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Relationship between larval habitat characteristics and malaria vectorial capacity of adult Anopheles dirus in Chanthaburi Province, Thailand.

Sangvorn Kitthawee
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RELATIONSHIP BETWEEN LARVAL HABITAT CHARACTERISTICS AND MALARIA VECTORIAL CAPACITY OF ADULT *ANOPHELES DIRUS* IN CHANTHABURI PROVINCE, THAILAND

A Dissertation Presented

by

SANGVORN KITTHAWEE

Submitted to the Graduate School of the
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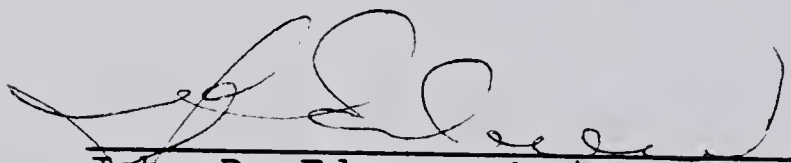
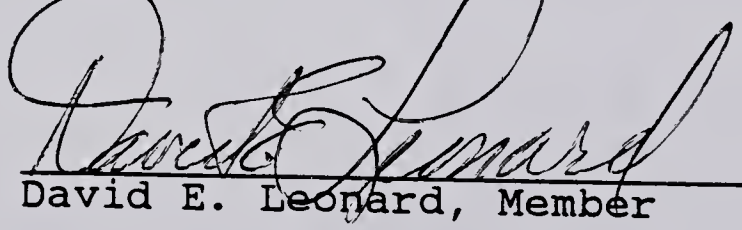
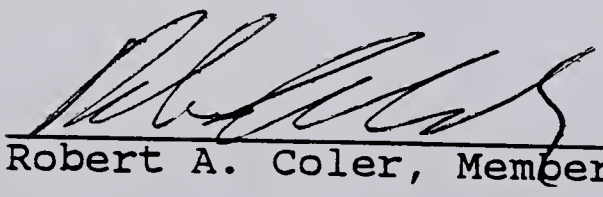


John D. Edman, Chairperson of Committee



David E. Leonard, Member



Robert A. Coler, Member

John D. Edman, Head
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To my parents who have shaped my life.

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ABSTRACT

RELATIONSHIP BETWEEN LARVAL HABITAT CHARACTERISTICS AND MALARIA VECTORIAL CAPACITY OF ADULT *ANOPHELES DIRUS* IN CHANTHABURI PROVINCE, THAILAND

FEBRUARY 1990

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Field studies were conducted in Tha-Mai District, Chanthaburi Province. Larval habitat characteristics of 42 gem pits were examined from November 1986 through June 1988. Larvae were found in pits containing clear water under full or partial shade. Relationships between habitat characteristics and density were tested by stepwise regression analysis. High dissolved oxygen and humidity and low pH were associated with higher densities. Populations fluctuated with rainfall. Stepwise logistic regression analysis was used to predict the occurrence of *An. dirus*. Populations were positively correlated with humidity and collection water temperature but negatively correlated with pH and minimum water temperature. Variations in occurrence and density also were related to predators (Notonectidae and fish).

High minimum water temperature and rainfall were correlated with decreases in wing length among both emerging males and females. High turbidity was associated

with increased size in male but not in females. Seasonal variation in the size of both sexes was related to rainfall. Minimum water temperature, pH, protein and rainfall were correlated with the survivorship of emerging *An. dirus*. Adult size was positively correlated with survivorship but not as strongly as in lab-reared populations.

Mean wing length of nulliparous females (3.035 mm) was not significantly smaller than parous females (3.039 mm). However, there was a significant difference when seasonal variation was considered. Since size variation was correlated with rainfall and minimum air temperature, females tended to be smaller in the rainy season and larger (with a higher parity rate) in the dry season. On average, >40% of dry season *An. dirus* live long enough to complete the extrinsic incubation period for *Plasmodium falciparum*. Both *P. falciparum* and *P. vivax* were detected and mosquito infection rates determined by ELISA. Malaria sporozoites were found in *Anopheles dirus*, *vagus*, *tessellatus* and *hyrcanus* group so all are potential vectors in this area.

Four size classes of *An. dirus* were reared from different larval densities. Higher densities produced smaller adults with lower survivorship. Larger females took larger blood meals and thereby were more susceptible to infection by artificial feeding with cultured *P. falciparum* gametocytes.

