LABORATORY STUDIES ON THE BIOLOGY OF

ANOPHELES STEPHENSI LISTON₁

By

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PREFACE

The need for more laboratory work on the biology of the malaria vector, <u>Anopheles</u> <u>stephensi</u> Liston, was brought to the attention of the author by Dr. R. A. Ward, Head of the Malaria Section, Department of Entomology, Walter Reed Army Institute of Research. Since no comprehensive work on certain aspects of the laboratory biology of this mosquito could be found in the literature, the author selected as a thesis problem a study on the biology of <u>A</u>. <u>stephensi</u> Liston and its susceptibility to infection, under laboratory conditions, with the simian malaria organism, <u>Plasmodium</u> <u>knowlesi</u> Sinton and Mulligan.

The author wishes to express his appreciation to his major advisor, Dr. D. E. Howell, for his guidance and encouragement throughout the study and in the preparation of this paper.

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CHAPTER I

INTRODUCTION

Many workers have reported on studies involving the urban mosquito

vector of malaria in the Near East and Southwest Asia, Anopheles

stephensi Liston. Most of the studies have dealt with the bionomics of

this mosquito and its relation to malaria. Other papers have considered

specific problems of rearing A. stephensi in the laboratory. However,

some of the basic biological questions encountered when attempting to

rear A. stephensi have not been answered in the literature.

Meller (1962) published the most comprehensive study of the labora-

tory biology of A. stephensi in a comparative study of A. atroparvous and

A. stephensi. Since this species has been used successfully for the

transmission of laboratory strains of human and simian malaria and has

potential as a research tool in other mosquito studies, it was desired

to find more efficient methods of rearing this species under laboratory

conditions; thus, this study was initiated. Answers to the following

questions were needed: Under laboratory conditions what percentage egg

hatch could be expected; what effect would the number of mosquito larvae

placed in a given volume of water have on the adult size; how long would

it take for the larvae and pupae to develop; which of several simple

diets would be best for the larvae; could the water level or number of

pupa within a container affect the pupal and emerging adult mortality;

how long could a majority of the mosquitoes be expected to live; was a

maintenance diet of 5% sucrose and apples as good as honey-water and

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apples; what effect do different numbers of blood meals have on adult longevity; what percentage of the females would become inseminated; what would the laboratory egg production of the female mosquito be; does a relationship exist between mosquito age and parasite development stage with the infection of Plasmodium knowlesi Sinton and Mulligan in A. stephensi. These questions needed to be answered so that a vigorous colony of A. stephensi might be maintained for use in the laboratory. Some of the questions had been answered by Meller (1962) and others; however, it was necessary to know if the same biological responses were obtainable under our laboratory conditions.

CHAPTER II

REVIEW OF LITERATURE

ANOPHELES STEPHENSI AS A MALARIA VECTOR - W. M. G. Liston (1901)
described the mosquito Anopheles stephensi Liston in a paper based on a
one-year study of Anopheles in Ellichpur, India.

Colonel S. P. James (1902) first recorded A. stephensi in Calcutta,
India; however, none of the mosquitoes he dissected were infected with
malaria. Liston (1908) found that 25% of the A. stephensi from Calcutta
that he dissected were infected with malaria. De (1923) conducted a
detailed survey of the anophelines of India and found that only 1% of
1460 anophelines collected were A. stephensi. Covell (1927) reported
that A. stephensi was an important malarial vector found throughout
India. Basu (1930) found that the main source of the "malaria-carrying
A. stephensi" breeding in Calcutta was in the reservoirs of filtered
and unfiltered water in the center of the city. Covell (1932) referred
to A. stephensi as "the notorious carrier of malaria in Bombay, Delhi
and other cities of India." Russell (1936) incriminated A. stephensi
as a vector of malaria in Bombay, India. Afridi and Majid (1938) found
a prevalence of A. stephensi in the Bahrein Islands, it being the only
naturally infected anopheline mosquito of the islands. Afridi, Majid
and Singh (1938) found A. stephensi to be the only naturally infected
anopheline among those anophelines collected in Kutch State, India.
Senior White (1940) found that 1.8% of the anophelines collected near
Calcutta, India, were A. stephensi. Naturally infected A. stephensi

were also collected by Siddons (1943) in Calcutta. A. stephensi was
listed by Faust (1944) as an urban dwelling vector with extensive dis-
tribution throughout India, and the importance of this organism as an
urban vector of malaria in Calcutta was reported by Siddons (1946). He
stated that there was a definite increase in transmission of malaria in
Calcutta and that A. stephensi was the vector, although he could not show
a definite increase in the population of A. stephensi. Rafi (1955) re-
ported on what he called the "two well-known and established vectors of
malaria, A. culicifacies and A. stephensi." He found that these mosqui-
toes rested uniformly on the walls in houses of the Punjab area of India
and Pakistan. In a study of the fauna of Uttar Pradesh state of India,
Srivastava (1955) listed A. stephensi as an important malarial vector
under rural conditions in Western and Northwestern India, as well as the
chief vector of malaria in Bombay and Bangalore, India. He also stated
that within the state of Uttar Pradesh it had been incriminated as a
vector in several malaria outbreaks in Lucknow. Ansari and Nasir (1955)
reported that A. stephensi was an important malaria vector in Lahore,
Pakistan, constituting about 30% of the mosquitoes collected there.
Daggy (1959) reported that A. stephensi was the main malaria vector in
the oases of eastern Saudi Arabia. In 1962, Neogy and Sen incriminated,
for the first time, A. stephensi as a vector of malaria in rural Bengal,
India.

FIELD ECOLOGY OF A. STEPHENSI - Distribution - The distribution of
A. stephensi in India was well established by James (1902), Liston (1908),
De (1923), Covell (1927), Basu (1930), Russell (1936), Senior White
(1940), and others. Covell (1931), Rafi (1955), and Ansari and Nasir
(1955) reported on A. stephensi in Pakistan. Afridi and Majid (1938)

found A. stephensi prevalent in the Bahrein Islands. Collections of A. stephensi were made in Kabul, Afghanistan by Iyengar (1954). Daggy (1959) reported on an extensive study involving A. stephensi in Saudi Arabia.

Breeding Sites - In 1926, Chalam found A. stephensi was one of four anophelines collected in sea water near the shoreline in India. The water in which A. stephensi was found was about 90% sea water. Covell (1927) reported that A. stephensi was the most common Indian anopheline which bred habitually in wells and cisterns. He also found larvae of A. stephensi in river beds and other fresh water bodies, as well as in salt water pools in the Bombay area. Basu (1930) reported A. stephensi had been found to breed in 20 different types of artificial containers. Roy (1931[b]) stressed the importance of artificial containers as breeding sites of A. stephensi. Afridi and Majid (1938) reported on finding A. stephensi breeding in brackish water. Bana (1942) found A. stephensi breeding in salt pans in the Bombay area and noted that their breeding was reduced a substantial amount when larvivorous fish were introduced into their breeding sites. Artificial containers, considered to be the prime breeding site of A. stephensi in India, were found to be unimportant as a source of this mosquito in the oases of Saudi Arabia by Daggy (1959). He found drainage ditches and other natural sources of water in the oases to be the most important breeding sites.

Biting and Resting Studies - Strickland, Roy and Chowdhuri (1936) indicated that A. stephensi did not leave the native houses when feeding on humans; however, the largest percentage of engorged mosquitoes were found in cattle sheds. Their work also indicated a marked preference of A. stephensi to bite Indians rather than Europeans. Roy, Chandra,

and Siddons (1938) revealed by means of precipitin tests that 96.6% of
the positive reactions were with cattle antisera and 3.4% with human
antisera. These tests confirmed their observations and those of Strick-
land, Roy and Chowdhuri (1933) that A. stephensi feeds more commonly on
cattle than on man. Rafi (1955) found a uniform distribution of A.
stephensi on walls of houses in Calcutta, from the ceiling to the floor.
Daggy (1959) reported that in Saudi Arabia, A. stephensi was abundant in
either houses or stables during the day. He found a preference for the
dark areas of the thatch shelters as did Strickland, Roy and Chowdhuri
(1936). Daggy found as many as 500 to 600 engorged females per 12 to
15 in^2 area. He also stated that feeding began after sundown and that
the greatest activity occurred just before midnight.

