larvae in mosquitoes with low residual parasite burdens to be lost with the same efficiency as that which occurs in high parasite burdens is probably due to a combination of factors. Infective larvae represent a heterogenous parasite poulation as may be demonstrated by

the variability seen in larval behaviour (Croll, 1971, 1972, 1973; Croll and Blair, 1973). Intuitively one would expect that some larvae will be less suited to migration within the mosquito than others. Thus the population of worms which remains in mosquitoes after a blood meal may consist of a high proportion of "born-failures".

However, if emergence is triggered only by the presence of at least one infective larvae in the proboscis another possibility exists. In mosquitoes with low worm burdens there is a greater proportion of mosquitoes which do not have larvae in the proboscis when compared to mosquitoes with high worm burdens. As a consequence fewer individuals with low mean burdens lose larvae during feeding.

CHAPTER 5

THE EFFECT OF *B. PAHANGI* ON THE SPONTANEOUS FLIGHT ACTIVITY OF *AE. AEGYPTI*

SUMMARY:

The circadian flight activity of infected and uninfected virgin females was recorded for twelve consecutive days using an acoustic actograph technique. The flight activity of uninfected mosquitoes rose to a maximum 2-3 days after a blood meal and then decreased to a plateau level for the remainder of the experiment. The flight activity of heavily infected mosquitoes (>12 larvae per mosquito) was temporarily depressed during the first few days after ingesting microfilariae. This was followed by a permanent suppression of flight activity in heavily infected mosquitoes, commencing 7 days post infection, and was associated with the migration of infective larvae out of the flight muscles.

INTRODUCTION

It was shown in the previous chapter that filarial parasitism could have a direct effect on mosquito survival. It was suggested that more subtle consequences of infection might increase the susceptibility of infected mosquitoes to predation and reduced their competitive fitness. Both effects would be of some importance as they act as

stabilising the dynamics of host-parasite associations (Anderson, 1978, 1981). The ability of the host to fly is, of course, fundamental to both these considerations.

Sharp (1928) appears to be the first worker to mention how heavily infected hosts were unable to fly. Since then many other workers have observed this effect including Townson (1970), Husain and Kershaw (1971) and Paige and Craig (1975). Previous attempts to quantify the degree of flight impairement have relied on methods which require tethered insects to be flown continuously until exhausted (Townson, 1970; Hockmeyer et al., 1975). However, such conditions must be recognized as being extremely artificial in relation to the mosquito's behaviour under field conditions. Therefore, an accurate assessment of any deleterious consequences of filarial parasitism on the flight capability of the vector would be of some importance.

Using an acoustic actograph technique, Jones, Hill and Hope (1967) developed an elegant method for continuously measuring the circadian rhythm of spontaneous flight activity in individual mosquitoes. The principle advantage of this instrument is that it continuously monitors the activity of "unrestrained" mosquitoes over long periods. The actograph has been used to investigate the nature of mosquito flight activity in relation to

different light regimes, starvation, mating and the gonotrophic cycle (Jones et al., 1967; Jones and Gubbins, 1978, 1979; Jones, 1981) but, until now, has not been used to study the effects of parasites on behaviour.

MATERIALS AND METHODS

The procedure for rearing mosquitoes was similar to that described earlier. Batches of 200 eggs were hatched in plastic bowls full of water and reared at a temperature of 30 ± 1 °C. Two cages of 3 day old mosquitoes, each containing approximately 100 adult females, were fed on either an uninfected cat (control mosquitoes) or a infected cat (test mosquitoes). Only virgin females were used in these experiments since Jones (1981) has shown that their spontaneous flight activity is considerably greater than that of inseminated females so that any change in flight activity would be easier to detect.

The procedure used for measuring spontaneous flight activity was similar to that described by Jones et al. (1967). Individual mosquitoes were placed in a flight chamber as shown in Plate 5.1. The glass chamber was 10 cm high with a bottom interior diameter of 6 cm. The base was constructed from a circular disc of filter paper (Whatman No. 1) and sealed with P.V.C. film ("Cling Film", C.E. Payne and Sons Ltd, London) through which the flight sounds could readily be transmitted to the underlying

Plate 5.1 A flight chamber.

microphone. A 1.5 ml plastic Eppendorf tube (Sarstedt, Beaumont Leys, Leicester) containing a 2% glucose solution, with a cotton wool "wick", provided the food supply. The relative humidity in the chamber was maintained above 80% by filling a 1 cm high by 2 cm diameter plastic cap (Regina Industries, Stoke) with water. Humidity was checked on several occasions using an Evaporimeter (Servo Med Evaporimeter, EB1). The mosquito was prevented from accidental drowning in the water by covering the top of the cap with sand-fly netting, secured with a small elastic band. Both the sugar solution and water were replenished 7 days after the blood meal.

Twenty eight similarly prepared flight-chambers were placed at random above microphones in one of two boxes as shown in Plate 5.2, i.e. 9-10 control mosquitoes and 18-19 test mosquitoes. In addition 5 control mosquitoes and 10 test mosquitoes were placed in flight-chambers and reserved. Any mosquitoes which died during the course of the experiment were replaced by a mosquito in a reserve flight chamber. The chambers were sealed with "cling film" to keep the humidity within constant and to allow the sound made by a flying mosquito to be transmitted to each microphone. Each microphone had a maximum response to the frequency of the sound produced by the wing-beat of a flying mosquito (i.e. 400-1500 cycles per second). The sound-insulated boxes had an internal volume of 67 cm X 67 cm X 67 cm.

Plate 5.2 The arrangement of flight chambers, inserted over microphones (see channel 21), in one of the sound- and light-insulated boxes.

They were constructed from 2 cm blockboard mounted on a 4.5 cm x 4.5 cm softwood timber frame and lined with 2.5 cm thick layers of foamsponge on all surfaces except the ceiling of the box. Illumination was provided from above by a fluorescent d.c. light-strip (Maximum light intensity approximately 50 Lux). The light-source was diffused through a layer of grease-proof paper (Safeway Food Stores Ltd) to eliminate shadows and clear perspex sheeting which separated the light-source from the flight-chambers. Both boxes were housed in a constant temperature room at 27 $\pm$ 0.5 ^{o}C.

The light-source for each box operated on a daily 12 hour light: 12 hour dark regime (i.e. light 08.00-20.00 hrs). "Dawn" and "dusk" periods were produced by two tungsten bulbs (15W, 250V). At 07.00 hrs when the bulbs were switched on, the light intensity increased from 0 to 2.5 lux. Thereafter the light intensity increased progressively from 2.5 lux to 50 lux during the next hour (i.e. 07.00 - 08.00 hrs). The gradual light off-on or on-off was controlled by an electronic device, as described by Jones and Gubbins (1979). At 08.00 hrs the tungsten bulbs were turned off and a fluorescent light strip (Ilford Camping Light, 13W, 12V, D.C.) was switched on producing a stable light intensity of 48 lux. At 19.00 hrs, the fluorescent-light was turned off and the light intensity of both tungsten bulbs gradually decreased to total darkness

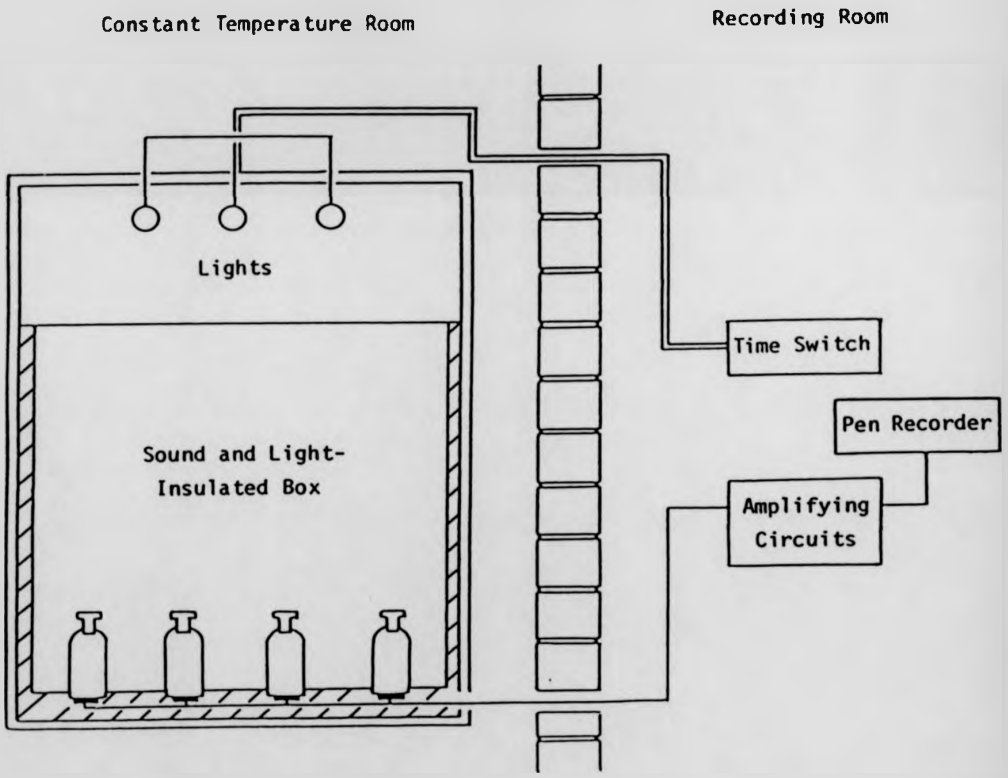
at 20.00 hrs.

The sound produced by a flying mosquito was picked up by a microphone under each chamber which was then passed through two amplifiers. The second amplifier also filtered out unwanted frequencies (Jones et al., 1967). The resultant signals caused deflections on a pen-recorder (Miniscript Z for D.C. low voltages, E.H.P. Technical Services Ltd, London) which inscribed lines on the paper travelling under the pen. The paper moved at a speed of 2 cm per hour. Each deflection represented a mosquito flying for all or a fraction of a minute. Therefore, the maximum score possible for one individual mosquito during a period of one hour was 60. The arrangement of the apparatus in the two rooms is shown in Figure 5.1.

Mosquitoes which died during the trial were all autopsied, as were all mosquitoes surviving until the 13th day following the initial blood-meal. The number and distribution of infective larvae within each mosquito was determined. The entire experiment was performed on two separate occasions.

The analysis of the actograph data was as follows. The total number of deflections, inscribed by the pen-recorders, for each individual mosquito, was counted for each hour for the duration of each 12 day trial. The mean hourly scores were then determined for four groups of

Figure 5.1 A schematic representation of the Actograph apparatus.



mosquitoes i.e.

1. Control; mosquitoes fed on an uninfected cat.
2. Uninfected test; mosquitoes fed on a microfilaraemic cat but which had no infective larvae.
3. 1-12 test; mosquitoes with 1-12 infective larvae.
4. > 12 test; mosquitoes with more than 12 infective larvae.

The recordings made by mosquitoes the day before death and the day on which death occured were not included in the calculations described above.

The Mann-Whitney U-test was used to compare the total daily flight activity for each mosquito in the control group with the total daily flight activity for mosquitoes in one of the test groups, for each day post blood-meal. In these, and all later statistical tests significance was assumed at the 5% level (i.e. $p < 0.05$).

Only virgin mosquitoes were used in this study, a condition which is rarely found in the wild. It was, therefore, of interest to determine whether the susceptibilty of virgin females to filarial infections differs from that of inseminated females. Two cages, one cage containing 80 female mosquitoes and 40 male adult mosquitoes (inseminated females) and the other cage containing 80 female mosquitoes only (virgin females) were fed 5-7 days p.i. on an infected

cat. The first 35 mosquitoes were taken at random from both cages on day 10 post infection. These mosquitoes were autopsied and their worm burdens determined. This experiment was performed on six separate occasions.