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CHAPTER I

INTRODUCTION

Malaria remains the most important arthropod-borne disease in Thailand. *Anopheles dirus* (as *balabacensis* Baisas) is known to be the primary vector in hilly or forest areas (Scanlon and Sandhinand 1965). This mosquito has proven difficult to control with residual sprays because it tends to avoid insecticide-treated surfaces or to not remain long enough to receive a lethal dose (Ismail et al. 1975).

Since transmission by *An. dirus* appears to take place primarily outdoors, the spread of rural malaria in forested and hilly areas poses a serious problem for malaria control programs. The ultimate aim of any vector-borne disease control strategy is the interruption of transmission. Much attention has been directed toward chemical and biological control methods, but little has been devoted towards understanding the ecology of vectors. Newly proposed integrated vector control strategies require an understanding of larval ecology, adult vectorial capacity, and transmission dynamics.

Vectorial capacity (VC) is the probability that a species population or individual arthropod will successfully acquire, incubate, and transmit a disease pathogen from an infected to susceptible human hosts. It

is usually expressed as the daily parasite inoculation rate.

Adult body size within a vector population may influence the probability of transmission; larger mosquitoes generally live longer (Hawley 1985). Vector survival rate is an important component of vectorial capacity (Macdonald 1957). If certain larval habitats produce longer-lived adults that have more contact with humans, these are the populations that should receive control priority. Consequently, this research emphasizes larval ecology and its relationship to the vectorial capacity of *An. dirus* for malaria. I proposed to answer the following questions:

1. What factors effect or regulate the population density of *An. dirus*? Which biotic (e.g., predators) and/or abiotic (e.g., temperature, pH, turbidity, dissolved oxygen, rainfall) factors are most important?

2. Does habitat quality relate to adult productivity in nature? Is it temperature or food abundance which determines the adult size and survivorship of *An. dirus*?

3. Is adult size related to seasonal and local variation in larval habitats?

4. Is body size an important measure of the vectorial capacity of individual females? How do large and small females vary in their natural survivorship and malaria infection rate?

5. Does female size effect survivorship and susceptibility to cultured malaria parasites (*Plasmodium falciparum*) in the laboratory?

Site Description

The study area is located at latitude 12^0 37′ N and longitude 102^0 07′ E or about 240 km southeast of Bangkok, the capitol of Thailand (Fig. 1). It comprises two villages in the Tha-Mai District of Chanthaburi Province (Fig. 2). The total study area was approximately 3.36 km^2 and contained a population of approximately 342 persons living in 61 houses (1986 data from Tha-Mai District Central Office).

Tha-Mai District receives a particularly heavy rainfall during much of the year. The major occupation of the people living in this area is fruit production. The orchard plantations of mixed trees are superficially similar to the original tropical rain forest. Hand digging of gems (sapphire, topaz, emerald, etc.) is often a secondary occupation carried out during the dry season. As a result, this area has numerous old gem pits which have filled with water (Fig. 4). Most of the pits are located near small permanent streams. The 42 gem pits and concrete-lined wells (Fig. 3) selected for this study were concentrated in hilly areas with many trees (durian, rambutan, coconut, mangosteen, banana, and beetlenut) within settlement 5 of Baupu village and settlement 8 and 9

of Sri-Phraya village (Fig. 2). They represented a broad range of sizes, water depths, levels of shade, etc.

Figure 1. Map of Thailand, showing the location of Chanthaburi Province.