Climatic Conditions and Flight Range - Strickland, Roy, and
Chowdhuri (1936) related seasonal prevalence of A. stephensi to high
humidity which followed rains in Calcutta. Afridi and Majid (1938)
reported the flight range of A. stephensi to be 1.5 miles, while Covell
(1944) stated that he believed it did not exceed 0.5 miles.

LABORATORY STUDIES ON A. STEPHENSI - Taxonomy - Sweet and Rao
(1937) found egg size differences and physiological differences, such
as adult longevity and willingness to feed on human blood in the labora-
tory in A. stephensi. On the basis of these differences, Sweet and Rao
established type B or A. stephensi type form and type M or A. stephensi
var. mysorensis. Rao, Sweet and Rao (1938) confirmed their previous
work on the existence of different types within A. stephensi. The
differences reported were that the mean ova measurements of A. stephensi
type form were 555 microns in length, 204 microns in breadth and there

were 18 ridges on one side of the float. Corresponding measurements of
A. stephensi var. mysorensis ova were 476 microns, 160 microns and 13
ridges per side. They also found that the type form colonized easily,
while they were unable to colonize A. stephensi var. mysorensis. Stone,
Knight and Starcke (1959) listed the two forms as A. stephensi Liston
and A. stephensi Liston and A. stephensi ssp. mysorensis. Also in 1959,
Foote and Cook, listed the two forms as A. stephensi stephensi and A.
stephensi mysorensis.

Rearing Procedures - The procedures used to rear A. stephensi in
other laboratories were reported by Trembley (1955) and Shute and Maryon
(1960).

Egg - Chalam (1927) found that the eggs of A. stephensi from the
Bombay, India area would remain viable for 12 days if left in harbor
mud and allowed to dry at room temperature. Roy (1931^a) collected eggs
on wet filter paper, wet cotton-wool, porcelain crucibles, earthen pots
and test tubes and allow them to be desiccated at 11° C, 24° C and room
temperature. Hatching did not occur after 144 hours in any of the
treatments. Eggs laid on both the filter paper and cotton-wool material
and held at 11° C remained viable the longest. Roy also showed that
when temperature effect was measured, A. stephensi eggs did not hatch
after an exposure of 164 hours at 11° C. Russell and Mohan (1939) re-
porting on a laboratory colony of A. stephensi type form, found that
they got equal number of eggs from both cow dung and tap water sources
of oviposition. Meller (1962), who studied the laboratory biology of
A. atroparvous and A. stephensi, carried out some tests similar to those
conducted by this author. Meller's work is the only wide scope labora-
tory biology study conducted with A. stephensi. He carried out these

studies at a temperature of 26° to 27°C (78.8° F to 80.6° F) and a relative humidity of 75% to 80%. The cages were 26 x 32 x 32 cm and the larval pan 35 x 35 x 5 cm in size. He collected 12,000 eggs of which 10,766, or 89.8% hatched. The average number of days required for hatching was 13.14 days.

Larvae - Roy (1931[b]) found earthen pots or enamel pans, in which grass had been placed, to be the most suitable larval rearing containers. He pointed out that the grass was used to maintain a cool water temperature, reduce cannibalism in the larvae, and to prevent scum from forming on the surface of the water. Powdered manure was found to be an excellent source of larval food. This study also showed the importance of not having the water in the containers deeper than several inches. He points out that the success of larval rearing depends on the density or crowding of the larvae, food source and depth of water in their rearing containers. Galliard and Golvan (1957) tested several diets for larval development. In their tests they used whole milk extract, thyroxine, vitamin B-12 and guinea pig feces, using powdered dog biscuits and powdered meat as controls. The whole milk extract was found to be the best diet for larval development. Ghosh and Bandyopadhyay (1958) increased the number of healthy and active adult A. stephensi by adding dried human blood to yeast powder in the larval diet. Meller (1962) found that under different levels of larval crowding, duration of the larval period varied from 8 to 13 days in the lower crowding levels (50 and 100 larvae per pan) and ranged up to 15 to 27 days (400 larvae per pan). At the 400 larvae pan level, a larval mortality of 32.3% occurred. Under the lower crowding levels, at which his pans were normally maintained, the number of days spent within the larval instars were: I, 1 to 3 days;

II, 2 to 5 days; III, 3 to 7 days; and IV, 5 to 14 days. He found an area of 1.8 cm^2 per larva was required as compared with 3.2 cm^2 found by Davidson (1958).

Adult - Mayne (1930) found the minimum effective relative humidity range following infection with malaria for A. stephensi to be 55% to 58%. Subba Rao, Sweet and Rao (1938) stated that there were differences in the breadth and length of wings in the two types of A. stephensi; however, these differences were so small as not to be practical as a tool in differentiation of the two types. Sweet, Rao and Rao (1938) found that cross-breeding between the types produced only a small number of females capable of laying viable eggs. Knowles and Basu(1942) found that "....at temperatures of 50° F to 80° F, a high degree of relative humidity does not seem to be essential for the longevity of A. stephensi...." adults, if sufficient food and water were provided. However, the highest survival rate occurred at 20° F and 50% relative humidity. Dakshinanurty and Sharma (1951[a] and 1951[b]) published on the temperature and humidity effects on A. stephensi. They found A. stephensi preferred a temperature of 25° C when gradients of 20° C to 25° C and 25° to 30° C were provided. They also indicated that when A. stephensi were tested at different relative humidity ranges of 20% to 60%, 40% to 60%, 60% to 80%, 40% to 80% and 80% to 100% at temperatures above and below 30° C, A. stephensi preferred the higher % in most ranges. In the 80% to 100% range, the A. stephensi chose the 80% relative humidity at temperatures above and below 30° C. Lal (1953) found the most favorable range of temperature for A. stephensi to be 25° C to 34° C, with 32° C being optimum. Bhatnagar, Bhatia and Krishnan (1958) found certain abnormalities to exist in the wings of the type form of

A. stephensi. The morphology and development of the salivary glands and their chromosomes in the type form of A. stephensi was reported by Rishikesh (1959). Ogden (1961), in testing an artificial method of providing blood for adult mosquitoes, found that A. stephensi fed more readily through an ox-cecum membrane than through a hog-gut membrane. Meller (1962) believed that the longevity of the female A. stephensi, regardless of nutrition, was dependent upon mating. The mean survival for mated females was 14 days and that for non-mated females was 17 to 18 days. Longevity of the males was also found to be dependent upon mating, with mated and non-mated males having a mean survival time of 10 days and 17 days, respectively.

Development and Mating - Russell and Mohan (1939) found that development from egg to adult took from five to nine days. Lal (1953) found the developmental period from egg to adult under his laboratory conditions to be 8.1 days. Roy (1931[a]) checked the insemination of mosquitoes held in different size containers and found a direct correlation between the size of container and the number of females which were inseminated. He reported no mating in test tubes, an 8.3% rate in 3.5- x 3.5-inch glass jars, 16.7% in a 8.5- x 6.5- x 5.5-inch cage, 33.3% rate in a 16- x 12- x 8.5-inch cage and 50% insemination rate in a 7.5- x 6- x 6-foot walk-in mosquito curtain container. Russell and Mohan (1939) discovered that ovarian development was not dependent on mating nor was a blood meal a prerequisite for mating.