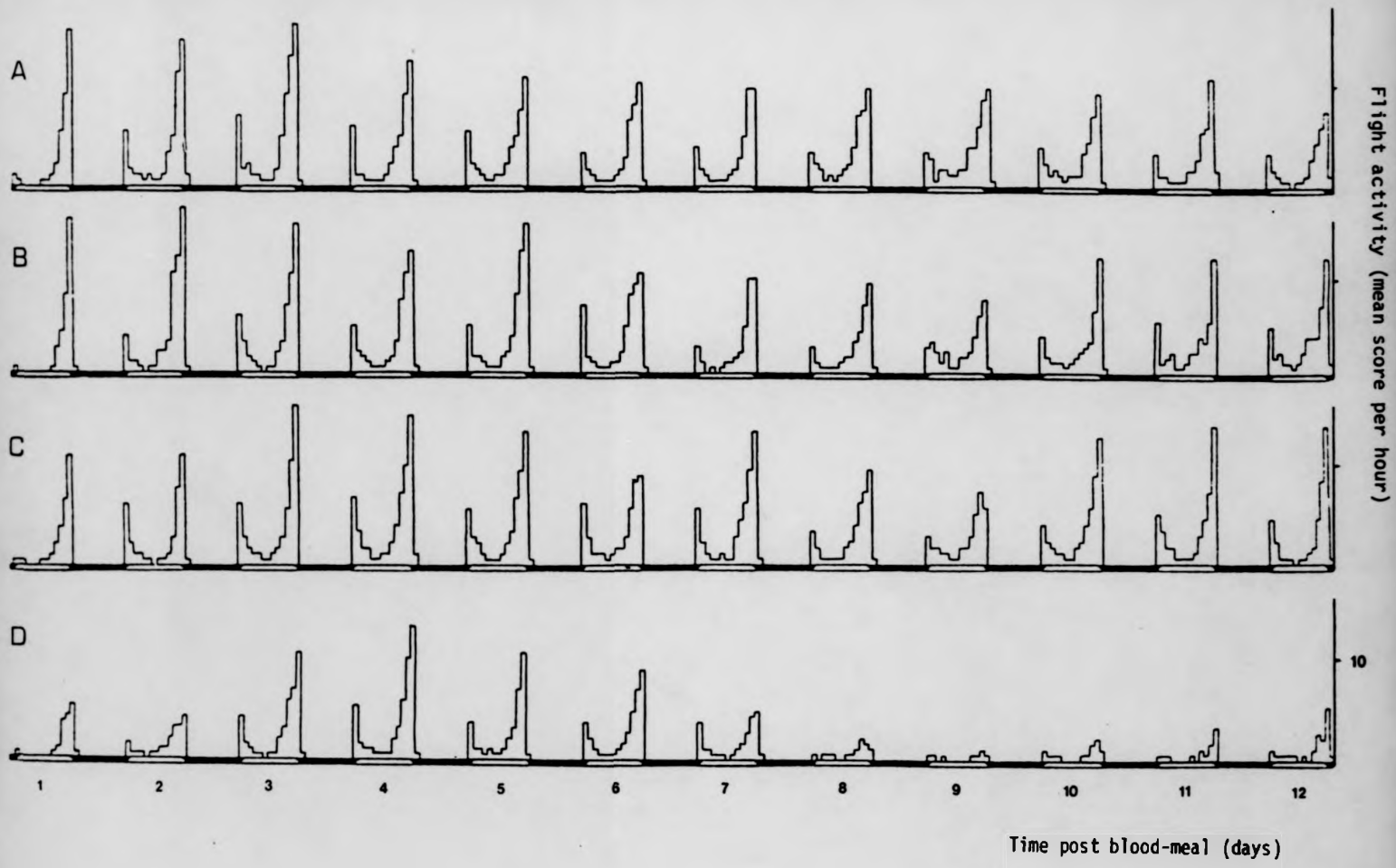
Comparisons between the individual worm burdens of virgin and inseminated female mosquitoes were made using the Mann-Whitney U-test.

RESULTS

The characteristic bimodal pattern of daily, diurnal flight-activity was similar for all mosquitoes (Figure 5.2). Peak activity occurred at the end of each light-phase, at "dusk". There was also a smaller peak of activity at "dawn" which increased in importance, relative to the "dusk" period, as the total daily activity decreased.

Virgin females were relatively inactive immediately following a blood-meal, but their activity increased to a maximum level during the following few days. Thereafter, the level of activity declined slightly but was maintained at a plateau level for the duration of the experiment. The activity of mosquitoes fed only on an uninfected cat did not differ significantly from the activity of mosquitoes which fed an infected blood-meal and which had no larvae. However, the activity of mosquitoes with more than 12

Figure 5.2 The mean flight activity of mosquitoes following a blood-meal. Where A are mosquitoes fed on an uninfected cat, B are mosquitoes fed on a microfilaraemic cat which had no infective larvae, C are mosquitoes with 1-12 larvae and D are mosquitoes with more than 12 larvae (see appendix 5.1 for values of n).


A
B
C
D
Flight activity (mean score per hour)
10
1
2
3
4
5
6
7
8
9
10
11
12
Time post blood-meal (days)

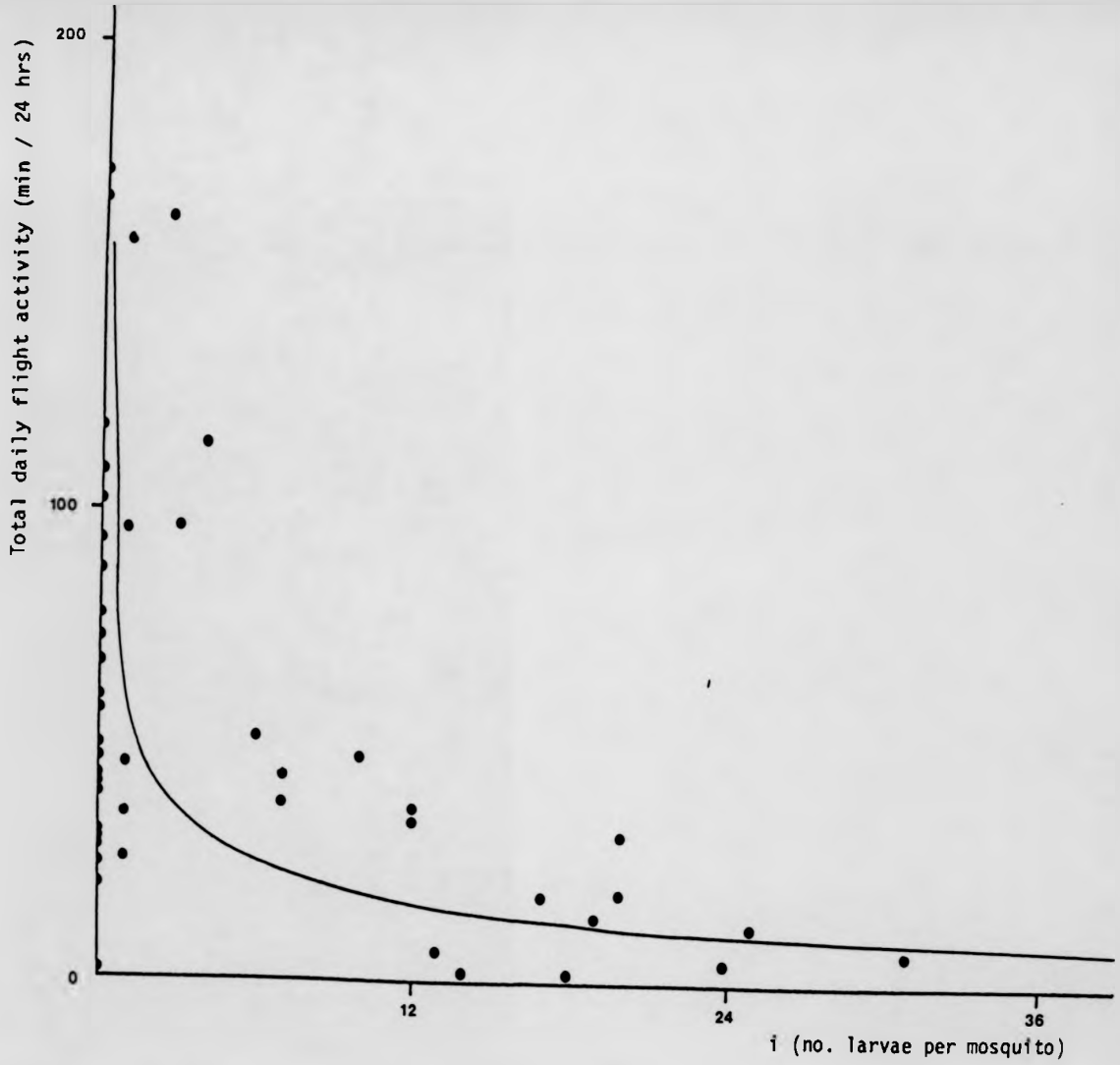
larvae was significantly depressed on days 1 and 2 p.i. and then again later between days 8-11 post infection.

The inability to demonstrate a significant difference in flight activity on day 12 p.i. between the control group of mosquitoes and the most heavily infected mosquitoes was an artefact. No recovery in flight-activity was seen in any of the individual flight-activity patterns of heavily infected mosquitoes. Parasite-induced host mortality is density-dependent and, therefore, mosquitoes with the heaviest infections suffer preferential mortality. Consequently, the mosquitoes with heavy infections which died during the trial were replaced by fitter individuals. This had the effect of raising the mean level of activity, an effect which was compounded by the relatively small sample size.

The density-dependent effect of parasite burden on spontaneous flight activity which occurs after day 7 p.i. (in this instance day 9 p.i.) is shown in Figure 5.3. The general trend shows how flight activity decreases as the worm burden in each mosquito increases.

There was no significant difference between the worm burdens of virgin mosquitoes ($\bar{x}$ = 8.1) compared with inseminated mosquitoes ($\bar{x}$ = 9.1)(z = 0.917).

Figure 5.3 The relationship between the total flight activity of mosquitoes 9 days after a blood meal and worm burden. The data was subjected to a general curve-fitting computer program where the line of best fit occurs with ln y on ln x (where $r = -0.6024$, $P < 0.001$, 51 d.f.).


Total daily flight activity (min / 24 hrs)
200
100
0
12
24
36
i (no. larvae per mosquito)

DISCUSSION

The intrinsic daily pattern of spontaneous flight activity in Ae. aegypti is diurnal and bimodal in character. This pattern of activity has also been demonstrated for Ae. aegypti by others working with an acoustic actograph (Taylor and Jones, 1968; Jones, 1981). It is not simply a product of laboratory conditions as this pattern has also been shown under field conditions for a number of different behaviours. Blood-feeding (Brengues, Subra and Bouchite, 1970; Trpis, McClelland, Gillett, Teesdale and Rao, 1973; Nelson, Self, Pant and Usman, 1978; Tinker and Olano, 1981) and sugar-feeding (Gillett, Haddow and Corbet, 1962) both show a similar activity pattern. Indeed it is a characteristic of animal circadian rhythms that many different forms of behaviour are cophasic (Brady, 1975, 1981). The inference is, these cophasic rhythms are controlled from one neural centre, the excitability of which oscillates in a predictable daily cycle.

The overall level of activity in uninfected mosquitoes is lower than that previously reported, illustrating the variability which can occur between different strains of the same species of mosquito.

There is a wide variation in flight activity within uninfected mosquitoes. It has been shown that the flying

ability of a laboratory strain of mosquito is not as good as that of a field strain, of the same species (Clarke, Rowley and Asman; 1983). Beckett and Townson (1982) showed that in laboratory strains of Ae. aegypti the statistical distribution of the number of flight-muscle bundles per individual was far wider than that found with wild strains of the same insect. From this it was argued that in the field there was selection for flies with an optimal number of flight-muscle bundles. Under cloistered laboratory conditions this selection pressure is of minimal importance or fails to operate altogether. It is possible that the number of flight-muscle bundles may directly affect the ability of a mosquito to fly. Consequently, the wide variation in flight activity seen in laboratory mosquitoes may be a direct result of the large variability in the number of flight muscle bundles.

The greatest daily flight activity occurred on the second or third day following the blood-meal in each group of mosquitoes. This is probably due to a combination of two effects. Firstly, the heightened level of activity associated with mosquitoes of this age (Rowley and Graham, 1968; Taylor and Jones, 1969) and secondly an increase in activity during "dusk" associated with oviposition behaviour (Haddow and Gillett, 1957; although it should be appreciated that virgin females retain most of their eggs indefinitely). The fall in activity to a plateau level,

which followed later, has not been previously shown and demonstrates that aging has little effect on flight activity, during this period of a laboratory mosquito's life.

The period at which spontaneous flight activity is depressed in heavily infected mosquitoes coincides with the periods of greatest mortality (Fig. 4.1). Clearly mosquitoes are under great stress at these times. The initial depression in flight activity, which follows an infective blood-meal, is associated with the migration of microfilariae from the mid-gut, and invasion of the flight-muscles. However, this effect is only temporary and by day 3 p.i. their activity returns to normal. One likely explanation for this phenomena could be that when microfilariae tear their way out of the mid-gut acidic digestive juices percolate from the mid-gut into the haemocoele. A resultant drop in haemocoele pH would reduce the contractile efficiency of the flight muscles. However, as the acids are excreted, the pH balance is redressed and flight activity returns to normal. This effect could also be due to damage to the gut wall and interference with absorption, entry of larvae into the muscle fibres and the secretion of an inhibitor of muscle contraction (e.g. acetylcholinesterase, as occurs with some gut nematodes, Ogilvie, Rothwell, Bremner, Schnitzerling, Nolar and Keith, 1973).

This resumption of activity is contrary to the findings of Hockmeyer et al. (1975), who working with a flight-mill, showed that flight activity in infected mosquitoes was depressed 3, 6 and 9 days post infection. However, flight-mills are a crude way of measuring the intrinsic flight-activity of mosquitoes. Firstly, individuals are flown to exhaustion and are, therefore, only used once. Secondly, a continuous recording of an individuals daily variation in activity over many days is impossible. Thirdly, given the chance, mosquitoes will not fly in one continuous burst until exhausted. Fourthly, as many as 80% of a sample of mosquitoes will not fly on a flight-mill (Clarke and Rowley, 1983), so flight activity will only be measured in a select group of mosquitoes. Lastly, what this technique may be principally measuring is the availabilty of energy for flight activity. Both glycogen and the number of mitochondria have been shown to be reduced in the flight muscles of infected mosquitoes (Kan and Ho, 1973; Lehane and Laurence, 1977).