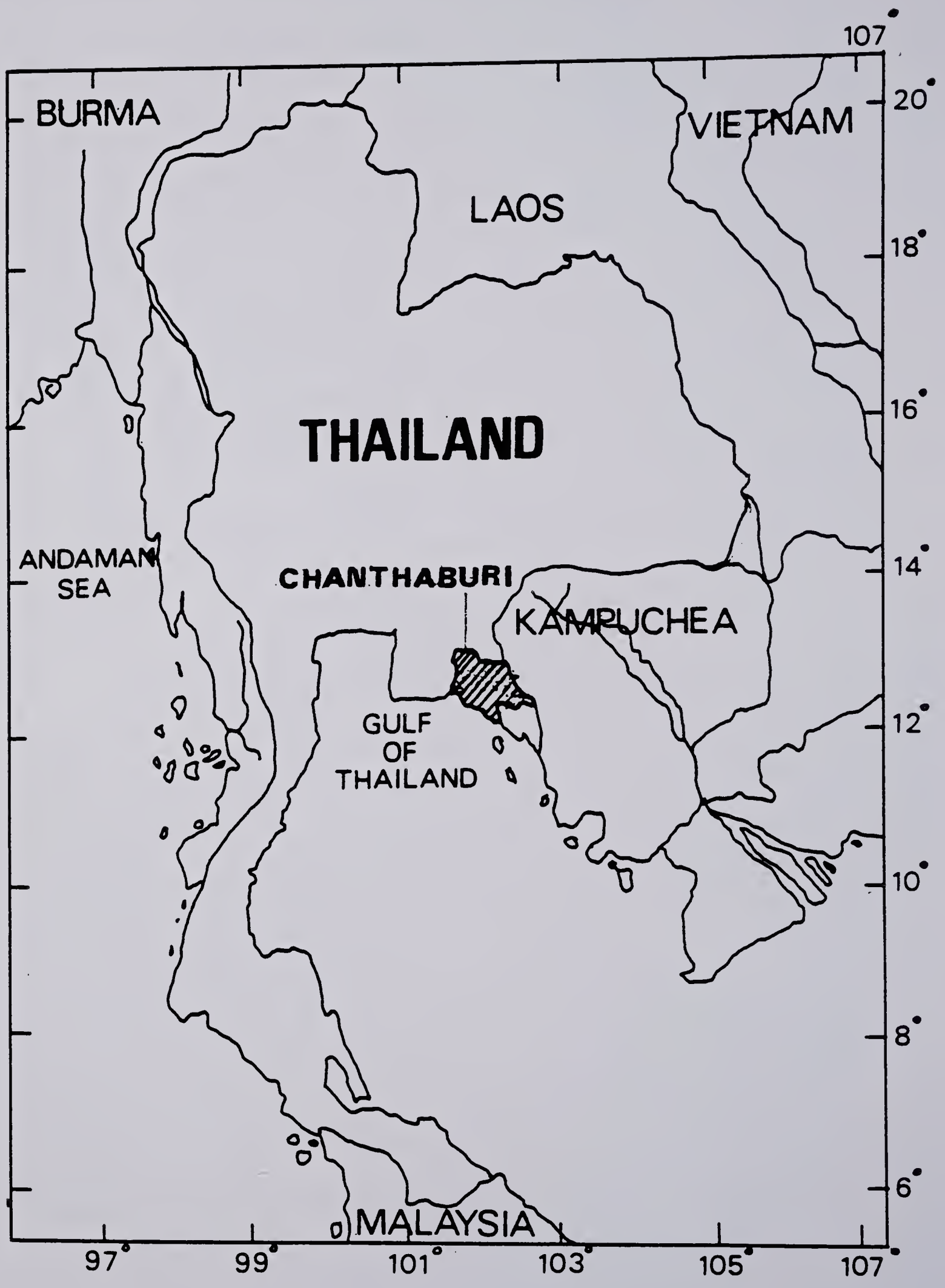
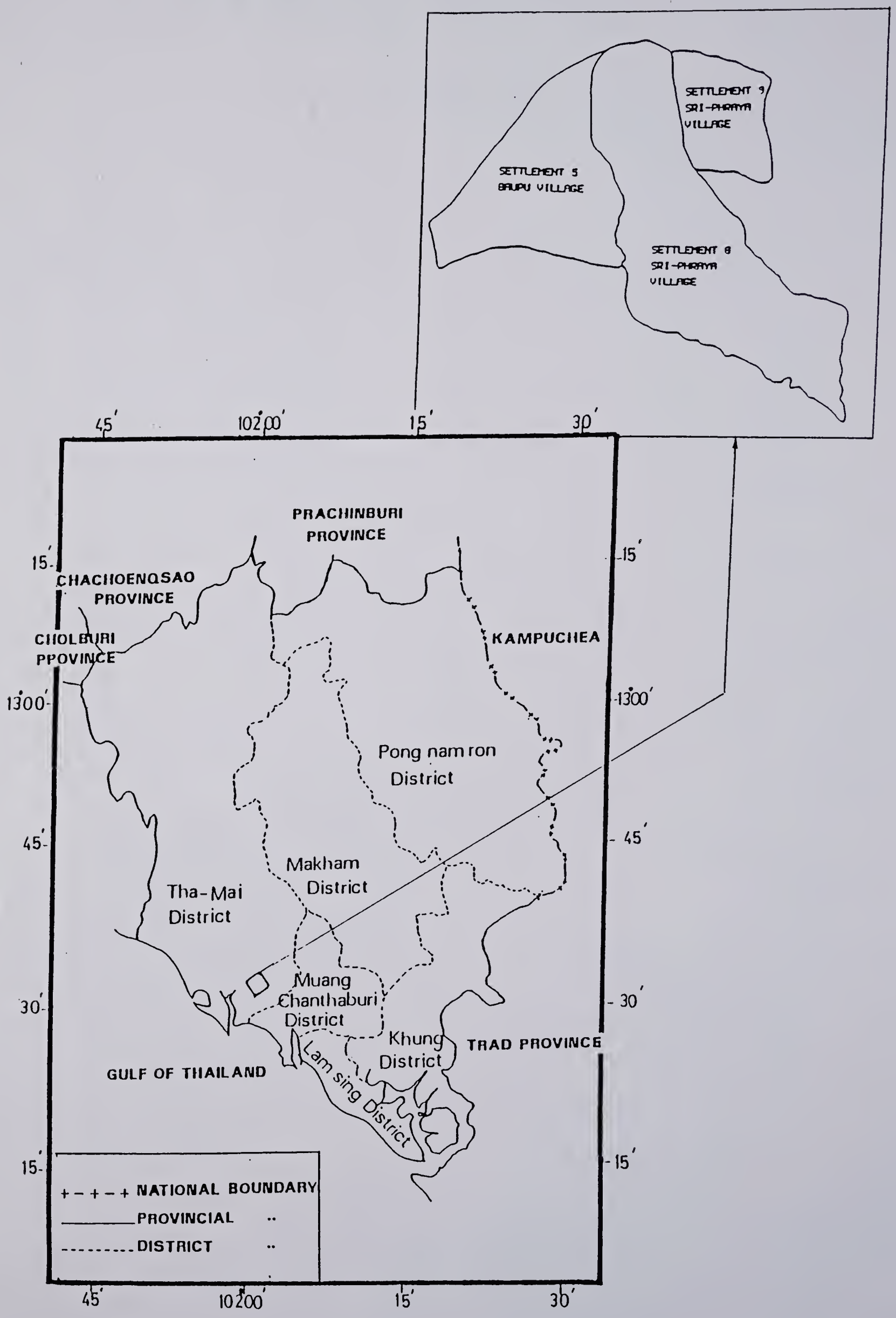