MALARIAL RESEARCH WITH A. STEPHENSI - Knowles and Basu (1942), reporting on the effects of temperature and relative humidity on the infection of A. stephensi with several strains of human malaria, found

that at 70° F the heaviest mosquito salivary gland infection occurred
when using <u>Plasmodium</u> <u>vivax</u>. When infections were attempted with
<u>Plasmodium</u> <u>falciparum</u>, the heaviest infections were reported at 80° F.
They found that the temperature had more bearing than the relative
humidity on the infections of <u>A</u>. <u>stephensi</u> with strains of human malaria.
Russell, West and Manwell (1946) stated that the "wall" <u>Anopheles</u>, which
Ross dissected and in which he observed the oocyst of a human malarial
organism for the first time, was possibly <u>A</u>. <u>stephensi</u>. Basu (1947)
found "black spores" or densely pigmented and degenerated oocysts in the
gut when <u>A</u>. <u>stephensi</u> was infected with <u>Plasmodium</u> <u>falciparum</u>, <u>P</u>. <u>vivax</u>
or <u>P</u>. <u>malariae</u>. Mohan (1955) in a comparative study of <u>A</u>. <u>fluviatilis</u>
and <u>A</u>. <u>stephensi</u> found that the <u>A</u>. <u>stephensi</u> was highly susceptible to
infection with <u>P</u>. <u>falciparum</u>, although the colony he tested had been
maintained for 17 years. The colony still showed an eagerness to feed
on humans and became readily infected. According to Singh, Ray and Nair
(1949), the use of <u>A</u>. <u>stephensi</u> in laboratory studies with <u>P</u>. <u>knowlesi</u>
has been attempted by many workers without success. The early workers
were unable to demonstrate oocysts or sporozoites in the <u>A</u>. <u>stephensi</u>.
A non-virulent strain of <u>P</u>. <u>knowlesi</u> was successfully transmitted
through <u>A</u>. <u>stephensi</u> by Singh, Ray and Nair (1949). Hawking, Mellanby,
Terry and Winfrith (1957) were able to transmit <u>P</u>. <u>knowlesi</u> in monkeys
by injecting intravenously, infected <u>A</u>. <u>stephensi</u> on four different
occasions. Garnham, Lainson and Cooper (1957) in their study on the
tissue stages and sporogony of <u>P</u>. <u>knowlesi</u>, used <u>A</u>. <u>stephensi</u> as the
laboratory vector. Infections were obtained in monkeys by injecting
sporozoites from both the salivary glands and rupturing oocysts.

CHAPTER III

MATERIALS AND METHODS

SOURCE AND MAINTENANCE OF COLONY - The Anopheles stephensi used in this study were type form obtained from the Malaria Reference Laboratory, Horton Hospital, Epsom, England. The original colony was formed of mosquitoes collected from the Delhi, India, area. This work was carried out in the insectary and laboratories at the Walter Reed Army Institute of Research, WRAMC, Washington, D. C. A temperature of approximately 27° C and a relative humidity of 65% to 75% were maintained in the insectary. Lighting in the insectary was supplied by overhead lights and controlled by an electrical timer, as described by Levin, Kugler and Barnett (1958), to give a 14-hour constant light period preceded and followed by simulated dawn and dusk. Larvae were raised in 10- x 16- x 2.5-inch enamel pans. Adult mosquitoes in the stock colony were maintained in a 2- x 2- x 2-foot cage. In the stock colony and in all cages of the study not dealing with the effects of a maintenance diet, a maintenance diet of 5% sucrose in sterile water and sliced apples was provided for the adult mosquitoes.

EGG HATCH - Eggs from the stock colony were divided into lots of 100 on two different dates. The number of first instar larvae hatching from eggs in 72 hours was counted. The number of larvae hatching from eggs collected on the two dates was consolidated. Seven hundred eggs were chosen at random from the stock colony and divided into seven lots.

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One lot was placed in a wide-mouth pint jar of distilled water and allowed to hatch. The remaining six lots were placed on moistened filter paper inside petri dishes sealed with masking tape and placed in a household refrigerator at a temperature between 0° and 2° C. At two-day intervals, a petri dish containing eggs was removed from the refrigerator and the eggs were placed in a wide-mouth pint jar half full of distilled water. The eggs were given an opportunity to hatch in the insectary and the number of eggs which hatched was recorded.

EFFECT OF CROWDING IN LARVAL PANS ON ADULT WEIGHT - Eggs obtained from the stock colony were placed in enamel pans for hatching. After the majority of the eggs had hatched, within 24 hours of setting the pans up, the first instar larvae were transferred into three pans to obtain density levels of 75, 150, 300 and 600 larvae per pan. Each crowding level was replicated three times and each pan was filled with 2000 ml of distilled water. The larvae were fed on a yeast-water solution prepared by mixing one 0.5 gm dried yeast tablet in 50 ml of distilled water. The amount of yeast-water provided in each pan was based on the crowding level of the pan. On alternate days, 2.5 ml of yeast-water was added for each 75 larvae within the pans. The pans were placed on a shelf in the insectary and the pans' positions were rotated within each series daily. A rough count of the number of larvae in each pan within each instar was made in each pan daily. Upon pupation, the pupae were placed in bowls within four 9- x 12-inch cages corresponding to the different crowding levels, and the adults allowed to emerge. Nine days after pupation had begun, 15 female mosquitoes were taken from each of the cages and anesthetized with chloroform. The weight of each

of the 15 females, weighed to 0.001 mg, was obtained by the use of a
Cahn electro-balance. These weights were then used as an indicator of
the effect of larval crowding conditions upon the adult.

EFFECT OF LARVAL DIET ON ADULT WEIGHT - The diets tested were yeast-
water, dog chow and a combination diet in which the yeast-water was fed
to the larvae during the first two instars and dog chow was fed during
the last two instars. Eggs from the stock colony were hatched in dis-
tilled water. Seventy-five first-instar larvae were placed in a pan
containing 2000 ml of distilled water per pan. Each diet was represented
by four pans. Yeast water, prepared by mixing one 0.5 dried yeast tablet
in 50 ml of distilled water, was fed to larvae at the rate of 2.5 mg per
day and the dog chow, Pard Crunchers[1], was distributed at a rate of 100
mg per day. Food was added to the pans on alternate days. The larvae
were observed during the larval period and the number of larvae in each
stage was counted daily. Pans were rotated from bottom to top in each
series daily. As pupation occurred, the pupae were placed in three
cages and allowed to emerge. Nine days after pupation began, 16 female
mosquitoes from each cage were removed, anesthetized and weighed to
0.001 mg on the Cahn electro-balance.

LARVAL LONGEVITY - Larval longevity was observed and the percentage
of the larvae in each instar was counted during the larval density and
larval food studies.

EFFECT OF THE TYPE OF WATER USED IN LARVAL REARING ON ADULT WEIGHTS
One hundred and fifty eggs obtained from the stock colony were divided
in lots of 75 and allowed to hatch either in distilled water or in tap

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[1]Swift & Co., Pet Food Div., Gen. Off., Chicago, Ill.

water. Upon hatching, 50 first-instar larvae were placed in each of two pans which contained 2000 ml of either distilled water or tap water. The two pans were then placed on a shelf in the insectary. The larvae were allowed to develop to pupal stage. The larval pans were provided with dog chow at a rate of 100 mg per pan on alternate days. When pupation occurred, the pupae were placed in two cages and the adults allowed to emerge. Five days after pupation started, six females from each of the cages were placed in a drying oven for 24 hours and then removed and weighed to 0.001 mg on the Cahn electro-balance. The dry weights of the adult mosquitoes were used as an indicator of the effect of either tap or distilled water, used in larval rearing, on the adult mosquito.

PUPAL PERIOD - The length of the time period during which pupation occurred and the length of the pupal period were observed and recorded in several of the tests in this study.

EFFECT OF WATER LEVEL AND CROWDING OF PUPAE DURING THE PUPAL EMER-GENCE PERIOD - Twelve wide-mouth pint jars were placed at random in a 2- x 2- x 2-foot mosquito cage. The jars were divided into two water-level groups with three pupal density levels within each group. The water levels used were one inch and three inches, and the density levels were 75, 150 and 300 pupae per jar. The pupae were placed into the jars on four consecutive days, since there were not enough pupae available to fill all the jars on a single day. The 1-inch, 75 pupae; 3-inch, 75 pupae; 1-inch, 150 pupae; and 3-inch, 150 pupae jars were all set up the first day. The 1-inch, 300 pupae and 3-inch, 300 pupae jars were set up the second day; the 3-inch, 300 pupae jar on the third day; and the 1-inch, 300 pupae jar on the fourth day. The pupal emergence time and mortality of both the non-emerging pupae and drowned adult mosquitoes were recorded.