Other pathological changes seen in parasitised flight muscles have included a disruption of muscle fibres, an increase in the number and size of nuclei surrounding the larvae and a disorganization of interfibrillar mitochrondria (Townson, 1970; Beckett and Boothroyd, 1970; Beckett, 1971b, 1973; Kan and Ho, 1973). Beckett (1971b) also maintained that damage to non-parasitised fibres may

occur, the number affected and the intensity of damage increasing during larval development (Beckett, 1973).

The permanent crash in activity which occurs 7-8 days p.i. is associated with the migration of infective larvae out of the flight-muscles (Figure 3.3). Indeed, Beckett (1973) has shown that the major damage to the flight-muscles occurs at this time and she also attributed this effect to the egress of larvae from the thorax.

The larvae of D. immitis develop in the malpighian tubules, in the abdomen, and not in the flight-muscles. It would be of interest to see whether in this parasite-host relationship a "crash" in flight-activity is associated with the migration of infective larvae. If this was found to be the case it would indicate that some mechanism other than just a physical rupture of the flight-muscles was responsible for decreased flight activity. It is possible that the presence of large and active migrating larvae within a mosquito would result in neurological disturbances, particularly when the larvae are in the head of the insect.

Clearly, when infective larvae commence migration, flight activity remains permanently depressed in heavily infected mosquitoes. This supports Beckett (1971b, 1973) who found that muscle damage at this time was irreversible in Ae. aegypti infected with B. pahangi. However, damage to

the flight muscles of a larger vector species, Ae. togoi, infected with B. pahangi, appeared to be reversible (Kan and Ho, 1973). It would, therefore, be of interest to determine whether a similar study on the effect of parasitism on the spontaneous flight activity of Ae. togoi would substantiate their claim.

Flight-activity is dependent on the worm burden of an individual mosquito, the greater the worm burden the lower the flight-activity. The grouping of mosquitoes with more than 12 larvae may have given the impression that there exists a level of parasitaemia above which the flight activity of an individual mosquito slumps and below which the mosquito is unaffected. More simply, the ability of a mosquito to fly is some function of its parasite burden. As a cautionary note the reader should not attach too much emphasis to the regression line as its shape is greatly influenced by the large variation in flight activity found in mosquitoes with no larvae and the low activity in highly infected mosquitoes. The analysis places less emphasis on the relatively few points found for mosquitoes with few larvae.

From the results it is clear that there is no difference in filarial susceptibilty between virgin and inseminated females. This confirms earlier work, where the development of filarial larvae have been shown to be independent of

host hormones (Yoeli et al., 1962; Gwadz and Spielman, 1974).

A reduced capacity to fly, particularly in the wild, is obviously of great importance. It results in a reduced ability to evade predation and to obtain resources when competing with other individuals. However, this density-dependent effect will have a stabilising influence on host-parasite population dynamics (Anderson, 1978, 1981). Furthermore, the poor flying ability of heavily infected insects may partly explain why such individuals are rarely found in field situations.

An interesting finding from this study was the erratic flight-activity of dying mosquitoes. In these instances flight-activity could even occur in total darkness and was often intense. However, it is unlikely that the increased activity would be coordinated sufficiently to enable an infective mosquito to infect a host. It might simply represent the uncoordinated "death throws" of the insect. A coordinated increase in activity could be advantageous to the mosquito if it represented a last attempt by the insect to oviposit and, therefore, to pass its genes on to future generations. However, if the increased activity represented an attempt to feed it would also allow an infective larva a greater chance of infecting a host, but it would be of no benefit to the dying insect.

As the overall level of flight activity decreases proportionately more of the activity is performed at "dawn" with less being performed at "dusk". This effect has also been observed in tsetse flies (Brady, 1975; Brady and Crump, 1978), which adopt a similar duirnal, bimodal pattern of activity. However, the mechanism behind this phenomena seems obscure. The "dusk" peak in flight-activity in Ae. aegypti is the only true circadian rhythm as only this peak of activity is manifest when the insect "free-runs" when in total darkness (Jones, 1981). The "dawn" peak in flight activity may simply be a "startle response" to the light coming on. Imagine two neural control systems; one which acts centrally only on circadian-rhythms and another which acts peripheraly on fright-flight responses. It would be more advantageous to a stressed insect if its short-term fright-flight responses (which act essentially as escape mechanisms) remain less affected than the less essential level of activity associated with day to day living. i.e. safety first, food and sex later. On these grounds it is easier to understand why the activity at dawn is less depressed. In tsetse flies, however, both peaks in flight-activity are tru ly circadian (Brady, 1975).

CHAPTER 6

OBSERVATIONS ON THE TRANSMISSION OF THE INFECTIVE LARVAE OF *B. PAHANGI* FROM BLOOD-FEEDING *AE. AEGYPTI*.

SUMMARY

A novel photomicrographic system was used to study the feeding behaviour of filarial-infected and uninfected mosquitoes. It was found that infected mosquitoes often exhibited aberrant behaviour when feeding. This was chiefly manifest by the difficulty exhibited when penetrating the skin surface of a host. Infective larvae typically escape from the tip of one or both mosquito labella , during blood-feeding. However, large numbers of larvae can result in the emergence of worms from a longitudinal split in the labial gutter. A mechanism for the emergence of larvae is proposed, based on these observations together with those made of larvae in a simple *in vivo* experiment. A number of simple *in vitro* experiments demonstrated that 1) infective larvae on the skin surface of the host probably use the surface-film of the enveloping droplet of haemolymph to gain purchase and penetrate their definitive host and 2) both positive thermotaxic and chemotaxic behaviours were shown by infective larvae, both of which were considered to improve parasite transmission.

INTRODUCTION

The transmission of an infective filarial larva, from a mosquito to it's definitive host, is one of the most vulnerable stages in the life-cycle of the worm. Infective larvae are incapable of penetrating intact skin and gain access to the host through the puncture wound made by the feeding mosquito (Bahr, 1912; Yokogawa, 1939; Menon and Ramamuti, 1941; Ewert, 1967). The filarial larvae live essentially an aquatic life and are especially vulnerable to dessication when exposed on the skin surface of a definitive host. Although never determined by direct observation, this may be illustrated by the large proportion of larvae which fail to penetrate the host (Ewert and Ho, 1967; Ho and Ewert, 1967; Zielke, 1973, 1976). Clearly a fuller appreciation of the processes involved during the escape of infective larvae from the mosquito is of importance.

Although the movement of infective larvae within the mosquito and their escape and subsequent invasion of the definitive host has been studied in the past, little is known about the behaviour of the larvae at this time. Therefore, the primary aim of this chapter was to describe the transmission process from a "worms'-eye" view.

Much of the work on the behaviour of filarial larvae has been inadequately described or not performed under strictly

controlled conditions or both. Confusion has also arisen because of an inconsistent adherence to the nomenclature presently used for describing orientation responses (reviewed by Croll, 1973). Thus, much of the early work refers, incorrectly, to larval movement as tropisms. Tropism is a term used to describe growth movements of plants and the bending movements of sessile animals and is, therefore, inappropriate when discussing larval movement. Much of the work on behaviour of filariae has dealt solely with the infective larvae of W. bancrofti. Whilst the emergence of infective larvae of different species of filarial worms from mosquitoes may require similar behavioural responses, it would be wrong to believe that the behaviour of all species of filarial larvae will be identical. Indeed, it should be appreciated that the behaviour of individuals within populations of a single species will be heterogenous.

Detailed accounts of the normal feeding behaviour of mosquitoes have been produced in the past by a number of workers including Gordon and Lumsden (1939), Robinson (1939), Griffiths and Gordon (1952), Christophers (1960) and Jones (1978). However, the effect of filarial parasitism on mosquito feeding behaviour has, to date, been neglected, a situation this study, in part, aims to rectify.

MATERIALS AND METHODS

The procedure for observing and photographing the feeding behaviour of infective and non-infective mosquitoes was as follows.

A photomicrographic optical system (Plate 6.1) was designed to produce an erect, unreversed image of the subject in the eye-piece of a 35 mm photographic camera with a maximum magnification of X 12. This design was based on the body of a Biolam student microscope stand. A right-angled prism, immediately below a X 3 objective lense (Nikon Ltd., NA 0.08), allowed the subject to be viewed in profile, not from directly above, as would occur with a more conventional assembly. The course focus of the microscope thus became a quick means of changing elevation.

A Beck tube-length corrector (an achromatic lens combination of variable power) was fitted above the objective. This provided both quick manual focusing and a range of magnification (and therefore working distances. e.g. at X 6.5 the working distance from the prism face is 30 mm, whilst at X 12 the distance is only 10 mm). In the absence of a tube-length corrector changes in magnification and working distance can only be obtained by draw tube extensions with a consequent reduction of image intensity.

The upper part of the imaging system, fitted to a monocular

Plate 6.1 The photomicrographic assembly for observing and photographing the feeding of mosquitoes (demonstrating focusing with the tube-length corrector).

draw tube (on minimum extension) employed a X 5 Huyghenian eye-piece and a modified 35 mm photomicrographic camera set-up. A beam-splitter directed 30% of the image forming light to a horizontal eye-piece which viewed an image projected into the plane of a graticule defining the photographic field.

The remaining 70% of the image forming light passed to the plane of a motor driven Nikon F camera body, operated by a foot switch. 64 ASA colour slide film (Ektachrome 64, Kodak Ltd.) was used throughout. Since light reaching the film plane undergoes one less reflection than the viewing image the resulting transparencies must be viewed from the emulsion side to read correctly. Both graticule and eye-piece could be rotated through 90° to allow vertical format photographs to be made without change of operator position.

Illumination for the photomicrography was provided by three flash-guns firing simultaneously. The subject was lit from the left, at a distance of 150 mm, with a 130M Miranda flash gun and from the right with a 133 Photax flash gun at a distance of 100 mm. A card provided the background colour and was illuminated with a 202 Metz flash gun (at half power), synchronised with the shutter release on the camera. This flash triggered the photo-electric cells which in turn fired the subject flashes.

Because of the narrow depth of field resulting from the optical assembly it was necessary to get each feeding mosquito both close, and at right-angles to the objective of the microscope. In order to accomplish this the insects was fed individually on the index finger of the camera operator and orientated parallel to the finger. This was done by holding the finger vertically and placing an inverted test tube containing a starved mosquito on the dorsal surface of the middle phalanx. Mosquitoes on a vertical surface tend to feed head uppermost. Fine-focusing was performed by a slight rolling of the finger on the microscope stage. Light from a fibre-optic cold-light source (Fibrox, Rank, Taylor and Hobson Ltd.) provided the illumination required for focusing.

The feeding of 40 uninfected and 20 infected mosquitoes was observed and photographed in this manner on three separate occasions. Feeding took place between 18.00-20.00 hrs at $24 \pm 1\ ^{o}C$.

The behaviour of larvae <u>in vivo</u>:

The movement of infective larvae within the labium of a mosquito was studied as follows. The wings, legs and stylets were removed from an infected mosquito. A glass slide (76 mm X 26 mm) was used as a base for an observation chamber with a square coverslip (22 mm X 22 mm) raised above the glass surface supported (on three sides by a

thin-layer of petroleum jelly). The space between the slide and the coverslip was then filled with PBS. The insect was secured on it's dorsal surface on the slide with a small quantity of petroleum jelly and it's labium positioned within the PBS-filled space under the coverslip. The labium was illuminated with a cold-light source and observed through a dissecting microscope at a magnification of X 20. The temperature was 24 $\pm$ 1 ^{o}C.

The behaviour of larvae <u>in vitro</u>:

The movements of 12-day old infective larvae were observed in a shrinking droplets of PBS. Infective larvae were obtained from mosquitoes fed 12 days earlier on a microfilaraemic cat. The insects were killed with a pyrethroid spray and crushed in a small quantity of PBS on a glass plate using a glass test tube. The bodies were then placed into a 75 um meshed brass sieve (Endecotts Ltd, London) in a Baerman funnel (Plate 6.2). After 30 minutes infective larvae were collected by releasing the tap at the bottom of the funnel and collecting the liquid in plastic universal tubes. Larvae were individually placed, using a cat's whisker brush, into a small droplet of PBS (5μl) on a glass slide. Illumination was by a cold-light source and the larvae observed through a dissecting microscope at X 12 magnification at 24 $\pm$ 1 ^{o}C.

Plate 6.2 Baermann funnel used for separating infective larvae from mosquito cadavers.

Response to temperature:

The base of a 5 cm diameter perspex petri dish was divided into ten strips by scoring a series of parallel lines, 0.5 cm apart. 5 mls of liquid 0.75% Noble Agar (Difco Ltd.) in PBS was added to each dish and allowed to solidify at room temperature.

Twenty-five, 12-day old infective larvae were individually placed on the agar, along the centre-line. A 40 ^{o}C point-heat source was then placed against one side of the dish, perpendicular to the centre of the line of infective larvae. After 30 minutes the number of larvae within each division were counted and the heat source removed and applied to the opposite side of the dish. After a further 30 minutes the number of larvae within each division were counted again. This procedure was performed on four separate occasions.