107°
BURMA
VIETNAM
20°
LAOS
18°
THAILAND
16°
ANDAMAN
SEA
CHANTHABURI
14°
KAMPUCHEA
GULF
OF
THAILAND
12°
10°
8°
6°
MALAYSIA
97°
99°
101°
103°
105°
107°

Figure 2. Location of settlement 5 of Baupu Village and settlements 8 and 9 of Sri-Phraya Village within Tha-Mai District, Chanthaburi Province.


SETTLEMENT 9
SRI-PHRAYA
VILLAGE
SETTLEMENT 5
BAUPU VILLAGE
SETTLEMENT 8
SRI-PHRAYA
VILLAGE
45'
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15'
30'
15'
CHACHOENGSAO
PROVINCE
PRACHINBURI
PROVINCE
CHOLBURI
PROVINCE
KAMPUCHEA
1300'
Pong nam ron
District
45'
Makham
District
Tha-Mai
District
Muang
Chanthaburi
District
30'
Khung
District
TRAD PROVINCE
GULF OF THAILAND
Lam sing District
15'
+ - + - + NATIONAL BOUNDARY
PROVINCIAL ..
DISTRICT ..
45'
10200'
15'
30'

Figure 3. Diagram showing approximate location of 42 test pits in settlement 5 of Baupu Village and settlements 8 and 9 of Sri-Phraya Village.


a
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Figure 4. Photographs of gem pits.
(A). Old, shaded pit under dense vegetation.
(B). Concrete-lined well in open area under shade trees.
(C). Exposed pit under full shade.
(D). Close up of same pit (C) when partially shaded.


A
B
C
D

CHAPTER II

LITERATURE REVIEW

Malaria in Thailand

Malaria is recognized as a major public health problem and socio-economic stress in most tropical countries. In Thailand, it was historically the greatest cause of death and sickness in the population (Harinasuta et al. 1976). In 1956, the World Health Organization (WHO) initiated a major campaign to rid the world of malaria. This campaign was based on the use of the cheap and effective, residual insecticide DDT. After a post-WW II period of significant reduction, malaria has resurged almost everywhere in recent years. The main causes for this increase in Thailand are:

1. The human host: Thailand has a large population migration precipitated by rapid growth and attendant economic pressures, coupled with low agricultural productivity in certain areas. The intensity of population movements varies from time to time. One particularly important consequence of this population instability is the penetration of Natural Forest Reserves for various purposes such as cultivation, search for timber, gem mining, hunting, and collection of fruit and bark. Man-mosquito contact involving infected migrants creates continuing difficulty in the eradication of malaria (Wessen 1972).