EFFECT OF MAINTENANCE DIET UPON ADULT LONGEVITY - Pupae obtained from larvae reared on dog chow were placed in six cages in emergence jars on six consecutive days. The test was divided into two treatments, a honey-water group and a sucrose-water group. The same number of pupae was placed in each of the corresponding cages on each day so that cage one of each treatment had 300 pupae placed in them over a 2-day period, cage two of each treatment received 300 pupae each over a 3-day period, and cage three of each treatment received 263 pupae over a 4-day period. The number of pupae and newly emerged adults dying within the pupal emergence jars was recorded until all pupation was completed. During the test, the number of adult male and female mosquitoes dying from all causes each day was recorded. Records were kept until 50% of the potential adults, minus those pupae and adults dying in the pupal emergence jars, had died. Twenty-four hours after the 50% mortality level had been reached, 20 adult females were removed from the cage and placed in a drying oven for 24 hours. The dry weights of 14 of these females were used as an indicator of the effect of the maintenance diet on the adult weight.

EFFECT OF THE NUMBER OF BLOOD MEALS ON THE ADULT AND ADULT LON-GEVITY - Pupae raised from stock larvae reared on a dog chow diet were collected over a 3-day period. Each day's collection of pupae was placed in individual holding cages. Fifty males and 50 females from the three holding cages were then placed in each of 12 cages. These cages were divided at random into four treatments of 0-3 blood meals per week groups. A rabbit was offered to the mosquitoes, as a blood source, on Monday, Wednesday and Friday. A maintenance diet consisting of a 5% sucrose solution and apples was provided in each cage during the test.

A record was kept on the number of males and females dying each day until 50% level of mortality had been reached. Twenty-four hours after the 50% mortality was reached, ten mosquitoes were removed from each cage and placed in a drying oven for 24 hours. The mosquitoes were weighed to 0.001 mg and the dry weights used as an indicator of the effect of blood meals on adult female mosquitoes.

INSEMINATION RATE - A small cylindrical cage, approximately 9 x 12 inches, was set up with 300 pupae obtained from the standard rearing pans of stock mosquitoes. These pupae were collected over a 4-day period. The number of non-emerging pupae and drowned adults was noted. Ten days after pupation had begun, mosquitoes in the cage were offered a blood meal from a rabbit. That same day, ten engorged and ten un-engorged females were removed, anesthetized, and placed on a depression slide in a drop of physiological saline. The tip of the abdomen was torn away from the body, by means of a dissecting needle, allowing for easy dissection of the spermatheca. The spermatheca was placed in a drop of physiological saline and covered with a cover slip. Gentle pressure was applied to the cover slip to cause the rupture of the sper-matheca. These slides were examined with the aid of a 43X objective and a 10X ocular to determine the presence of sperm in the spermatheca, and the presence or absence of sperm was recorded.

EGG PRODUCTION - A large number of pupae obtained from routine re-population of the stock colony was placed in an cylindrical holding cage approximately 9 x 12 inches in size and allowed to emerge. Ten days after emergence began, a rabbit was offered to the mosquitoes as a source of blood. One hundred engorged females from the holding cage

were placed into another cylindrical cage and provided with a small bowl of water for oviposition. This cage was kept on a shelf in the insectary. An additional 36 engorged females were placed in individual pint jar cages, the type described by Eldridge and Gould (1960). Each jar was provided with 5% sucrose solution and a small pill cup of water for oviposition. These jars were placed in an incubator box and kept at a temperature of 27° C. The cage of mosquitoes in the insectary was observed daily and the total number of eggs laid each day was removed and counted. Mosquitoes in individual jars were destroyed after ten days, and the number of eggs laid by each mosquito recorded.

SEX RATIO - Twenty pupae, picked at random from the repopulation pans for the stock colony, were placed in small pill cups within each of three jars. This procedure was repeated during the first six days of pupation. The number of males, females and non-emerging pupae was recorded.

INFECTION WITH PLASMODIUM KNOWLESI - Eggs obtained from the stock colony were allowed to hatch in tap water and reared on a dog chow diet. All of the pupae obtained were placed in an 18- x 42-inch cage. The pupae were allowed to emerge for three days and all of the remaining pupae were then removed. This procedure was repeated two times at 3-day intervals and once again seven days later in order to obtain mosquitoes of different ages. A rhesus monkey, Macaca mulatta Shaw, was injected intravenously with 1.0 cc of monkey blood infected with P. knowlesi at a parasitemia level of approximately 40 parasitized cells per 1000 RBC. The blood had been frozen after being heparinized and mixed 1:1 with 20% glycerol in Alsever's solution. The strain had been passed by intravenous inoculations six times since its procurement from

the Laboratory of Parasitic Chemotherapy, National Institute of Allergy and Infectious Diseases. The monkey was checked daily by means of blood smears prepared from blood obtained from the monkey's ear. Beginning when the parasite was seen for the first time, blood was checked several times daily using the same method and the level of parasitemia was determined. Two smears were prepared each time the blood was sampled. The slides were stained with Giemsa's stain and observed through the oil immersion lens of the microscope. The RBC, within the boundaries of an ocular grid, were counted in a total of 25 fields of vision on the two slides prepared in each sample. When a desired parasitemia level of 10 to 20 parasitized cells per 1000 RBC was reached the mosquitoes of different ages were placed in small feeding cages and allowed to feed on the abdomen of the infected monkey. Each feeding cage was filled with in excess of 100 pre-starved adult female mosquitoes. The mosquitoes in each cage were given the opportunity to feed on the infected monkey for one hour. After the feeding, the mosquitoes were anesthetized lightly with ether and the males and unengorged females removed. The cages containing engorged females were then placed in incubator boxes and held at 27° C. The engorged mosquitoes which were alive at the end of seven days were preserved for later oocyst determination by the method described by Ward (1962). Oocyst development was determined by first dissecting out the mosquito midguts and placing them in a drop of weak methylene blue stain on a slide. The slides were then examined at a magnification of 430X. The number of oocysts on each midgut was counted and recorded. The same general procedures were repeated on two additional attempts.

CHAPTER IV

RESULTS AND DISCUSSION

EGG HATCH - Under the conditions of this study, the mean % hatch of 1800 Anopheles stephensi eggs was 71.25 ± 3.71. All hatching was completed within 92 hours from the time of oviposition. The egg hatch was not as high as that reported by Meller (1962) who obtained an 89.8 % hatch. Differences in fertility and the formation of scum in two of the samples may have lead to a hatch rate which was 18.5% lower than that found by Meller. When cold storage at 0° to 2° C was attempted as a method of holding eggs of A. stephensi, decreased hatch occurred after four days. In the trial of cold storage as a means of holding eggs, the percentage of the eggs hatching in each of the 100 egg samples, after different length of cold storage, varied. The % hatching was 70% after 2 days, 74% after 4 days, 42% after 6 days, 5% after 8 days, 2% after 10 days and no hatch after 12 days of cold storage, as compared with a 75% hatch on the day the eggs were collected. It appears from this limited trial that cold storage is ineffective for holding A. stephensi eggs, except for short periods up to approximately 96 hours.

EFFECT OF CROWDING IN LARVAL PANS ON ADULT WEIGHT - The effect of larval crowding might possibly be measured in many ways. This author chose the use of the weight of the adult female mosquito because it tends to be more responsive to environmental changes during its develop-

mental period than does the male. Other authors, Davidson (1958) and Meller (1962), expressed their results in terms of a surface area requirement per larva, their findings being 3.2 and 1.8 cm^2, respectively. When expressed in this manner, the levels of larval crowding used in this test would be 5.41 cm^2 per individual mosquito at the 75 larvae per pan level, 2.71 cm^2 at the 150 level, 1.35 cm^2 at the 300 level, and 0.68 cm^2 at the 600 level. While the larval time within each instar tended to be relatively constant and the overall pupation times were very close at all crowding levels, the rate of pupation was different (Fig. 1). A direct bearing on the adult female weight was also noted. The mean weights of the female mosquitoes were 1.49 mg $\pm$ .06 for the 150 level, 1.31 mg $\pm$ .04 for the 300 level and 1.13 mg $\pm$ .04 for the 600 level. Statistically (Table 1), the F value for the linear response in adult weights for the groups was significant at the 0.1% level.