Response to chemicals:

Three mls of 0.75% Noble Agar in PBS were added to a 35 mm diameter perspex petri dish. Two 6 mm diameter wells were cut from the agar when solid. The wells were situated at the edge of the dish 2 mm apart, equidistant from the centre. 25 µl of the test solution was added to one well and 25 µl of PBS to the other well (control well). The dish was then incubated for 2 hrs at 37 ^{o}C. Fifteen, 12

day-old infective larvae were then placed on the centre-line of the dish. The dish was incubated for a further hour in total darkness and the numbers of infective larvae in each well were counted.

The test solutions used in the chemotaxis assay were jird sera, rat sera, cat sera, human sera, rat lymph and human sweat. The sera were prepared as follows. Blood was allowed to clot for 30 minutes at room temperature and for a further 1.5 hrs at 4 ^{o}C before being centrifuged for 10 minutes at 2,000 r.p.m. Rat lymph was collected in a heparinised tube by cannulation of the thoracic lymph duct. Human sweat was obtained from a full-piece plastic suit worn by me for 20 minutes of vigorous exercise on a cycle ergonometer at a temperature of 50 ^{o}C. All test solutions were stored in 2 ml plastic screw-capped tubes at -70 ^{o}C, until required. The experiment was performed on ten separate occasions for each test solution.

An index of chemotaxic activity (CA) for each test solution was calculated as follows.

$$CA = \frac{\text{no. larvae in test well}}{\text{no. larvae in test well + no. larvae in control well}} \times 100$$

RESULTS AND DISCUSSION

Infected mosquitoes showed unusual forms of feeding behaviour when compared with non-infected mosquitoes. They were generally less coordinated than uninfected mosquitoes. Uninfected mosquitoes fed almost immediately on settling on the skin surface and in a characteristic fashion, with little variation between individuals. In contrast, infected mosquitoes took much longer to both settle and feed. They also showed great difficulty in penetrating the skin surface with their stylets, presumably due to a loss of flexibility in the labium because of the often large numbers of infective larvae contained therein. Infected mosquitoes showed a greater frequency of probing, perhaps as a direct consequence of having an inflexible labium or, indirectly, due to an irritant effect caused by the active movement of such large worms.

Probing has been considered to be an important stimulus for larval emergence for many years (Grassi and Noe, 1900; Fulleborn, 1908; Bahr, 1912; Brug and de Rook, 1930; Yen, 1938; Menon and Ramamurti, 1941). Later Wharton (1957a), Jordan (1959), de Meillon *et al*. (1965) and Lavoipierre and Ho (1966) showed that escape of larvae was due to some factor or factors associated with blood-feeding. More recently, it has been shown that the release of larvae is primarily associated with the bending back of the labium (Ho and Ewert, 1967; Lavoipierre and Ho, 1973; McGreevy

et al., 1974). Moreover, the rate of escape is related to the time spent probing in a logarithmic fashion (Ho and Lavoipierre, 1975). Prima facie, this could be interpreted as being yet another example of a parasite manipulating the host's behaviour of it's host to it's own advantage. However, although the probing activity of infected mosquitoes was often frantic, it appeared that the skin was rarely, if at all, punctured deeply by the stylets of the mosquito. Unless puncture wounds are made this behaviour would be disadvantageous to the worm. Although the chance for a larva to escape from a mosquito would be increased it would be unable to penetrate the definitive host since it's usual portal of entry, the puncture wound made by the feeding insect would be lacking.

These studies are by their nature preliminary and suffer from the failure to provide quantitative data on probing and biting behaviour and are also based on a relatively small sample size. The electronic recording of mosquito bites using a "bitometer" (Kashin and Wakeley, 1965; Kashin, 1966) together with a visual recording system would be a suitable way of quantifying the data in order to extend this line of research. An important field of further investigation would be the effect of sublethal doses of insecticide on the probing responsiveness of filarial vectors, especially since some insecticides have been shown to increase the probing of both tsetse flies and blowflies

(Chadd and Brady, 1982).

The difficulty shown by infected mosquitoes when attempting to penetrate the skin surface is further illustrated by the angle at which the stylets pierce the skin. In uninfected mosquitoes, the stylets usually penetrate perpendicular to the skin surface. However, many infected mosquitoes push their proboscises across the skin surface at an angle of 45^{o} (Plate 6.3) until the tip of the proboscis becomes secured in a shallow trench or gryke and the stylets can penetrate the host. Even uninfected mosquitoes often feed in grykes (Plate 6.4A, 6.4B). Intuitively, one would expect this behaviour to benefit the parasite for two reasons. Firstly, a worm which emerges into a gryke would be better protected from dessication because of the smaller surface area of exposed haemolymph. Secondly, the shape of the gryke would assist the location of the puncture wound by the worm by restricting the area available for lateral movement.

During penetration the stylets themselves may bend out of the comparatively straight labium to such an extent the labium and stylets separate (Plate 6.5). The mosquito then rearranges it's mouthparts using it's front legs, before it continues feeding. This behaviour is an example of proboscis cleaning in mosquitoes and has been described in detail by Goldman, Callaghan and Carlysle (1972).

Plate 6.3 An infected mosquito feeding at an angle of 45° to the surface of the skin. Note the emergence of an infective larva (arrowed) from one of it's labellae.

Plate 6.4A An uninfected mosquito feeding in a gryke.

Plate 6.4B An uninfected mosquito feeding in a gryke.

Plate 6.5 An infected mosquito feeding. Note the separation of the stylets from the labium at the point of entry into the skin (c.f. Plate 6.4B).

That larvae usually emerge from the tip of the proboscis already been recorded by a large number of workers including Yamada and Komori, 1926; Rao and Iyengar, 1932; Feng, 1936; Roubaud, 1936; Phillips, 1939; Galliard, 1941; Menon and Ramamurti, 1941; Highby, 1943; Pratt and Newton, 1946; Ewert, 1967; Zielke, 1973 and McGreevy et al., 1974) although originally Low (1900b) thought that the infective larvae pushed through the base of the labium and the hypopharynx amongst the stylets, at the junction with the head. Bacigalupo (1941) reported the emergence of one D. immitis larva from the maxillary palps of Taeniorhynchus titillans (= Mansonia titillans). Although this cannot be considered a common occurrence, it does illustrate the unusual sites in which one can find infective larvae.

More specifically, McGreevy et al. (1974) stated that the precise point of emergence is through the inner membranous wall of the labellae. However, from these studies the split appears to originate from the tip of one or both labellae and runs along the ridge dividing the two halves of a labellum. The shape of a labellum is analagous to that of a Bishop's mitre, the weakest point being the tip and the seams. It is this area which usuallly ruptures first when the proboscis of an uninfected mosquito is crushed beneath a coverslip. Bancroft (1901) and Yamada and Komori (1926) also recognised that the larvae emerge from the labellae simply because they are the most fragile

points of the proboscis, easily ruptured by any slight pressure. However, larvae do not invariably emerge from the tip of the labellae. In Plate 6.6 the split has occurred on the outside edge of the right labella, at its junction with the labium.

Annett et al. (1901) proposed on theoretical grounds that larvae emerge from Dutton's membrane, a delicate layer which separates the paired labella from the labium. They went on to describe how, during feeding, the labellae swing apart and rotate downwards so that the inner surfaces are in contact with the skin, stretching the membrane. Based on Manson's (1901) observations that the anterior end of the larvae had been noticed in this position. It was argued that the extra stress on the membrane would cause its rupture. This hypothesis was supported by Fulleborn (1908), Bahr (1912), Brug and de Rook (1930) and Yen (1938). However, Robinson (1939) and Christophers (1960) maintained that the labellae are always kept pressed close together, although Vogel (1921) and a few other early authors reported otherwise. In this study the labellae were always seen kept together. Indeed, they appear to act as a guide for the stylets during feeding, analagous to the resting hand of a snooker-player (Plate 6.7).

My study supports the finding that exceptionally large numbers of worms within the proboscis can result in a longitudinal split in the labial gutter (Grassi and Noe,

Plate 6.6 An infected mosquito feeding. Note the split on the outside edge of the mosquito's right labella, at it's junction with the labium (arrowed).

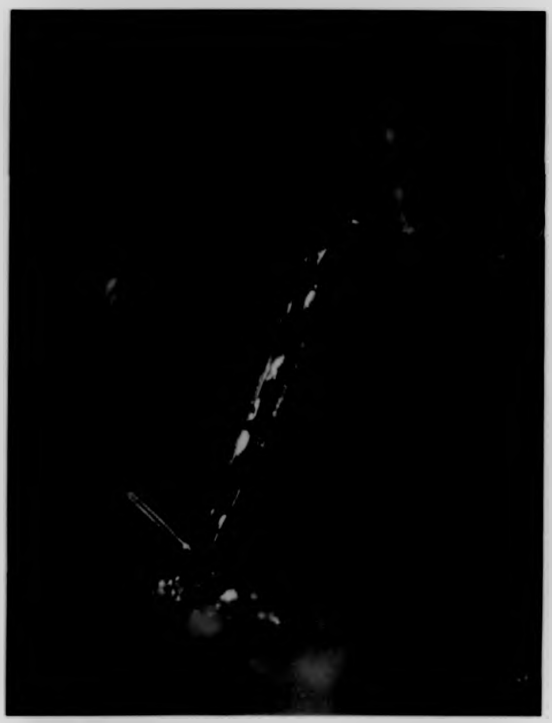
Plate 6.7 An uninfected mosquito feeding, illustrating the gripping action of the two labellae around the stylets.

1900; Noè, 1901; Fulleborn, 1908; Phillips, 1939; Bacigalupo, 1941; Galliard, 1941). In Plate 6.8 the labium has split in this manner. The gutter has filled up with haemolymph, which is highlighted by the light from the flash-guns. However, the labium has a remarkable capacity for expansion and Feng (1930) reports that even as many as 25 B. malayi larvae have failed to burst the labium in this manner. Ho and Ewert (1967) have even suggested that large numbers of larvae packed within the proboscis may be unable to escape.

The typical forward locomotion of infective larvae, as is seen within the labium, is due to the propagation of sinusoidal waves directed backwards along the length of their bodies. Within the labella , this behaviour is manifest as "tapping" or "pushing" behaviour. Such action would of course increase the likelihood of larval escape, particularly when this appendage was stressed during blood-feeding. Reversing is a less common occurrence and results from the propagation of a forward directed wave. Occasionaly larvae may completely withdraw from inside the labium and return into the head.

It was not possible to determine from casual observation whether larvae exhibited a positive thigmotaxic response. No increase in activity was observed when the proboscis was agitated, although this has been claimed in the past

Plate 6.8 An infected mosquito feeding, where the labial gutter has ruptured due to the presence of a large number of infective larvae.



(Yamada and Komori, 1926; Rao and Iyengar, 1932; Menon and Ramamurti, 1941).

Clearly the bending of the labium initiates larval emergence, but how? During feeding the labium of the mosquito appears to be pinched shut at one or possibly two points at the junction of the labium with the head (Plate 6.9). One of these points can also be clearly seen in Plate 6.10. Kadri and Lavoipierre (1978) deduced from histological sections that the labium becomes virtually closed off during feeding. However, such evidence should be treated with caution because of the difficulties inherent in preventing slight tissue movement during histological preparation. Further bending of a sealed and stressed labium during blood-feeding may result in it's rupture.

Larval recruitment from other areas of the mosquito's body is likely to occur as a result of the abdomen filling with blood due to an increase in intracoelomic pressure (de Meillon et al., 1965). This increase in intracoelomic pressure was also once considered to act as an important stimulus for the initial emergence of larvae from both mosquitoes (Fulleborn, 1908; de Meillon et al., 1965) and fleas (Stueben, 1954). However, Lavoipierre and Ho (1973) demonstrated that mosquitoes fed on honey until fully engorged lost relatively few larvae, despite the uptake of

Plate 6.9 A S.E.M. of Ae. aegypti at the junction with the head. The two arrows indicate the position at which the labium becomes sealed off.

Plate 6.10 An uninfected mosquito feeding, illustrating the likely sealing of the labium at it's junction with the head (arrowed).

large volumes and an increase in intracoelomic pressure. L. loa larvae have been observed emerging from the mouthparts of Chrysops silacea, even though the flies had not taken up any fluid (Gordon and Crewe, 1953). Bain (1967) provided histological evidence to show that the migration of D. viteae in the tick, Ornithodorus tartakovskyi, occurs well before blood enters the gut and is, therefore, independent of intracoelomic pressure.

Intense activity can be seen within one or both labellum, before the larvae emerge. Rupture of a labellum may ocur within a few seconds of the mosquito penetrating the skin surface with larvae pouring out of the proboscis, often thrashing vigorously in the air (Plate 6.3).

Larvae on the surface of the skin lie enveloped in a pool of liquid. Originally this liquid was thought to arise from condensation, larval secretions, perspiration or from serum leaking from the tiny wound made by the feeding mosquito (Fulleborn, 1908; Yokogawa, 1939; Menon and Ramamurti, 1941; Dissanaike, Dissanaike, Niles and Surendrathan, 1966), however, McGreevy et al. (1974) later showed that the liquid was haemolymph.