2. The vector: The major vector in Thailand at the present time is *Anopheles dirus* (as *balabacensis* Baisas) (Peyton and Harrison 1979). Its prevalence coincides with the distribution of forests and forest fringes, which provide favorable humidity, shade, and aquatic sites (seepage pools) for development (Scanlon and Sandhinand 1965). Transmission of malaria by this species is extremely difficult to control by insecticide spraying due to its exophilic and exophagic behavior. Epidemiological data from Ismail et al. (1975) showed a decrease in malaria incidence after house spraying in cleared zones but not in the forest fringe and deep forest zones.

3. The parasite: *Plasmodium falciparum* parasites have developed resistance to conventional antimalarial drugs such as chloroquine (Powell et al. 1964) and sulfadoxine with pyrimethamine (= Fansidar) (Bruce-Chwatt et al. 1981).

4. Other factors: Importation of malaria cases (and possibly vectors) from neighboring countries (e.g., Kampuchea) where control programs do not yet exist can further increases the complexity of the problem, especially in border areas.

Malaria and Its Eradication

The malarial control organization of Thailand has divided the country into non-transmission areas, consisting of the major part of country with a population of approximately 38.5 million, and transmission areas, consisting of forest hills, mountains, border areas, and unsecured areas containing about 10.5 million (Prasittisuk 1985). The proportion of cases by each parasite species in 1982 were 65.3 % *P. falciparum*, 34.4 % *P. vivax* and 0.02 % *P. malariae*. Chloroquine resistant strains of *P. falciparum* first appeared in 1962 (Prasittisuk 1985) and this was followed by Fansidar resistant strains in many parts of the country beginning in 1975 (Harinasuta et al. 1976).

The goal of most malariologists is to contribute directly or indirectly to the control and eventual elimination of disease. Malaria eradication programs were built upon the use of residual insecticides sprayed upon the surfaces of human and animal shelters where anophelines rest. DDT was the insecticide of choice because of its sustained residual properties indoors and its low cost. During the last two decades, DDT has fallen out of favor because its agricultural use outdoors led to biomagnification in food chains and caused it to become a major environmental pollutant. More important to malaria eradication programs, mosquito resistance became

widespread. Other insecticides such as Malathion have gradually replaced DDT but none are as effective or inexpensive.

In 1969, WHO revised the strategy of malaria eradication by stressing the need for greater involvement of the general health services. WHO also stressed the need for research on new insecticides, improved surveillance, development of new antimalarial drugs and alternative methods for malaria control such as vaccines and biological control of *Anopheles* larvae.

The original control program in Thailand began in 1949 with a WHO/UNICEF Malaria Control Demonstration Project employing house-spraying with residual insecticides. Thailand embarked on an eradication program in 1965 with the assistance of the WHO and the U.S. Government (Harinasuta et al. 1976, Malaria Division 1982).

One difficult problem in Thai malaria control is in areas where *An. dirus* is the vector because of the nature of this mosquito as well as human ecological factors. Scanlon and Sandhinand (1965) reported that limited collections in houses that had been sprayed indicated that females avoided the sprayed wall or spent a relatively short time resting on them. They tended to move away from areas treated with DDT and to remain in untreated areas.

Chemotherapy became the method of choice for both treatment and prevention. Chloroquine was introduced as an antimalarial and has been widely use for curing malaria and

in prophylactic prevention. The occurrence of drug resistance was perhaps a predictable consequence of this strategy (Young et al. 1963, Bourke et al. 1966).

Even though a program to eradicate malaria based upon the use of insecticides and chemotherapy has been carried out for several years, malaria is still a great problem in some provinces. The Thai Government has spent large and increasing amounts of money each year trying to solve these problems but the results are still unsatisfactory. In order to develop better tools for interrupting transmission, additional information about malaria vectors and malaria parasites is needed.

Malaria Vectors

In 1973, the Ministry of Public Health concluded that there are five vectors of malaria. This list is duplicated below in the order of their decreasing importance (Malaria Division 1982).