Since the slope of the linear response line is significantly different from zero and the quadratic and cubic curvatures are not significant, one might conclude that the response is linear with respect to the effect of crowding on adult weight. The 150 larvae per pan level was adopted for stock maintenance of the colony for practical purposes. This would indicate that water surface area required per larva is at least 5.41 cm^2 which is greater than that reported by either Davidson (1958) or Meller (1962). High larval mortality was noted in two of the three pans at the 600 level. The percentage of larvae dying in each of these pans was 50.0% and 36.2%. The third pan at the 600 larvae per pan level had a lower mortality rate of 28.9%. In only one pan was the mortality as high as that reported by Meller (1962) in which he had only 40.4% of
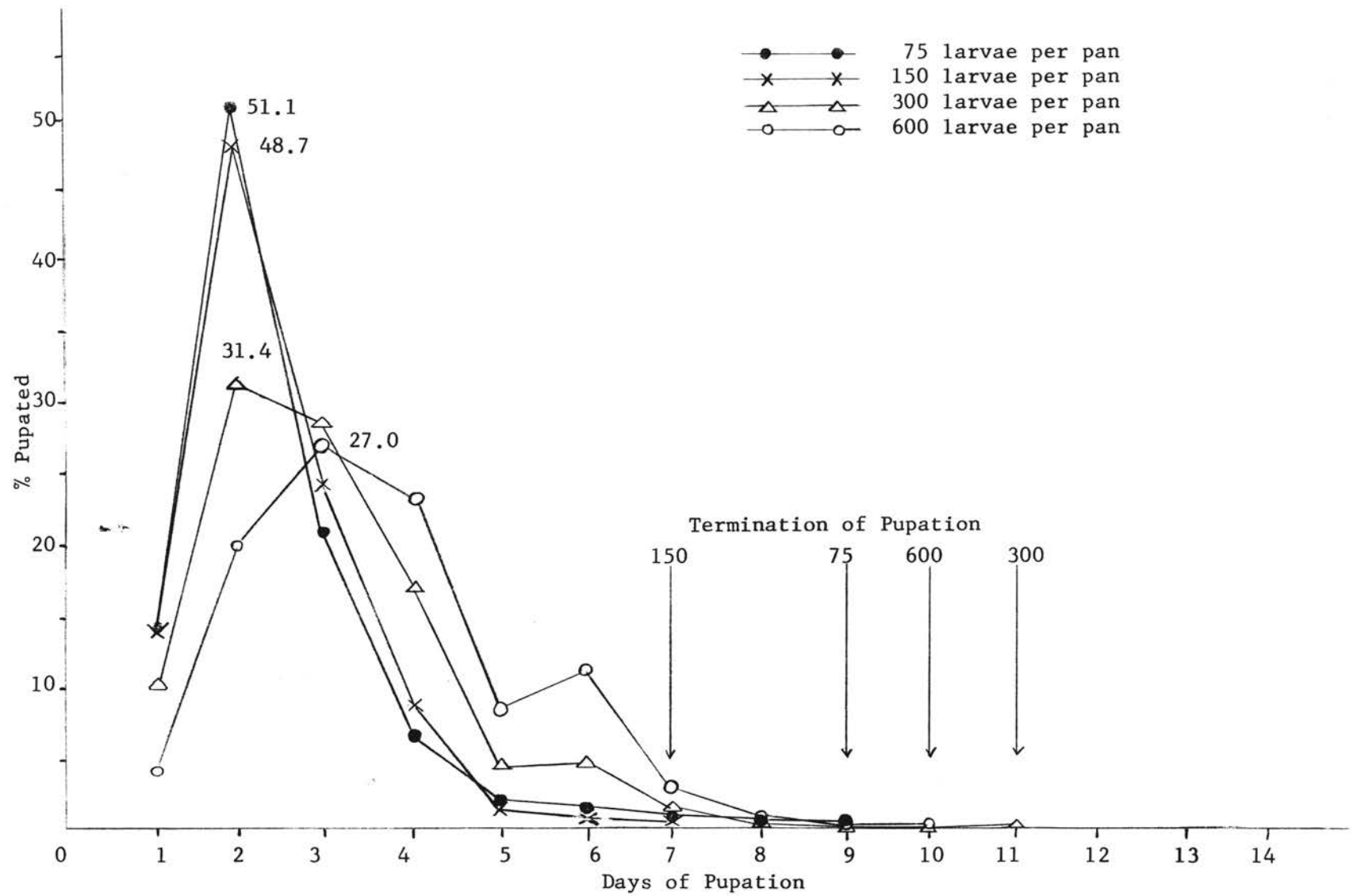
Fig. 1. Effect of larval crowding on pupation period.

Table 1. Effect of larval crowding on adult weight.

Source of Variation	df	SS	MS
Among groups	3	1.471	
linear	(1)	1.385	1.385
curvature	(2)	.086	
Within groups	56	1.894	0.032

400 larvae, reared in a 35- x 35- x 5-cm pan, reach the pupal stage of development.

EFFECT OF LARVAL DIET ON ADULT WEIGHT - The effect of the larval diets tested on the rate of development was so slight as to be impossible to determine by the observations made. There were some differences in the number of larvae dying during the larval period, a 45.3% larval mortality occurring among those larvae receiving the yeast-water diet and a 24.1% mortality rate among those fed the yeast-water for the first two instars and the dog chow the last two instars and an 18.3% mortality among the dog chow fed larvae. These mortality rates are much higher than those reported by Meller (1962) at either 50 or 100 larvae per pan levels. The effect of the diet on the length of the time over which pupation occurred (Fig. 2) did not appear to be different in the combination and dog chow diets. The yeast-water fed larvae had a slightly more extended pupation period. Using the weights of the female adult mosquito as an indicator, a significant difference occurred at the 1.0% level only between the weights of those mosquitoes obtained from larvae fed on the yeast-water diet and those obtained from larvae fed on dog chow (Table 2).
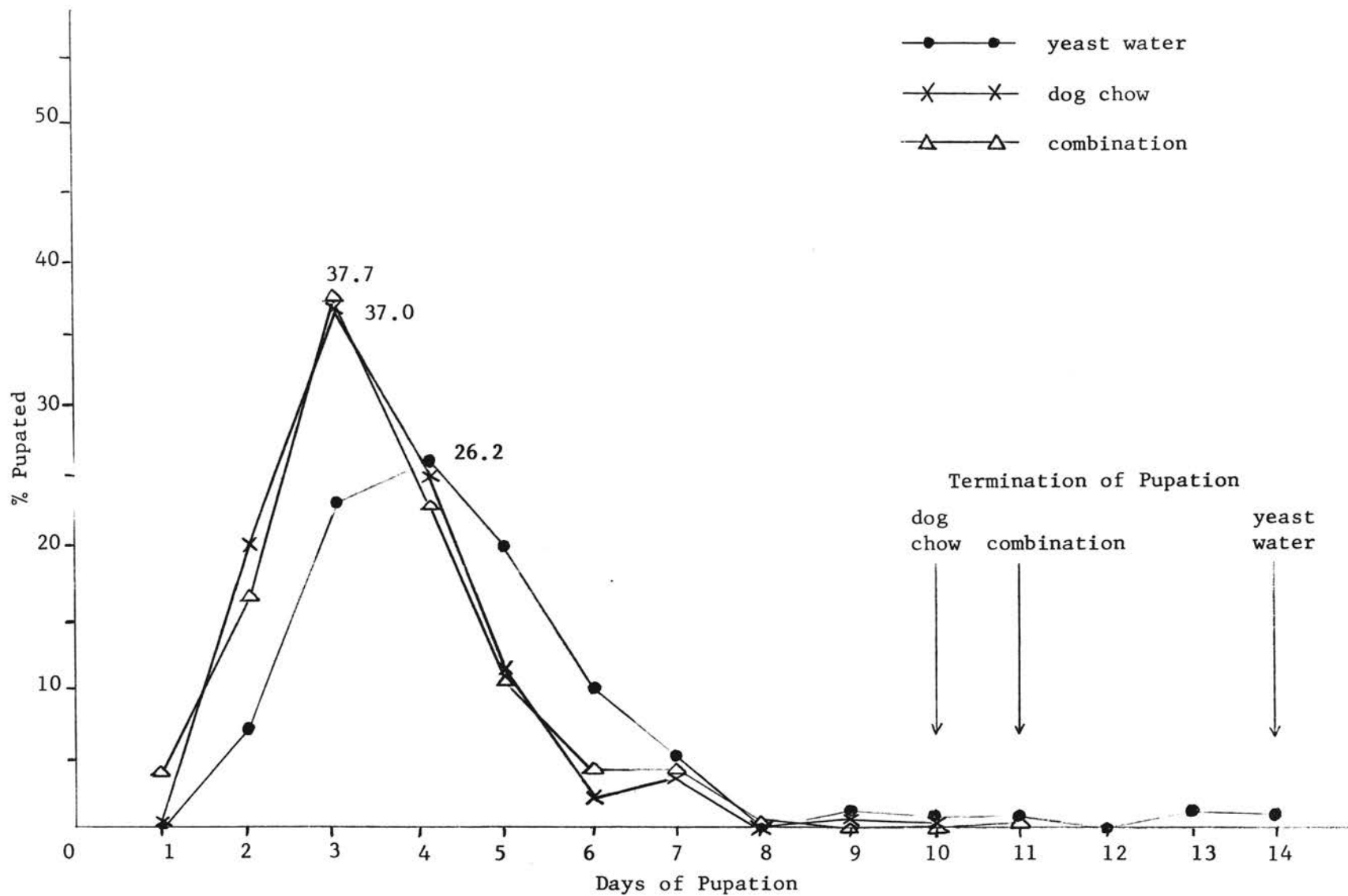
Fig. 2. Effect of larval diet on pupation period.