It would be misleading to believe that larval emergence is always accompanied by the production of a haemolymph droplet of standard volume. Photographic evidence shows

that the quantitiy of haemolymph produced is extremely variable, ranging from a thin film (Plate 6.11) to a large droplet (Plate 6.12). Presumably only small volumes are produced if the labium is pinched-shut or when the larvae are so tightly packed in the head capsule and labium or both that the flow of haemolymph from the mosquito is impeded. A large volume will be produced when the flow of haemolymph from the posterior of the mosquito, caused by imbibing blood, is not impeded. Survival data (Chapter 4) indicates that the loss of haemolymph could effect the subsequent survival of these mosquitoes.

Connal and Connal (1922) witnessed that the emergence of L. loa larvae from the proboscis of Chrysops spp. could be assisted by the insect "milking" it's labium with it's fore legs. "Milking" was also seen on two occasions in this study. This behaviour is unlikely to be of much assistance to the worm, unless the worms are deposited on the skin surface in direct contact with the puncture wound, which would seem unlikely. Indeed, on one occasion part of a larva looped out from a longitudinal-split in the labial-gutter. The mosquito, on trying to rid itself of this nuisance, became ensnared, with it's front leg caught in the loop for several minutes.

Larval emergence appeared, not surprisingly, to have an irritant effect on mosquito feeding behaviour. In many instances the emergence of larvae caused the insect to

Plate 6.11 An infected mosquito feeding, where very little haemolymph covers the infective larvae on the skin surface.

Plate 6.12 An infective mosquito feeding, where a large quantity of haemolymph covers the infective larvae on the skin surface.

withdraw it's mouthparts from the host, often while larvae were still streaming from the proboscis. Mosquitoes would often attempt to continue feeding but were never seen to succesfully repenetrate the skin, instead they repeatedly and frantically tapped the skin surface. Consequently, as many of the larvae continued to escape, they became separated from the puncture wound and thus failed to penetrate the host.

Penetration of the puncture wound by larvae can be rapid and can occur within a few seconds of the mosquito withdrawing it's mouthparts from the host.

The movement of larvae within the haemolymph pool has not been described although their activity in less viscous solutions of saline or water has. For example, Manson (1884) described the movement of W. bancrofti infective larvae in a drop of water placed on a glass slide under a cover slip "... it wriggles, twists, bends, extends and lashes about in all directions ... often it pauses in the midst of its contortions, and suddenly extends itself, remaining outstretched for a moment its body quivering in tetanic spasm ... frantically rushes forwards and backwards and in every direction ... frequently it [the larva] turns its mouth towards the observer, and seems to be endeavouring to uplift the cover-glass". However, no qualitative study has been carried out on larval movement in general or, more specifically, during penetration of the

host.

From the *in vitro* observations of larvae in shrinking droplets of PBS it was seen that the worms obtained purchase from the sides of the droplet and in particular the top of the dome. This invariably resulted in attempts by the larva to penetrate the substratum either using a persistent tapping or pushing motion of the anterior end. Again this is a manifestation of the worm's intrinsic behaviour and is not strictly penetration behaviour *per se*. As the droplet shrinks the worm uses more of its body to gain leverage from the sides of the droplet. Even in thin films, the larvae make repeated attempts to penetrate the substratum but in larger droplets the larvae have more time and a greater freedom of movement with which to locate the puncture wound. This behaviour is unlikely to be influenced by gravity since the force exerted by surface tension on a nematode in an aqueous film is 10^4 to 10^5 times stronger (Crofton, 1954). Thus, the process of larval penetration will be the same irrespective of whether the droplet hangs from, or rests on a skin surface.

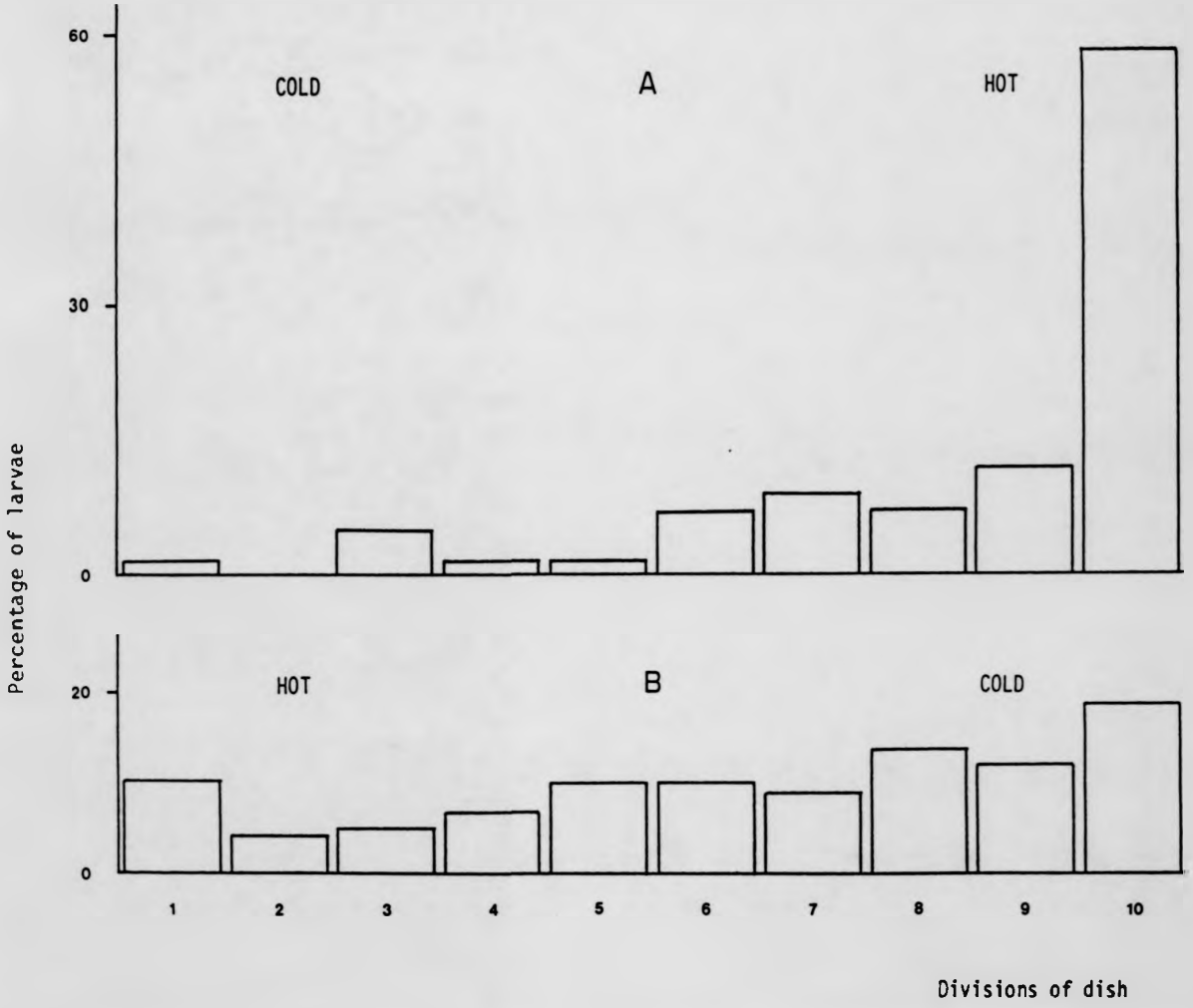
These *in vitro* observations also demonstrated that larvae are not constrained by the limits of the droplet and can move rapidly across a surface when covered by a thin film of liquid. Work by Wallace (1958) with *Heterodera schachtii* in different film thicknesses of water showed

that the maximum speed of this nematode occured when the water-film was slightly less than half the thickness of the worm. It is, however, improbable that filarial larvae, moving across a skin surface will locate the puncture wound unless there is direct contact with serum exuded from the puncture wound. It is possible that the larvae may use slight differences in skin-surface temperature as orientation cues, but in any case such movement would be comparatively short-lived due to the evaporation of the thin-film and trail-wetting. Moreover, the lack of continuity between a haemolymph droplet and serum from the puncture wound would also reduce larval penetration for reasons that are discussed below.

The results of the response of infective larvae to a point-heat source are shown in Figure 6.1. Clearly, as with most nematodes (Croll, 1973), the infective larvae exhibit a positive thermotaxic response. This may though be simply a direct effect of temperature increasing larval activity. As Croll and Smith (1972) have written, "thermopositive behaviour should not be seen in the static context of temperature per unit distance, but rather as the rate of temperature change". An infective larva on moving toward a point heat source will travel faster towards that point (and will, therefore, tend to accumulate there) due to an increase in it's base level of activity as a direct result of the increasing temperature gradient. The steeper the gradient the higher is the burst of activity. The rapid

Figure 6.1 The response of infective larvae to temperature. The distribution of infective larvae is shown when A, a point heat source was applied to the edge of the dish (Division 10) for 30 min and B, when the dish was rotated through 180°, the point heat source then being adjacent to Division 1, also for 30 min.

Distance between divisions 0.5 cm.
Total number of larvae, 100.


60
30
0
COLD
A
HOT
Percentage of larvae
20
0
HOT
B
COLD
1
2
3
4
5
6
7
8
9
10
Divisions of dish

change in temperature causes short-duration metabolic changes which are superimposed on the normal Q10 relationship (this phenomenon is known as "physiological overshoot", Grainger, 1956, 1960). Furthermore, larvae will tend to remain at the point heat source because of the characteristic stimulus-response behaviour of nematodes. That is, when there is a change in ambient conditions (e.g. temperature) the activity of an individual or a proportion of the population may increase. A maximum level of activity will be attained (measured in seconds, minutes or hours, depending on the species and physiological conditions) which will then gradually tail off (Croll and Al Hadithi, 1972). If the same stimulus is repeated the same behavioural response may be elicited, but at a lower level.

Bancroft (1899) initially thought that temperature might play a role in the escape of larvae from the mosquito. He believed that warm blood entering the stomach of a feeding mosquito stimulated larval emergence. An increase in larval activity does, as one might expect, occur as temperature rises. It has been claimed that emergence can be stimulated at high temperatures (Lebredo, 1905; Bahr, 1912; Yamada and Komori, 1926; Fulleborn, 1929; Galliard, 1941), although later work has failed to confirm this (Rao and Iyengar, 1932; Menon and Ramamurti, 1941; Stueben, 1954). Menon and Ramamurti (1941) were unable to demonstrate any response to slight variations in

temperature although they, like Stueben (1954) found that extremes of temperature (i.e. 40 °C) applied to the insect's proboscis caused the larvae to migrate posteriorly. But at 36 °C larvae made frequent attempts to escape the proboscis (Menon and Ramamurti, 1941).

The ingestion of a warm solution is certainly not a prerequisite for larval emergence as many species of cold-blooded animals can act as definitive hosts for filarial worms. Gordon and Crewe (1953) showed that L. loa larvae would emerge from the proboscises of tabanids, despite probing on a cold, dry surface, as did Kadri and Lavoipierre (1978) with B. pahangi in Ae. aegypti. There are also many accounts of larvae being lost whilst mosquitoes fed on sugar solutions or dried fruit (Grassi, 1900; Fulleborn, 1908; Bahr, 1912; Yamada and Komori, 1926; Sharp, 1928; Galliard, 1941, amongst many others).

The results of the responses of infective larvae to a variety of natural body solutions are shown in Table 6.1. Clearly there is a component of sera, lymph and sweat which elicits a positive positive chemotaxic response from infective larvae. Zietse, Klaver-Wesseling and Vetter (1981) have demonstrated that the infective larvae of Ancylostoma caninum respond in a similar way to a wide variety of sera from different hosts.

Serum also increases larval activity (James, 1900; Menon

Table 6.1 The chemotactic activity (C.A.) of a number of biological media (see page 136).

Test Solution	No. worms in test well	No. worms in control well	Total no. larvae	C.A.
Jird sera	70	0	150	100.0
Rat sera	66	1	150	98.5
Cat sera	70	1	150	98.6
Human sera	26	1	150	96.3
Rat lymph	65	0	150	100.0
Human sweat	35	0	150	100.0

and Ramamurti, 1941; Stueben, 1954), so it is possible that the positive chemotaxic response of larvae may be wholely due to an increase in activity as they move up the concentration gradient (in a similar way to which temperature affects activity).

Warmed serum has also been shown to induce the emergence of O. volvulus larvae (Blacklock, 1926) and D. arbuta larvae (Highby, 1943) from the mouthparts. However, Lavoipierre and Ho (1973) consider that "blood per se probably does not contain an emergence factor", and they stated that the bending of the labium is the most important factor for reasons described earlier. This is further supported by evidence that few larvae are lost from mosquitoes fed on blood-soaked pellets where the labium is kept comparatively straight (Lavoipierre and Ho, 1966) and that many worms are lost when infected mosquitoes fed on dead mice when the mosquitoes did not inbibe any fluid (Kadri and Lavoipierre, 1978).