1. *Anopheles balabacensis* Baisas, 1936
   (Peyton and Harrison 1979 = *dirus*)
2. *Anopheles minimus* Theobald, 1901
3. *Anopheles maculatus* Theobald, 1901
4. *Anopheles sundaicus* (Redenwaldt), 1925
5. *Anopheles aconitus* Donitz, 1902

Between the two most important species, *An. balabacensis* (= unknown *An. dirus*) and *An. minimus*,

Wilkinson et al. (1972) found that *An. dirus* was the most susceptible to *P. falciparum* infection.

Anopheles dirus has been reported from the southern border to the northern provinces of Chiengmai and Nan, and from the east to the west of Thailand. This species occurs only in areas where stands of forest remain (Scanlon and Sandhinand 1965, Wilkinson et el. 1978). Peyton and Harrison (1979) reported that *An.balabacensis* s.l. in Thailand should be named a new species of the *Leucosphyrus* group, *An. (Cellia) dirus*.

Anopheles dirus in Thailand now consists of 5 forms: A, B, C, D and F which are all common in parts of Thailand (Baimai et al. 1981, 1984, 1987, 1988, Peyton and Ramalingam 1988). Recently, Peyton and Ramalingam (1988) named form F *Anopheles nemophilous*. At my study site in Tha-Mai District, Chanthaburi Province, most *An. dirus* are form A.

Larval ecology

Anopheles dirus larvae have been collected in many areas of Thailand. The larval stages live in standing water. Scanlon and Sandhinand (1965) first found *An. dirus* and described an unusual larval site in gem pits in Southeastern Thailand, Tha-Mai District, Chanthaburi Province. In 1978 Wilkinson et al. observed *An. dirus* in Ban bu Phram and Ban Tablan in Prachinburi Province, 250 km Northeast of Bangkok. The largest numbers of *An. dirus*

were collected in small villages during June and July. Peyton and Harrison (1979) reported that the favored habitats of *An. dirus* appeared to be elephant footprints, pools in dry stream beds, pools at the edge of streams and springs, rock pools, ground pools, seepages, wheel ruts, bamboo stumps, human and domestic animal footprints, bases of large fallen trees, and longitudinal depressions in hollow logs. Many of these habitats can have considerable organic matter and may become quite turbid at times. When this happens (i.e. habitats become highly polluted or turbid), this species will not be encountered.

Recognition of the involvement of *An. dirus* in the transmission of human disease led to investigations into its basic biology to find effective control strategies. The most critical aspects of larval ecology include the interaction with the biotic and abiotic factors in the water, the nutritional requirements, adult production, and survivorship. Nayar and Sauerman (1970) demonstrated the effect of larval nurture in the laboratory on adult characteristics at emergence. Size variation is also a naturally occurring phenomenon (Terzian and Stahler 1949). Adult of *Aedes triseriatus* from nutritionally stressed larvae have reduced size and longevity (McComb 1980). Nutrition can be estimated by measuring total nitrogen (APHA 1985) or, preferably, total protein (Van Handel 1986). Temperature also appears to be a major factor

affecting the size of some adult mosquitoes (Bock and Milby 1981).

Several investigations have suggested that environmental factors effect the distribution and occurrence of mosquitoes (Axtell 1979, Udevitz et al. 1987). Hagstrum and Gunstream (1971) demonstrated that salinity, pH, and organic nitrogen of water were related to the presence of mosquito larvae. Dissolved oxygen levels in the water of *Coquillettidia perturbans* larval habitat have been found to be significant (Batzer and Sjogren 1986). Lunt and Peters (1976) showed that rainfall influenced the distribution of *Ae. triseriatus*.

Adult vectorial capacity

Body size is indirectly related to vectorial capacity because it is correlated with survivorship and blood-feeding success. Haramis (1983) first showed a correlation between adult size and age (i.e. parity) in field populations of *Ae. triseriatus*. Recently, Nasci (1986a, b) confirmed that variation in adult body size was related to survival and blood-feeding success in several species.

Detinova (1962, 1968) and Ungureanu (1974) reviewed the significance of parous rates to the epidemiology of mosquitoes-borne disease. Small females could have reduced vectorial capacity for several reasons. They probably fly less (Nayar 1969, Nayar and Sauerman 1970, Schief et al.