Table 2. Effect of larval diet on adult weight

Source of Variation	df	SS	MS
Among groups	2	0.413	
yeast-water vs dog chow	(1)	0.407	0.407
(yeast and chow) vs combination	(1)	0.006	0.006
Within groups	45	1.578	0.035

The mean weights obtained in the diet studies were 1.29 mg $\pm$ .04 on the yeast-water diet, 1.51 mg $\pm$ .06 on the dog chow diet and 1.38 mg $\pm$ .04 on the combination diet.

LARVAL LONGEVITY - The length of the larval period of A. stephensi varied only slightly under the different levels of crowding or types of larval diet. In the tests of different crowding levels, using yeast-water as a diet, the highest crowding levels of 600 larvae per pan showed a 24-hour lag in reaching the fourth instar; however, the first pupae appeared on the same day in all pans. The length of time over which pupation occurred was not appreciably longer in the higher crowding levels (Table 3). At neither the 300 nor 600 level was the larval period extended to the lengths reported by Meller (1962), who found at the 400 larvae per pan level that the larval period ranged from 15 to 27 days. In the diet test, having 75 larvae per pan, the larval period was extended somewhat, especially in the case of those larvae feeding on yeast water (Table 4). Under conditions such as existed during this study, a larval period of 10 to 20 days can be anticipated.

EFFECT OF THE TYPE OF WATER USED IN LARVAL REARING ON ADULT WEIGHT - When the eggs of the stock colony were obtained from the Malaria

Table 3. Length of larval period at different crowding levels.

Crowding level Larvae per pan	Larval period range (days)
75	10 - 16
150	10 - 14
300	10 - 18
600	10 - 17

Table 4. Length of larval period among larvae fed different larval diets.

Diet	Larval period range (days)
Yeast-water	12 - 24
Dog chow	11 - 20
Combination	11 - 21

Reference Laboratory, it was advised that the egg should be hatched and the larvae raised in distilled water. For this reason, distilled water was used without question at the time the study was initiated. When the size of the stock colony became large enough, the effects of distilled water and tap water on adult weight were determined. Two pans of larvae were raised in distilled water and tap water respectively. Using adult female weights as an indicator of the effect the different larval rearing media might have on the adult, no significant difference was observed.

PUPAL PERIOD - The length of time over which pupation occurred, in the larval crowding and larval food tests, differed somewhat (Fig. 1 and 2). In the 75-larvae-per-pan series of the crowding studies, the length of time over which pupation occurred in the three pans was 13, 16 and 17 days. In the comparable yeast-water series of the larval diet test, the pupae in the four pans of 75 larvae per pan took 11, 14, 14 and 15 days for all the larvae to pupate. In the crowding test the peak pupation of the 75-larvae series occurred on day two, when 51.1% of the surviving larvae pupated. The peak day in the diet test occurred in the comparable yeast-water treatment on day four, when 26.2% of the surviving larvae pupated. The time required for adults to emerge from pupae remained rather constant. In all observations, the surviving pupae became adults within 48 hours after they were taken from the larval pans. From observations in other tests, this author believes that the length of the pupation period varies from generation to generation; but the peak period occurs in the first several days. The length of time required for the adult to emerge appears to be a more constant factor, occurring between 24 and 48 hours after pupation.

EFFECT OF WATER LEVEL AND CROWDING OF PUPAE DURING THE PUPAL EMERGENCE PERIOD - It had been suggested that two factors might influence the number of pupae that fail to develop to adults and the number of adults which fail to escape from the pupal container. One factor, suggested as influencing pupal death, was crowding. A possible factor which might affect the adult death rate was suggested to be the distance from the water surface to the top of the jar which the newly emerged mosquito must negotiate. This was called water level. When the test had been concluded, the percentage of adults surviving at the

different pupal crowding and water levels were approximately the same (Table 5).

Table 5. Percentages of surviving adults from pupal emergence containers with different pupal crowding and water levels.

Water level	Pupal density		
	75	150	300
1 inch	97.3	98.7	98.3
	94.7	99.3	95.0
3 inches	98.7	98.7	97.7
	97.3	96.7	96.0