Little is known about the behaviour of infective larvae during penetration. With reference to the in vitro behaviour of larvae, one would expect that both the presence of serum and, to a lesser extent, temperature would be important stimuli for the location of the puncture wound by infective larvae. If the haemolymph covering the larvae on the skin surface and the serum leaking from the puncture wound are in continuum both a serum and

temperature gradient would be set up within the droplet. Both gradients may then be used as cues by the larvae to discover the site of the puncture wound.

CHAPTER 7

THE EFFECT OF HUMIDITY ON THE TRANSMISSION OF THE INFECTIVE LARVAE OF *B. PAHANGI* BY *AE. AEGYPTI*

SUMMARY

The transmission of *B. pahangi* from *Ae. aegypti* into jirds was compared at low and high humidity. There was no statistical difference between the number of infected mosquitoes feeding or the egress of infective larvae from these mosquitoes at high or low humidity. The penetration of the host by the infective larvae was significantly greater ($p < 0.05$) at a high humidity than at a low one.

INTRODUCTION

Many authors have noted that mosquito-transmitted human filariasis is limited to humid areas of the tropics. Earlier work indicated that high humidity was important for the successful development of infective filarial larvae (Rao and Iyengar, 1930; Acton and Rao, 1931; Basu and Rao, 1939) whilst McGreevy *et al*. (1974) suggested that humidity might directly affect transmission.

When an infected mosquito feeds on a host, the emergence of filarial larvae from a mosquito's proboscis is accompanied by a small volume of the insect's haemolymph which envelops the worms on the surface of the skin (see Chapter 6).

McGreevy et al. (1974) considered that the rate at which the haemolymph evaporated could be a major factor governing the successful penetration of the infective larvae into the puncture wound made by the mosquito's stylet. They proposed that at low humidity the haemolymph evaporates quickly and so might prevent a significant proportion of larvae from penetrating the puncture wound made by the feeding mosquito. Conversely, at high humidity the rate of evaporation would be slower and fewer larvae would succumb to desiccation on the surface of the host.

In the present study an attempt was made to determine the success of mosquito transmitted Brugian filariasis at a low (i.e. 40-50% RH) and a high humidity (i.e. 90-100% RH). These two values of humidity were based on extremes of relative humidity likely to be encountered in areas of endemic filariasis. The success of transmission is dependent on three factors: how many infected mosquitoes feed, the loss of larvae from the mosquitoes and larval penetration of the host. The experiments were performed by feeding infected mosquitoes on jirds.

MATERIALS AND METHODS

Dried fruit was removed from a cage of infected mosquitoes 24 hours prior to feeding and equal numbers of between 50-100 females placed into three separate 20 x 20 x 20 cm gauze cages (one control cage and two test cages).

Experiments were conducted 10-12 days post infection when a large number of larvae have accumulated in the proboscis (Figure 3.4).

After removal of the water pots, each test cage was equilibrated for 3 hours in a glass tank before feeding at a temperature of 28 $\pm$ 1 ^{o}C and a relative humidity between 40-50% or 90-100%. The lower humidity was obtained using a solution of potassium hydroxide (75 g/100 ml) and the saturated environment by using distilled water alone (Buxton and Mellanby, 1934). The humidity was checked with a hair hygrometer (B. and T. Ltd., Romford, Essex, U.K.).

Each jird was anaesthetized and it's ventral surface shaved with electric clippers. The animal was then placed on one or other of the test cages. The mosquitoes were allowed to feed for 15 minutes through a rectangular area 5.0 X 2.5 cm cut from a paper towel, on the abdomen of the jird (Plate 7.1).

Immediately after feeding the area on which the mosquitoes had fed was rinsed repeatedly with PBS and then dried with paper tissues to minimize further larval penetration. The jird was then killed by exsanguination, the blood collected and lysed. The ventral portion of the pelt and body wall

Plate 7.1 The test assembly. A cage of infective mosquitoes feeding on an anaesthetised jird in a humidity chamber.

were removed separately and placed on a Baermann funnel, such that only the pelage remained dry. This procedure was completed within 5 minutes after the mosquitoes had ceased feeding. The heart and lungs were removed, gently teased apart in PBS and searched for larvae. After discarding the alimentary canal the remainder of the cadaver was left soaking in PBS overnight. The following morning the blood, washings and Baermann concentrate were all examined for the presence of larvae.

All mosquitoes were kept at 12°C after feeding to restrict the movement of larvae within the mosquito as I had found previously, *in vitro*, that infective larvae become tightly coiled and do not undergo translatory movement at this temperature. Mosquito dissections were performed, as described in Chapter 3, on 80-100% of both cages of test mosquitoes and until twenty infected mosquitoes were obtained from the non-feeding control cage. The number of larvae within each mosquito and whether the insects had fed was recorded.

The procedure was performed on six separate occasions. The order in which the test cages were fed was changed between each replicate.

An estimation of the efficiency of larval recovery from jird autopsies was made as follows. Using a skin-prick

technique (Ewert and El Bihari, 1971) to s imulate mosquito-feeding, 50 larvae were placed on 0.2 - 0.3 ml Medium 199 over an area punctured 100 times with a fine pin on the exposed ventral surface of each of the five jirds. After 15 minutes, the jirds were autopsied as described earlier and the percentage larval recovery calculated.

The percentage of mosquitoes feeding was estimated by dividing the number of blood-fed mosquitoes dissected by the total number of mosquitoes dissected from that cage, namely

$$\% \text{ mosquitoes feeding} = \frac{n_1}{n_1 + n_2} \times 100$$

where n_1 is the number of fed mosquitoes and n_2 is the number of unfed mosquitoes.

This value would represent a crude estimate of how heavily a jird would be infected. Ho and Lavoipierre (1975) demonstrated that the ingestion of blood is not essential for the release of larvae from the mosquito and that probing alone results in the release of larvae, although to a lesser extent. Therefore, in these experiments, mosquitoes which did not imbide blood could have infected the jird. Though few, if any, mosquitoes landing and probing on the jird failed to take a blood-meal.

The percentage of larvae lost from mosquitoes during

feeding was calculated by finding the difference in the mean larval load before (control mosquitoes) and after feeding (blood fed test mosquitoes) and expressing this as a percentage of the mean larval load before feeding (control mosquitoes). This resulted in the following formula:

$$\text{Percentage loss of larvae} = \frac{(m_1 - m_2)}{m_1} \times 100$$

where m_1 is the mean number of larvae in the control mosquitoes and m_2 is the mean number of larvae in the fed test mosquitoes.

It was assumed that after feeding no additional larvae were lost from the ruptured proboscis and that unfed mosquitoes had not probed the skin of the jird.

The success of larval penetration of the jird was determined by expressing the number of larvae recovered from the jird autopsy (L) as a percentage of the number of larvae lost from the mosquito as follows:

$$\text{Percentage of larvae penetrating jird} = \frac{L}{n_1(m_1 - m_2)} \times 100$$

Using the Wilcoxon's matched-pairs signed-ranks test for each set of calculations the significance of the differences between the results at low and high humidity was determined. When $p < 0.05$, the difference was considered significant.

It has been shown that mosquitoes with high worm burdens are less likely to feed (Chapter 4). If mosquitoes with heavy infections did not feed fewer larvae would be lost from the feeding mosquitoes than one would expect and consequently the calculated values of larval loss and larval penetration would be underestimated. Using the Mann-Whitney U-test the significance of the difference between the number of larvae in each non-feeding test mosquito was determined. When $p < 0.05$, the difference was considered significant.

RESULTS

The results of feeding infected mosquitoes on jirds are presented in Table 7.1. There was no statistically significant difference between the feeding avidity of the mosquitoes at high or low humidity. Before feeding the mean worm burdens in the mosquitoes from the six trials were 2.2, 2.5, 2.8, 2.2, 1.2, and 3.6 respectively. The egress of larvae from feeding mosquitoes was also independent of humidity (Table 7.2).

Table 7.1 The percentage of mosquitoes feeding at a low and a high humidity.

Expt.	n	40-50% RH	n	90-100% RH
1	98	92.8	86	51.5
2	65	95.4	52	48.1
3	90	92.2	82	58.5
4	56	80.3	72	84.7
5	78	96.3	60	91.7
6	59	94.9	72	86.7
$\bar{x}$		92.0		70.2

Table 7.2 The percentage loss of larvae from mosquitoes feeding at a low and a high humidity.

Values of mean worm burden for the control groups are given on page 172.

Expt.	40-50% RH	90-100% RH
1	85.6	88.3
2	73.3	76.1
3	82.8	82.5
4	67.7	71.8
5	85.6	92.4
6	69.9	97.3
$\bar{x}$	77.5	84.7

More larvae penetrated jirds at high rather than at low humidity (Table 7.3). Statistical analysis revealed that this difference was significant.

The mean efficiency of larval recovery, as estimated by the pin-prick technique, from jirds was 53%. Although this technique is a crude simulation of mosquito transmission, these results indicate that the real values for percentage larval penetration are greater than those found.

There was no statistical difference between the number of larvae within the control group and the number of larvae in the non-feeding individuals of the test group ($p = 0.24$). This would imply that in these experiments mosquitoes feeding on jirds do so regardless of their parasite load.

DISCUSSION

Overall, the results indicate that hungry *Ae. aegypti* feed regardless of humidity. This is in agreement with Lumsden (1947) who found that with *Ae. aegypti* the relative humidity of the environment at the time of biting is of little or no importance but in contradiction to Christophers (1960) who considered that at 25 °C the feeding of *Ae. aegypti* was maximal at a relative humidity of 70-90%.

That larval egress from feeding mosquitoes is independent

Table 7.3 The percentage of larvae penetrating jirds at a low and a high humidity. For method see page 172.

Expt.	40-50% RH	90-100% RH
1	21.0	38.5
2	12.4	28.3
3	4.8	18.4
4	0.0	24.9
5	0.0	26.0
6	5.1	9.5
$\bar{x}$	7.2	24.3

of humidity supports the widely held view that the larval escape mechanism is purely mechanical, brought about by the bending of the labium (Lavoipierre and Ho, 1966; McGreevy et al., 1974; Ho and Lavoipierre, 1975, amongst others). The egress of around 80% of larvae from mosquitoes feeding on jirds is similar to that found by Lavoipierre and Ho (1973) and Ho and Lavoipierre (1975) who worked with Ae. togoi infected with B. pahangi. However, more recently, Zielke (1979) estimated only 46% of B. pahangi were lost from Ae. aegypti feeding on jirds.

Larval penetration was directly affected by the humidity of the environment; at high humidity more larvae penetrated the host. These results support the view that evaporation of the protective drop of haemolymph is a major factor governing larval penetration of the host. This is of some practical importance, as increasing the rate of evaporation at the skin surface would decrease transmission and thus lower the risk of infection.

Mosquitoes which bite before dawn, when the relative humidity is at a maximum (often > 90%) or when it is foggy would be potentially more efficient vectors. Interestingly, the natives of Fiji and Tahiti (where day-biting Aedes spp. are filarial vectors) believed that filariasis came from "walking in the early morning through dew in the gardens" (Hoeppli, 1959) but the Polynesians

believed that the frequent contact with water, rather than high humidity was responsible for elephantiasis while Saffre (1884) thought it was due to sleeping on damp ground.

The potential for transmission in rural areas compared with urban areas would also be greater as relative humidity in cities at night can be 30% less than corresponding rural areas (Barry and Chorley, 1976) due to the lack of vegetation cover, absence of large areas of standing water and the removal of surface water by drains in cities. However, during calm, clear weather, particularly where buildings are tall, the streets will trap warm air and retain more moisture as less dew is deposited on the warm street surfaces.

CHAPTER 8

THE EFFECT OF TEMPERATURE ON THE TRANSMISSION OF THE INFECTIVE LARVAE OF *B. PAHANGI* BY *AE. AEGYPTI*

SUMMARY

The transmission of *B. pahangi* by *Ae. aegypti* into jirds was compared at 21°C, 28°C and 35°C. Significantly, greater numbers of infective mosquitoes fed at 28°C than at 35°C. Lower temperatures resulted in a greater loss of larvae from feeding infective mosquitoes and a greater percentage of larvae penetrating the host after they had been deposited on the skin.

INTRODUCTION

The major inference drawn from examining the effect of humidity on transmission was that the rate of evaporation of the haemolymph which surrounds the infective larvae whilst it is exposed on the surface of the skin is a crucial factor governing the success of filariasis transmission.

Temperature will also effect the rate of haemolymph evaporation. However, although a rise in temperature will increase the rate of haemolymph evaporation and consequently reduce larval penetration it will also increase the activity and, therefore, the ability of

infective larvae to penetrate the host.

This chapter describes how ambient temperature effects the transmission of infective larvae by mosquitoes at 21°C, 28°C and 35°C. 28°C is close to the optimal temperature for filarial development (Rao and Iyengar, 1930; Rodriguez and Thompson, 1974), the other two values represent temperature extremes likely to be encountered in areas of endemic filariasis.

MATERIALS AND METHODS

The experimental protocol was similar to that described in Chapter 7. Equal numbers of between 60-100 infective moquitoes were placed in four separate cages (one control cage and three test cages). Each test cage was equilibrated at 21 ± 1°C, 28 ± 1°C or 35 ± 1°C for 3 hrs at 70% relative humidity in glass tanks. Mosquitoes in the test cages were then allowed to feed on a jird for 15 minutes between 11.00-12.00 hours.