1973), ingest less blood, produce fewer eggs, and have reduced survival (Reisen 1975). They also could be less efficient transmitters of disease pathogens. In contrast, Grimstad and Haramis (1984) showed that small *Ae. triseriatus* from nutritionally stressed larvae had a higher threshold of infection to LaCrosse virus in the laboratory. Patrican and DeFoliart (1985) confirmed these findings and demonstrated higher transovarial transmission rates in small *Ae. triseriatus*. Landry et al. (1988) noted that there was significant seasonal variation in body size of females, but found no strong evidence that increased size was advantageous to survival. There are no data on *An. dirus* body size and its relationship to survival and malaria susceptibility. However, I hypothesized that larger females would live longer take larger blood meals, and, therefore, have a greater chance of developing gut oocysts and salivary sporozoites.

Artificial feeding

The fundamental considerations in artificial blood-feeding techniques is to attract the insects to the fluid being offered. The success of this technique is based largely upon keeping warm, heparinized or defibrinated blood in a glass vessel covered at the bottom with a thin membrane that the mosquitoes can probe through. It is well known that most mosquitoes are positively thermotrophic. Greenberg (1949) designed an artificial feeding apparatus

employing an electrically heated resistance wire as a source of heat. The apparatus was made to accommodate six lantern globe cages, each having its own heating element to warm the feeding tube. It is important not to heat the nutrient fluids much above normal body temperatures. Russell et al. (1963) found feeding was encouraged by keeping the solution at 41°C and moistening the surface of the membrane with saliva. Rutledge et al. (1964) modified the feeder of Greenberg (1949) by adding a stirring mechanism to prevent sedimentation of the blood suspension. Their feeder temperature was 38±1°C.

Baudruche membranes (bovine intestine preparation) have worked quite well and are widely used for artificial feeding (Greenberg 1949, Collins et al. 1964a). Rutledge et al. (1964) found that chick skin was superior to the Baudruche membrane. Behin (1967) reported that the most satisfactory membrane was one prepared from the diverticulum or crop of the chicken. Baily et al. (1978) used natural lamb skin membranes (prophylactic condom) to successfully feed a colony of *Anopheles albimanus* Wiedemann on defibrination bovine blood.

Collin et al. (1964b) studied the infectivity of *Anopheles freeborni*, *Anopheles quadrimaculatus*, and *An. albimanus* to a strain of *P. falciparum* from Panama using both membrane feeding and patient feeding techniques. Results indicated that the latter two species had heavier infections from membrane feeding than from feeding directly

on the patient. This finding suggested the feasibility of using this technique to compare infection and transmission rates among different species.

Parasite identification and measurement

Enzyme-linked immunosorbent assay (ELISA) is a simple and easy method for parasite species identification and for determination of the sporozoite rate. This method was developed by preparing monoclonal antibodies (MABs) developed against the immunodominant peptide repeat portion of the circumsporozoite (CS) protein of *P. falciparum* and *P.vivax* sporozoites (Burkot et al. 1984, Wirtz et al. 1985). The basic two-site ELISA was later modified by Wirtz (1987) to increase test sensitivity. The ELISA has proven to be a specific and accurate way to detect sporozoites in mosquitoes and to estimate their number (Burkot et al. 1989).

CHAPTER III

MOSQUITO LARVAE AND ASSOCIATED MACROORGANISMS OCCURRING IN GEM PITS AND *ANOPHELES* ADULTS BITING AND RESTING IN SOUTHERN THA-MAI DISTRICT

Introduction

Tha-Mai District is in Chanthaburi, a province in southeastern Thailand with a serious malaria problem. In 1980, the Malaria Division reported that the greatest number of cases (>30,000) in Thailand occurred in this Province (Malaria Division 1982). Human migration is an important factor in understanding the epidemiology and control of malaria in this region. Control activity has been concentrated along the Thai-Kampuchea border. This consists of active case detection and drug treatment of all positive cases. DDT is also used for controlling adult *Anopheles* resting on the walls of human dwellings. However, malaria has not been eradicated and both drug-resistant malaria and DDT-resistant mosquitoes are now widespread in this region. Newly proposed integrated vector control strategies require better understanding of vector biology, especially of larval populations.

Anopheles dirus is the primary vector of malarial disease in hilly, forested areas in Thailand (Scanlon and Sandhinand 1965) including Tha-Mai District in Chanthaburi Province. *Anopheles dirus* larvae are commonly found in the

numerous pits which have been dug for the recovery of gems in this district. Old gem pits are ideal habitats for larval studies since they hold water for several months of the year and are easy to sample and manipulate. In 1983, some pits in this area were used for biological control studies with guppies and *Tilapia* (Chanthaburi Malaria Center staff, pers. commun.).