EFFECT OF MAINTENANCE DIET UPON ADULT LONGEVITY - In most laboratories rearing mosquitoes, a maintenance diet is fed to the mosquitoes until a blood meal or experiments require the elimination of the diet. At Walter Reed Army Institute of Research, the standard maintenance diet consisted of 5% sucrose sugar water in cotton-filled petri dishes and slices of apples. It was reported by personal communication to a member of the staff that a honey-water diet was being used with greater success than sucrose in water. When the test was set up, there were not enough pupae available on a single day to start all the replications simultaneously, so pupae from several different days during pupation were used. This procedure proved to have more effect on the results than the treatment itself (Fig. 3). The test was terminated when 50% mortality was reached. Beyond this level the researcher is working with a minority of his original population. In longevity tests conducted until all the members of a population are dead, one or more
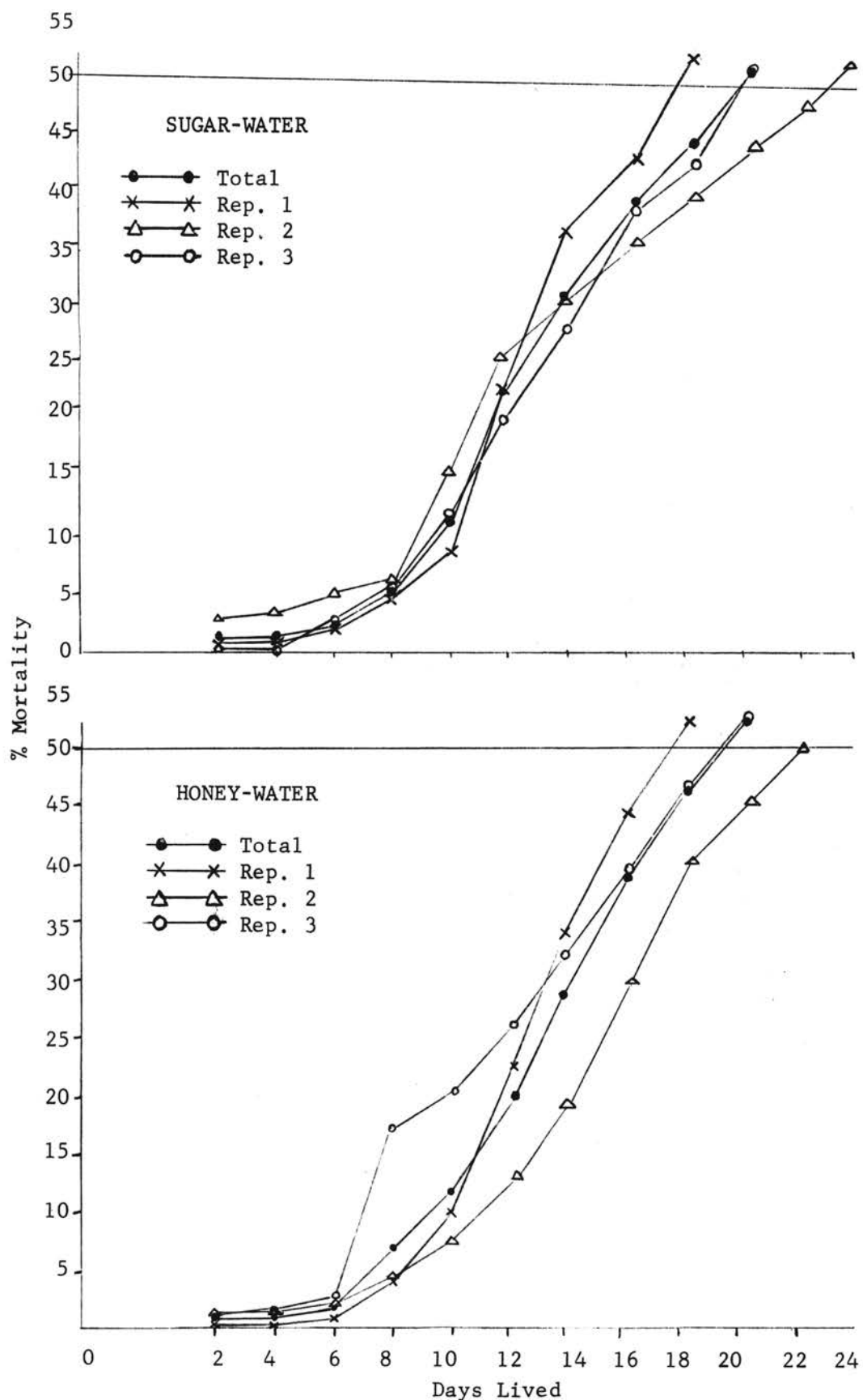
Fig. 3. Effect of maintenance diets on adult longevity.

individuals may live unusually long, thus distorting the mean longevity.
The average days to the 50% mortality level was reached in both treat-
ments within 24 hours of each other. It appears that neither honey
water nor sugar water extended the life of adult mosquitoes to any
appreciable degree over the other treatment. However, throughout the
test, the honey-water feeders developed mold within 24 hours after pre-
paration. Mold developed on both the petri dish and plug-type feeders.
Since the water and dishes used were sterilized, mold development in
the honey-water series, was possibly due to some contamination of the
honey. Under the conditions existing in this laboratory, the sugar
water, due to the lack of mold development with routine changing, is
to be recommended as the main portion of a maintenance diet.

EFFECT OF THE NUMBER OF BLOOD MEALS ON THE ADULT AND ADULT
LONGEVITY - The effect of the number of blood meals on the longevity of
the adult mosquito was desired for use in the maintenance of a colony
or its use in experiments of which blood meals are a part. Since the
female is the sex involved in the blood feeding on a host, the records
were kept separately for males and females. The mean number of days
required to reach the 50% mortality level in the females was the same
in the two blood meals per week, three blood meals per week, and the
controls (Table 6). Females that received one blood meal per week
reached the 50% mortality level somewhat faster than those in the other
treatments. Within this group, one of the three replicates was much
lower than the other two. The response of the males to the different
conditions under which their mates were placed was very erratic, and no
relationship between blood meals and male weight could be shown.

Table 6. Days to the 50% mortality level in mosquitoes that received
different numbers of blood meals per week.

Blood meals per week	Day 50% mortality obtained	
	Males	Females
0 - Controls		
a.	17	25
b.	21	22
c.	17	22
Mean	18.3	23.0
1		
a.	14	17
b.	14	24
c.	13	21
Mean	13.7	20.7
2		
a.	13	23
b.	16	24
c.	13	22
Mean	14.0	23.0
3		
a.	12	23
b.	10	24
c.	16	22
Mean	12.7	23.0

INSEMINATION RATE - In an attempt to see if there might be an in-
dication of mated mosquitoes tending to feed when a blood meal was
offered, the insemination of engorged and unengorged females was checked.
A check of 20 females, 10 engorged and 10 unengorged from a population
obtained from 300 pupae, for three consecutive days, revealed the in-
semination rate to be from 40 to 50% in both the engorged and unengorged
groups.

EGG PRODUCTION - The potential egg production of a female mosquito
which had fed and mated once, was checked by the methods indicated in
the material and methods section. The mean number of eggs laid per
female in the 36 randomly picked, engorged females was 50.4 $\pm$ 6.5. When
discounting those mosquitoes which, for lack of insemination or some
other factor, did not lay eggs, the mean number of eggs laid was 75.7 $\pm$
3.3. The overall mean of the 100 engorged females in a single cage was
51.3 varying 0.9 from the mean number of eggs derived by the individual
per jar method.

SEX RATIO - The sex ratio of males to females in the laboratory
strain of A. stephensi was presumed to be almost equal. Since it is im-
practical to maintain pans after the bulk of the larvae have pupated, it
was desirable to check the sex ratio of mosquitoes emerging from pupae
picked over a 6-day period. The graph (Fig. 4) shows the expected ratios
of more males than females at the beginning of pupation and more females
than males at the end of the period. Due to the small sample size in
this test, no conclusions could be drawn without further testing.