Jird autopsies were performed to determine how many larvae had penetrated the animal. Fifty mosquitoes were taken from each cage at random as were 30-57 from the control cages, and then dissected to determine the number of infective larvae within each mosquito. In addition, the abdomens of the remaining mosquitoes from the test cages were dissected to determine whether they had fed.

The experiment was performed on eight separate occasions. The order in which the test cages were fed at the different temperatures was changed at random between replicates.

The percentage of mosquitoes feeding, the percentage loss of larvae from those mosquitoes and the percentage of the larvae lost from those mosquitoes and the percentage successfully penetrating the host were calculated using the formulae described in Chapter 7. The data was analysed using the Friedman two-way analysis of variance by ranks and the Wilcoxon matched-pairs signed ranks test.

The Mann-Whitney U-test was used to determine whether heavily infected mosquitoes were less likely to feed. The worm burdens of non-feeding mosquitoes in the test cages were compared with worm burdens of mosquitoes from the control cages. When $p < 0.05$ the difference was considered significant.

RESULTS

The results of feeding infective mosquitoes on jirds at the different temperatures are shown in Table 8.1. Although not statistically significant ($p = 0.149$) more infective mosquitoes fed at 28°C than at 21°C or 35°C. However, significantly greater numbers of infective mosquitoes fed

Table 8.1 The percentage of mosquitoes feeding at different temperatures. 60-100 mosquitoes per test group.

Expt.	21 °C	28 °C	35 °C
1	70.4	80.0	67.3
2	80.6	100.0	82.7
3	96.0	69.2	62.0
4	41.2	92.1	93.0
5	91.0	88.9	84.1
6	82.9	82.1	64.9
7	56.1	63.4	36.8
8	63.1	72.2	72.0
$\bar{x}$	72.7	81.0	70.4

at 28°C compared with 35°C.

The loss of larvae from feeding mosquitoes (Table 8.2) was statisticaly similar ($p = 0.079$). However, larval loss does demonstrate a trend, being least at the highest temperature and visa versa. When the loss of larvae at 35°C is compared with the combined data at 21°C and 28°C this difference is significant.

Although the percentage of larvae which penetrated the jirds is reduced at higher temperatures (Table 8.3) there is no statistical difference between the groups ($p = 0.079$).

There was no statistical difference between the number of larvae within mosquitoes from the control group and the number of larvae in the non-feeding mosquitoes from the test groups (Table 8.4). These results support those of the previous chapter.

DISCUSSION

The feeding avidity of infective mosquitoes at various temperatures mirrors that of uninfected mosquitoes. That is, this species of mosquito will feed whenever hungry, although the optimal temperature for feeding is generally

Table 8.2 The percentage loss of infective larvae from mosquitoes feeding on jirds at different temperatures.

For method see page 172.

Expt.	21 °C	28 °C	35 °C
1	90.1	91.1	90.9
2	93.3	91.4	93.1
3	89.5	88.3	86.7
4	89.7	90.3	74.5
5	92.8	73.7	81.4
6	90.6	86.8	65.3
7	89.7	76.4	65.8
8	87.5	89.3	78.1
$\bar{x}$	90.4	85.9	79.5

Table 8.3 The percentage of infective larvae penetrating jirds at different temperatures.

For method see page 172.

Expt.	21 °C	28 °C	35 °C
1	24.3	16.9	6.6
2	13.5	11.1	0.0
3	18.2	38.2	15.8
4	8.2	10.8	1.7
5	6.6	5.7	1.6
6	43.3	3.1	10.4
7	16.4	16.1	10.1
8	15.8	13.8	25.8
$\bar{x}$	18.3	14.5	9.0

Table 8.4 Comparisons between the parasite burdens of a group of Control mosquitoes and non-feeding mosquitoes from the groups of Test mosquitoes.

Group	No. mosq.	$\bar{x}$ no. larvae	p
Control	335	4.3	
21 °C	91	3.7	0.78
28 °C	62	3.9	0.34
35 °C	91	3.2	0.28
21 °C + 28 °C + 35 °C	244	3.6	0.26

considered to be around 28°C (Christophers, 1960).

Prima facie it might appear paradoxical that fewer larvae are lost from mosquitoes when feeding at higher temperatures. Higher temperatures will, as with all poikilotherms, increase larval activity and one might expect that this would increase their ability to escape from the mosquito. However, this trend could be a reflection of the positive thermotaxic behaviour of infective larvae. Croll and Smith (1972) suggested that it is the rate of temperature change which is the most important stimululs for initiating a burst of larval activity. Thus, at the highest air temperature used in this study (i.e. 35°C, which is equivalent to the temperature of the nematode) the temperature gradient between the skin surface (i.e. approximately 34-35°C) and the air will be least steep. Hence the relatively small loss of larvae at the higher temperature. It is noteworthy that at an ambient temperature of 35°C a temperature gradient may not exist or be negative during the first few minutes at which feeding occurred, which would further explain why few larvae are lost from mosquitoes under these conditions.

Evaporation of the haemolymph droplet on the skin surface will be greatest at 35°C because the "saturation deficit" of the air is greatest at the higher temperature (Ingram and Mount, 1975). However, although fewer larvae do

indeed penetrate the host at the higher temperatures, this difference is not statistically significant.

These results further demonstrate how the environment effects the transmission of Brugian filariasis by mosquitoes. Perhaps the biggest determinant governing successful transmission would be the combined effect of humidity and air temperature on larval penetration. Low humidity and high temperature resulting in high haemolymph evaporation would represent the most unsuitable conditions for larval penetration of the host.

Together with humidity and air temperature, wind speed and solar radiation (sunshine) also determine the rate of evaporation. Sunshine is a major influence on evaporation, particularly in the tropics, and is the key to what would appear to be two contradictory facts, namely that areas of endemic filariasis are limited to areas of the tropics where the rate of evaporation is greatest. This is because filariasis is predominantly transmitted by mosquitoes which feed at night, when because of the absence of sunlight, lower temperatures and higher humidity, evaporation is reduced.

Evaporation can be increased locally by increasing the wind speed through dwellings and across the body surfaces of the occupants. This may be achieved where buildings are placed on exposed positions, the surrounding vegetation cleared

and the building area opened up to breezes and orientated across the line of the prevailing wind. Such considerations though have been characteristic of tropical architecture for many centuries simply because it results in a more comfortable environment in which to live. However, with space at a premium in many cities these objectives have been less easily obtained.

Shading in dwellings, another major consideration in tropical architecture, would on the other hand reduce evaporation and could provide suitable sites for resting mosquitoes as well as improving the comfort of the human occupants. Such architectural dilemmas would be worth considering in areas of endemic fialiasis.

Together with data on the prevalence and behaviour of vector species, climatic factors governing evaporation (i.e. humidity, air temperature, and to a lesser extent solar radiation and wind speed) might be useful for forecasting filariasis. Identifying both areas and months of high transmission risk would be advantageous to control schemes and, at a time when the disease is increasing worldwide (W.H.O., 1984), could be used to predict areas which would be highly susceptible to incursions of filarial infection.

CHAPTER 9

THE EFFECT OF DIFFERENT TYPES OF SKIN SURFACES ON THE TRANSMISSION OF THE INFECTIVE LARVAE OF *B. PAHANGI* BY *AE. AEGYPTI*

SUMMARY

The transmission of the infective larvae of *B. pahangi* from *Ae. aegypti* into jirds was compared for four different types of skin surface. Infective mosquitoes were reluctant to feed on a hairy surface. The loss of larvae from infective mosquitoes was greatest when these insects fed on "exposed" skin surfaces (i.e. clean and bald or greasy and bald). Significantly fewer infective larvae penetrated the host when infective mosquitoes fed on a jird through a thin-layer of cloth.

INTRODUCTION

From the preceeding two chapters it was demonstrated that the vector-host interface is of vital important in the transmission of infective filarial larvae from mosquitoes to mammalian hosts. This study was designed to investigate how the nature of the skin surface on which the mosquito feeds affects filarial transmission.

Haemolymph acts as a medium for larval locomotion as well as protecting the worms from exsiccating on the skin surface of the definitive host. McGreevy *et al*. (1974) considered

that penetration would be optimal when the haemolymph formed a dome-shaped droplet with its basal centre directly over the puncture wound. Spreading of the haemolymph would, therefore, reduce transmission not only because the increased surface areas would result in a greater rate of evaporation but also because of the less suitable droplet shape.

The experiment was performed by feeding infective mosquitoes on jirds presenting four different types of skin surfaces, illustrated in Figure 9.1. These were normal (HAIRY), clean and bald covered with a thin-layer of cotton (CLOTH), clean and bald (CLEAN) and greasy and bald (GREASY). The two "covered" surfaces (HAIRY and CLOTH) were used chiefly to test the important of absorption of haemolymph in relation to filarial transmission and the two "exposed" surfaces (CLEAN and GREASY) to test the effect of droplet shape on larval penetration.

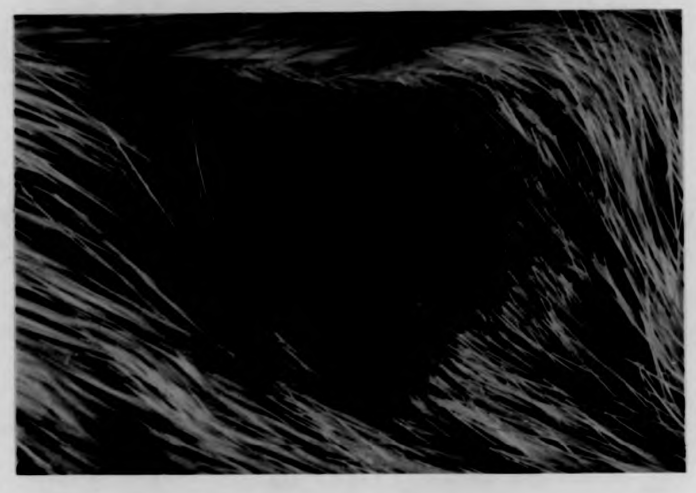
MATERIALS AND METHODS

The experimental protocol for transmission was similar to that described in Chapter 7. Equal numbers of between 60-100 infective mosquitoes were placed into five separate cages (one control cage and four test cages).

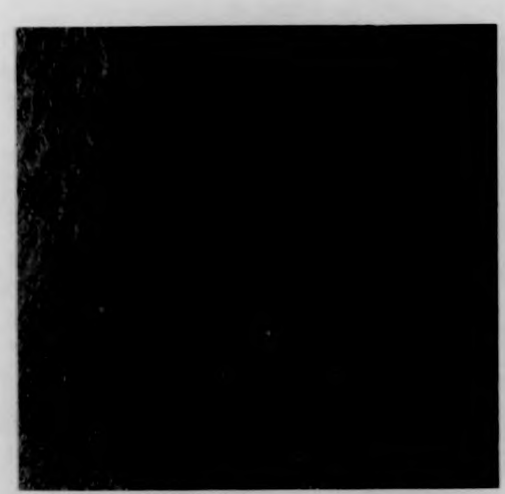
The different jird skin surfaces upon which the mosquitoes fed were prepared as follows. The HAIRY surface was the

Plate 9.1 Three types of skin surface under investigation
A, HAIRY; B, CLEAN and C, GREASY.

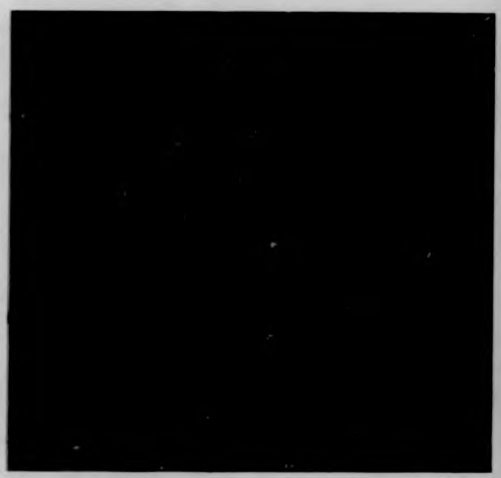
A



B



C



normal hairy skin of a jird. The remaining three skin surfaces were shaved with electric clippers and then depilated with "Nair" (Carter-Wallace Ltd, Folkestone, Kent, U.K.) three days prior to feeding. These surfaces were rinsed with 70% ethanol and dried with paper tissues 10 minutes before feeding. In addition a small quantity of petroleum jelly ("Vaseline", Chesebrough Ponds Ltd, London) was rubbed into the GREASY surface. The material used for the CLOTH surface consisted of a clean, white, cotton handkerchief, with fibres 0.11-0.20 mm thick, spaced at 0.09-0.15 mm intervals.

The mosquitoes in the test cages were then allowed to feed on a jird for 15 minutes between 10.30-12.30 hours at 28 $\pm$ 1 ^{o}C and 70-80% relative humidity.

Jird autopsies were performed to determine how many larvae had penetrated the animal. Fifty mosquitoes were taken from each test cage at random as were thirty from the control cage, and then dissected in order to determine the number of infective larvae within each mosquito. In addition, the abdomens of the remaining mosquitoes from the test cages were dissected to determine whether they had fed.