INFECTION WITH PLASMODIUM KNOWLESI - An attempt was made to see if
the erratic infection rate of A. stephensi with the simian malaria,
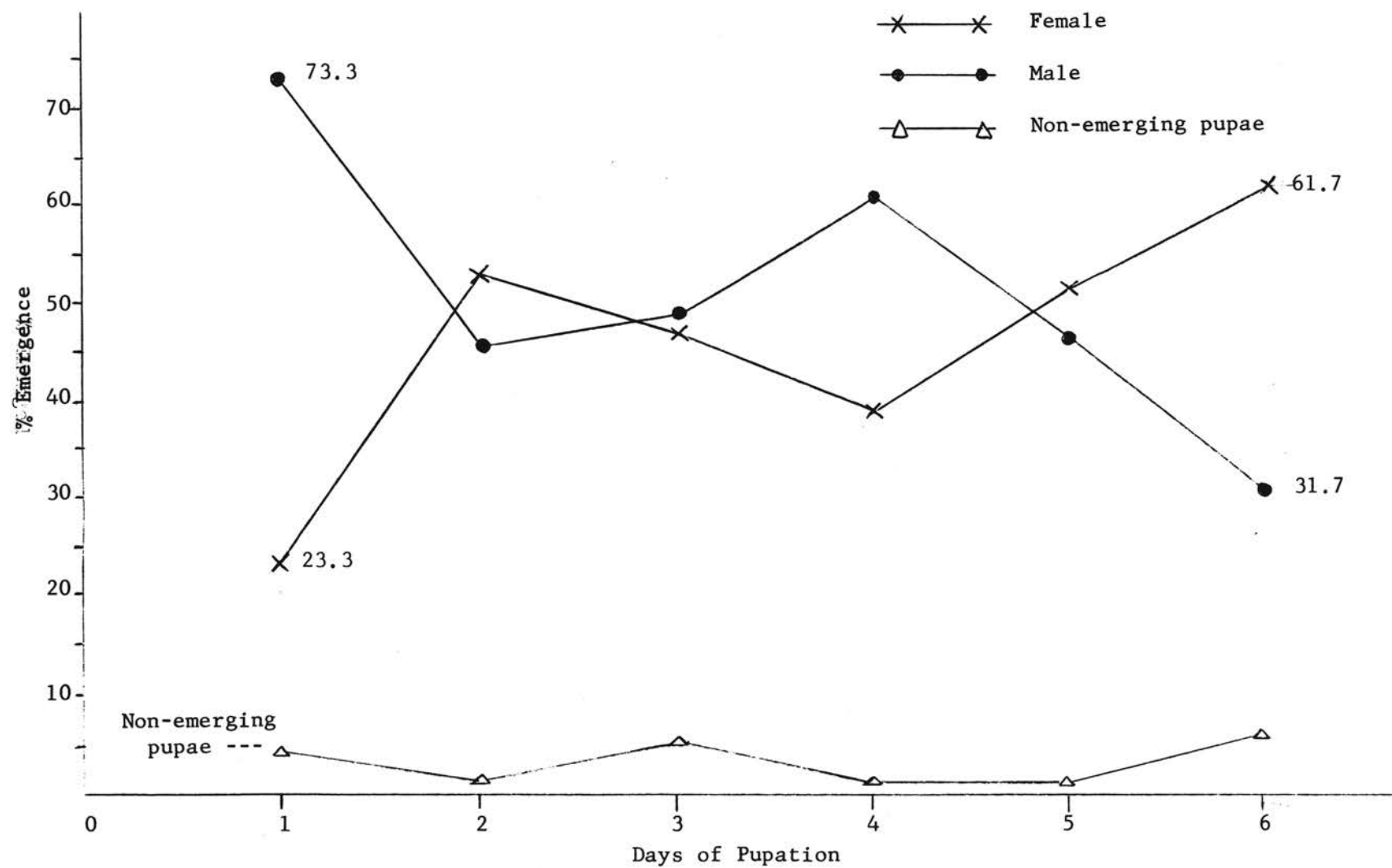
Fig. 4.   Daily sex ratio of pupae.

Plasmodium knowlesi Sinton and Mulligan, could be related with the age of

the mosquito feeding on the monkey or the stage of the parasite develop-

ment at the time the mosquiotes fed. Three attempts were made to get an

indication of some relationship, but with each attempt the majority of the

engorged mosquitoes failed to live long enough for oocyst formation.

Further studies of this nature would have to be at a much larger scale,

so as to have sufficient numbers of mosquitoes of all ages. The problems

of maintaining infected A. stephensi in the incubators over the period

required for oocyst development would also have to be solved. Light in-

fections occurred in the 1- to 4-day-old mosquitoes in each of the four

parasitemia levels, at which the mosquitoes were allowed to feed in the

first attempted test. The oocysts were found on the midguts of 7 of 26

2-day-old mosquitoes fed at a parasitemia of 16.48 parasitized cells per

1000 RBC, with the range of the number of oocysts per gut being 2 to 14;

6 of 23 2-day-old mosquitoes fed when the parasitemia was at 43.40, para-

sitized cells per 1000 RBC, with a range of 1 to 16 oocysts per midgut;

and when 3-day-old mosquitoes fed at a parasitemia level of 109.91 para-

sitized cells per 1000 RBC, 1 of 3 surviving mosquitoes developed 45

oocysts. Three of 40 4-day-old mosquitoes developed from 2 to 7 oocysts

when fed at a parasitemia level of 277.18 parasitized cells per 1000 RBC.

In the second attempted test, no infection was observed at a parasitemia

level of 31.35 parasitized cells per 1000 RBC. At a parasitemia of 52.06

parasitized cells per 1000 RBC, mosquitoes of 3, 8 and 14 days all devel-

oped oocysts. The heaviest oocyst development observed in any of the

attempts occurred with the 3-day-old mosquitoes at this level, with 23 of

26 surviving mosquitoes having oocysts ranging from 3 to 159 oocysts per

midgut. The 8-day-old mosquitoes group had oocysts ranging from 1 to 121 in 8 of 16 surviving mosquitoes and the 14-day-old mosquitoes group had oocysts ranging from 1 to 71 oocysts in 3 of 7 surviving mosquitoes. Only very light infections were observed in the third attempt. The predominance of any one stage of parasite development at the time the mosquito fed could not be correlated to the unusually heavy oocyst development observed at the 52.06 parasitized cells per 1000 RBC level. This was 1 of 2 parasitemia levels at which feeds were made when the mature trophozoites outnumbered the ring-stage trophozoites. The other parasitemia level at which this was observed was at the 277.18 parasitized cells per 1000 RBC level, in the first attempt. At that level only three of 40 4-day-old mosquitoes developed 2, 4 and 7 oocysts per midgut.

CHAPTER V

SUMMARY

Laboratory studies were conducted to determine certain aspects of the biology of a newly established colony of Anopheles stephensi Liston at the Walter Reed Army Institute of Research.

Measurements were made on the egg hatch, effect of larval crowding and larval diet on the adult, the length of larval and pupal periods, effect of distilled and tap water as the larval rearing media on the adult, and the effect of pupal crowding and water level of pupal emergence container on mortality of pupae and newly emerged adults. The effect of two different maintenance diets on adult longevity, effect of different numbers of blood meals per week on adult longevity, the rate of insemination, egg production, sex ratio of the colony, and certain responses to infection with Plasmodium knowlesi were also studied. The mean % egg hatch of an 1800 egg sample was 71.25 ± 3.7. Cold storage 0° to 2° C for holding of the eggs was found to reduce the hatch rate of A. stephensi after 96 hours. The effect of crowding on the larvae in the larval rearing pans was shown to be linear in response. This would indicate that water surface area required per larva to be at least 5.41 cm^{2}, which is greater than that reported by either Davidson (1958) or Meller (1962). The larval diet test, in which yeast water (the diet this strain of A. stephensi had been maintained on at the time it was obtained), dog chow and a combination of the two were tried, showed the dog chow diet

produced heavier mosquitoes with less larval mortality. Larval longevity in the laboratory ranged from 10 to 18 days. When larvae were raised in tap water or distilled water, no difference was observed in the mean weights of female adults. The pupal period ranged between 24 and 48 hours in all observations. Neither the crowding level of pupae nor the water level within the pupal emergence container had any effect on the number of pupae which failed to develop or newly emerged adults which drowned. When 5% sucrose in water or 5% honey in water were tested as the main portion of a maintenance diet, the sugar water proved to be more suitable than honey water, due to mold formation on the latter. No effect on the longevity of the mosquitoes was noted. No apparent effect due to the number of blood meals was noted in tests in which 0 to 3 blood meals per week were offered the mosquitoes. The insemination rate of the colony was found to be between 40 and 50% in both engorged and unengorged fe- males. The number of eggs laid per female for the colony as a whole was 50.4 ± 6.5 and 75.7 ± 3.3 when considering only those mosquitoes actually laying eggs. The sex ratio of mosquitoes emerging from pupae collected daily for six days showed a heavy male population on the first day, de- creasing to a minority of the daily population on the sixth day of pupa- tion, with the opposite response for the females. No conclusions could be drawn from the attempts to correlate infection of Plasmodium knowlesi Sinton and Mulligan in A. stephensi with the age of the mosquito or the stage of parasite development at the time the mosquito fed. In one of the trials, an unusually large number of oocysts developed on the midguts of a group of the mosquitoes.

The information obtained in these tests gives an indication of certain biological responses of A. stephensi Liston under insectary and

laboratory conditions.

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