The experiment was performed on six occasions. The order in which the test cages were fed on the different skin surfaces was changed at random between replicates.

The percentage of mosquitoes feeding, the percentage loss of larvae from those mosquitoes and the percentage of the larvae escaping from those mosquitoes and successfully penetrating the jirds were calculated using the formulae described in Chapter 7.

The data was analysed using the Friedman two-way analysis of variance by ranks and the Wilcoxon matched-pairs signed-ranks test. In this, and all subsequent statistical tests, when $p < 0.05$ the difference was considered significant.

The Mann-Whitney U-Test was used to determine whether heavily infected mosquitoes were less likely to feed. The worm burdens of non-feeding mosquitoes in the test cages were compared with the worm burdens of mosquitoes from the control cages.

RESULTS

The results of feeding infective mosquitoes on the different skin surface are presented in Table 9.1. The null hypothesis that all groups were similar was rejected ($0.05 < p > 0.02$). However, the percentage of mosquitoes feeding on CLOTH, CLEAN and GREASY surfaces were similar ($p = 0.252$). Significantly fewer infective mosquitoes fed on the HAIRY surface than on the other three surfaces.

Table 9.1 The percentage of mosquitoes feeding on different skin surfaces.

60-100 mosquitoes per test group.

Expt.	HAIRY	CLOTH	CLEAN	GREASY
1	54.2	78.9	65.7	73.9
2	51.4	42.9	55.6	63.3
3	40.5	74.3	79.6	56.2
4	47.6	70.7	76.3	62.3
5	51.6	86.4	92.9	82.5
6	83.3	86.0	89.5	76.8
$\bar{x}$	54.8	73.2	76.6	69.2

The loss of larvae from infective mosquitoes feeding on the four surfaces as shown in Table 9.2 was not the same and the null hypothesis was rejected ($0.05 < p > 0.02$). Significantly more larvae were lost from infective mosquitoes feeding on the two "exposed" skin surfaces (CLEAN and GREASY) than from those feeding on "covered" skin surfaces (HAIRY and CLOTH).

The percentage of larvae on the skin surface which successfully penetrated the host (Table 9.3) was not the same for each group and the null hypothesis was once more rejected ($0.01 < p > 0.001$). However the larval penetration of CLEAN, GREASY and HAIRY surfaces was similar ($p = 0.43$). The shape of the haemolymph droplet, therefore, has no significant effect on the ability of filarial larvae to penetrate the host. When the data for these three groups were combined and compared with the results for the CLOTH surface a significant difference was found. This means that larval penetration was significantly reduced when infective mosquitoes fed through cloth.

Non-feeding mosquitoes which were given the opportunity to feed on HAIRY and CLEAN jirds both had significantly higher worm burdens than mosquitoes from the control group (Table 9.4). The other two groups, CLOTH and GREASY, had worm burdens which were not significantly different from the

Table 9.2 The percentage loss of infective larvae from mosquitoes feeding on different skin surfaces.

For method see page 172.

Expt.	HAIRY	CLOTH	CLEAN	GREASY
1	80.3	80.6	86.7	80.4
2	86.4	88.2	95.3	90.2
3	66.1	50.2	91.1	98.5
4	89.5	56.2	85.6	96.1
5	90.1	76.4	91.9	88.6
6	77.9	44.1	83.8	87.4
$\bar{x}$	81.7	66.0	89.1	90.2

Table 9.3 The percentage of infective larvae penetrating different jird skin surfaces.

For method see page 172.

Expt.	HAIRY	CLOTH	CLEAN	GREASY
1	35.5	2.0	6.0	42.6
2	4.0	0.7	5.5	14.2
3	39.5	5.0	35.7	28.9
4	30.7	8.3	21.6	25.9
5	19.4	8.5	14.7	14.8
6	12.7	4.0	14.2	41.1
$\bar{x}$	23.6	4.8	16.3	27.9

Table 9.4 Comparisons between the parasite burdens of a group of Control mosquitoes and non-feeding mosquitoes from the groups of Test mosquitoes.

Group	No. mosq.	$\bar{x}$ worm burden	p
Control	204	6.2	
HAIRY	123	7.8	0.002
CLOTH	59	7.6	0.061
CLEAN	60	7.8	0.046
GREASY	73	8.3	0.087
CLOTH + CLEAN + GREASY	192	7.9	0.008

group of Control mosquitoes (Table 9.4).

It should be appreciated that because many mosquitoes with heavy infections did not feed the real percentage loss of larvae from feeding mosquitoes and percentage of larvae entering the host are greater than indicated from the results.

DISCUSSION

Hairy skin partially deters mosquitoes from feeding on a host, but it is not fully protective. Although Lewis (1933) was unable to feed mosquitoes on hairy guinea-pigs it is my experience that this is possible. A heavy growth of hair on man may also deter feeding, but again it is not protective (Gordon, 1922).

The greatest loss of larvae from mosquitoes feeding on exposed surfaces occurs when the labium is most severly bent (Lavoipierre and Ho, 1966, 1973). However, direct observations of mosquitoes feeding through cotton cloth show that the labium is bent more acutely than when feeding on an exposed surface; the tip of the labium (labellae) being positioned on the surface of the fibres. Intuitively, one would expect that mosquitoes feeding on the skin through material would have to sink their stylets deeper in order to feed on the same capillaries as they would on an uncovered surface. As a consequence the

mosquito pushes its head closer to the surface and thus the labium is bent more acutely. In such a position the labium may be pinched-shut at one or possibly two points at its junction with the head capsule (Plate 6.2). If this is so it is likely that only those larvae in the proboscis are lost during feeding; the pinched labium effectively preventing recruitment of larvae from other areas of the body into the labium.

Larval penetration is clearly affected by the nature of the skin surface on which the infective mosquito feeds. The failure of larvae to penetrate the host through the CLOTH surface is probably due to a combination of factors. Possibly the most important is that the haemolymph is absorbed by the cotton fibres, because in the other "covered" surface (HAIRY), where larval penetration is relatively good, absorption of haemolymph by the pelage is minimal. Moreover, the lack of continuity between the haemolymph droplet and serum from the puncture wound would also reduce larval penetration for two reasons. Larvae have the ability to move up both a serum and temperature gradient (Chapter 5), as occurs when the haemolymph comes into contact with the warm serum from the puncture wound. Thus, the lack of contact between the haemolymph and serum results in the larvae going "blind".

Any proposals to encourage individuals in areas of endemic filariasis to keep their bodies covered when vectors are

more likely to feed must be tempered by two factors. Firstly, in a tropical environment body coverings are kept to a minimum simply because it is more comfortable. Secondly, mosquitoes preferentially feed on exposed areas of the body, so efficient protection cannot be given to an individual unless that person is fully covered by an absorptive material.

These results demonstrate further that filariasis transmission is a subtle biological phenomena and that entry of larvae into a host is not guaranteed when an infective mosquitoe feeds. In general the risk of infection is increased when the host's skin surface is "exposed".

The ability of mosquitoes with heavy infections to feed has been investigated in Chapters 4, 7, 8 and 9. However, the results have conflicted and it has not always been shown that high worm burdens depress the feeding avidity of mosquitoes. This is a result of the statistical analysis employed and the large number of mosquitoes with no larvae. It is impossible to directly compare the worm burden of non-feeding mosquitoes with the worm burden of Control mosquitoes, because there will also be a potential number of non-feeders in the Control group. Also some mosquitoes will not feed even if uninfected. In populations of mosquitoes where there is a large proportion of uninfected

mosquitoes their influence on any statistical comparison will be disproportionately large.

CHAPTER 10

SYNOPSIS: AN OVERVIEW.

The migration and transmission of the infective larvae of B. pahangi is a good example of the remarkable adaptability shown by a large number of parasites to a changing environment. For an infective larva, it is a journey which starts from the flight-muscles of a mosquito and finishes, if it is successful, in the lymphatic system of a suitable vertebrate host. The worm is well adapted to survive the rigours imposed by this changing physiological environment but it also uses these changes, indirectly, to it's own advantage. For example, changes in serum concentration and, to a lesser extent, temperature may both be used as navigational cues to locate a suitable portal of entry into a vertebrate host.

This thesis contains an account of the behaviour of infective larvae during their migration within their mosquito hosts and also during transmission, when the insects are blood-feeding. It was indicated that the accumulation of infective larvae in the proboscis of a mosquito may arise simply from the "undirected" movement of the worms, the directional force of migration coming from the internal anatomical characteristics of the mosquitoes. It may be a little disconcerting to think that the successful migration of infective larvae within a

mosquito relies on such a fortunate accident. Such a simple hypothesis may later be shown to be a gross over-simplification of the way in which filarial infective larvae actually arrive in the proboscises of mosquitoes. It is possible that infective larvae use physiological cues with which they navigate within a mosquito. The anterior to posterior circulation of haemolymph or the greater concentration of a neurological substance or substances in the head may themselves act as cues. Nonetheless, this basic model of migration would still act as a template for more sophisticated and efficient navigational systems. Indeed, the behaviour described by this model alone may well have sufficed as a primitive method of migration for the evolutionary ancestors of the present "modern" worm.

Although the movement of the worm is essentially non-directional, the inherent tendency of larvae to move forwards not only assists migration within the mosquito but also aids transmission. For transmission, this is seen as a "tapping" or "pushing" action which probably enables the larvae to break out of the proboscis more easily and also assists skin penetration when the worms are on the surface.

But what of the part played by the mosquito in this relationship? For these insects, it is not simply a case of always being a helpless victim of a parasite as mosquitoes have an arsenal of anatomical and physiological mechanisms

which operate against the establishment of an infection. Nor is the mosquito a villain, benefiting itself by infecting some unfortunate vertebrate. Filarial infections of mosquitoes should be thought of as a disease of the insect, in the same way as which we think of disease in humans. Thus, the severity of the disease will be determined not only by the density of the infection but also by the hosts' individual physiology. In it's severist form a filarial infection of a mosquito will result in the death of the host. Less dramatically, it may lead to morbidity, effecting the "fitness" of an individual insect. In this study, flight and feeding behaviour were both shown to be effected by filarial parasitism and it would not be surprising to discover other behavioural responses which are similarly effected.

It should be stressed that one should not consider that there exists a clearly defined mortality or morbidity level relative to the intensity of infection, above which a detrimental effect always results and below which the mosquito is uneffected. More simply, both mortality and morbidity should be considered to be a density-dependent function of the parasite burden.

The detrimental consequences of parasitism do serve a function, analogous to, and often complimentary, to the role of predators or a limited resource in constraining the growth of a population of mosquitoes. The stability of this

host-parasite relationship is improved because the distribution of larvae within a population of mosquitoes is over-dispersed. However, a parasite-induced reduction in mosquito reproduction and time delays in the development of infective larvae in heavily infected mosquitoes will both tend to counteract this stabilising effect.

The effects of filarial parasitism on the morbidity and mortality of laboratory mosquitoes kept under near ideal conditions with low level infections (as occurs commonly in the field) is not marked. However, given the stresses present in the field, the effects of low level infections are likely to be more overt and of much greater significance.

It has been demonstrated in these studies that the efficiency of filarial transmission is delicately balanced and is strongly dependent on a number of environmental factors such as humidity, temperature and the nature of the biting surface upon which the mosquito feeds. When one considers the nature of both larval migration and transmission it seems even more remarkable that not only does this unusual relationship exist at all but that the prevalence of the disease is increasing, despite attempts to control it. A century ago, Sir Patrick Manson (1884) perceptively wrote "the journey from the uterus to the spot where maturity is attained is beset with dangers, the

measure of these dangers being the prodigious numbers that start on the journey."

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APPENDICES

Appendix 3.1 The number of mosquitoes autopsied 7-30 days post infection. The corresponding mean parasite burden (no. of larvae) and range of parasite numbers is also shown.

Time p.i. (days)	No. mosq.	mean no. larvae	range
7	162	6.3	0-57
8	188	5.6	0-49
9	120	4.3	0-28
10	194	3.2	0-31
12	206	2.6	0-26
15	199	1.9	0-23
20	139	1.1	0-17
25	140	0.9	0-11
30	91	0.5	0-11

Appendix 3.2 The number of mosquitoes autopsied with a low (1-3 larvae per mosquito), medium (4-9 larvae per mosquito) and high parasite burden (>9 larvae per mosquito) per host.

Time p.i. (days)	No. mosq. autopsied with respect to their parasite burden		
	Low	Medium	High
7	25	32	32
8	22	36	43
9	11	24	23
10	31	32	24
12	32	33	19
15	33	26	9
20	17	16	3
25	23	10	2

Appendix 5.1 The number of mosquitoes used for determining values of mean flight activity.

Time post blood meal (days)	Uninfected Control (A)	Infected Control (B)	1-12 larvae (C)	>12 larvae (D)
1	19	12	13	13
2	19	12	13	13
3	19	12	13	13
4	19	12	13	13
5	19	11	13	13
6	19	11	12	13
7	19	9	11	12
8	19	9	8	11
9	19	9	8	10
10	16	9	8	9
11	15	8	7	4
12	14	7	6	3

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