My objective was to study the environmental requirements of *An. dirus* and other mosquito larvae associated with it in these gem pits. In addition, I wished to compare the adult *Anopheles* emerging from pupae collected in these pits with those that were biting people living in surrounding houses.

Materials and Methods

Collection of pupal mosquitoes

Gem pits and concrete-lined, open wells which contained rain or ground water were inspected for mosquito larvae. Each habitat was catalogued and marked with a permanent identification code. Initially, forty-one pits were selected but after five months, one pit (pit o′) was destroyed and had to be replaced by a new pit (p′) with similar characteristics (Fig. 3). Routine studies were conducted continuously for 13 and 20 month periods between November 1986 and July 1988. Some pits could not be studied for the entire 20-month period because of new mechanized mining operations entering the study area.

Pupal mosquitoes were collected from pits with a standard dipper (800 ml) and a standardized dipping protocol (10 dips per pit per day for 10 consecutive days each month). Specimens were placed in vials (one per vial), and allowed to emerge. After emergence, all *An. dirus* were provided with distilled water to drink. Survivorship was checked at 12 hr intervals and the wing length of each specimen was measured (as described in Chap. 5). For all species, the first 20 adult mosquitoes from each pit for each monthly sample were kept for identification. The remainder were identified to genus (if obvious) or were simply recorded as unidentified mosquitoes. Identifications were verified by Mr. Kol Mongkolpanya at the AFRIMS laboratory. Other aquatic macroorganisms collected in the first 4 dips per pit per day were observed and recorded from November 1987 through June 1988. These data were collected for 4 consecutive days during each month.

Collection of adult *Anopheles*

Three types of adult collections were made: indoor human-biting, outdoor human-biting, and indoor resting collections. These night catches of *Anopheles* were conducted for 18 months (October 1986 through March 1988). Catches were scheduled from dusk (1800 hr) until midnight on 6 consecutive days each month. Human-biting collections were made by 4 men (2 for indoor and 2 for outdoor

collections), armed with flashlights and aspirators or vials. The feet, legs, arms and heads of the collectors were bare. From January 1987 through March 1988, outdoor human biting collections were extended to include the period from midnight to 0600 hr on 4 nights of the 6 collection days (see Appendix 1 for data on diel patterns of biting activity).

Indoor, outdoor and resting collectors exchanged positions every 2 days. During each hour, they took a 15 minute break. All *Anopheles* captured biting or resting were kept individually in glass vials. Early the following morning, mosquitoes were identified at the field laboratory situated in the living quarters associated with the Village Temple.

Results

Anopheles dirus were found in large numbers in gem pits during the rainy season. Most pits were partially or fully shaded but a few were exposed to direct sunlight most of the day (see Fig. 4). Daytime water temperatures in the pits ranged from 22 to 28^{o}C. Pits were between 70 and 190 cm in diameter; all were less than 230 cm in depth, except for the concrete-lined, open wells (depth ranged from 250 to 610 cm) which contained underground water used by villagers for their daily water supply. The mosquitoes identified from study pits are listed in Table 1. The type

and number of mosquitoes collected from each pit each month are shown in Table 2.

Attempts to collect *An. dirus* pupae from study pits during the dry season failed, so collections were attempted in several neighboring pits during March and April 1988. A few larvae and pupae were found in 3 heavily shaded pits with fairly clean water that were hidden under dense vegetation (Fig. 4a). These pits are listed as extra pits in Table 7.

The aquatic fauna observed represented 5 orders of insects (Ephemeroptera, Odonata, Hemiptera, Coleoptera and Diptera) as well as other aquatic macroorganisms, i.e. copepods, fish (Guppy), tadpoles, snails, prawns and leeches (Tables 3 and 4). Each of these group of organisms include possible competitors or predators of *An. dirus*.

Mosquito pupae were present in every pit except pit e (Table 2). *Anopheles dirus* pupae were collected from every pit except pits e, t, x, n' and p'; larvae which appeared to be *An. dirus* were found in all pits (Tables 2 and 4, and in the presurvey). Predaceous Notonectidae and fish (Guppy) appeared to directly influence the number of mosquito larvae (Tables 5 and 6). *Anopheles dirus* pupae were rarely collected from pits having predators and never at the same time that predators were present (Table 5).

Pit u was the most common pit and provided the largest variety of mosquito species; few predators were ever found in this pit (Tables 2 and 4). Overall, *An.*

dirus was the most widely distributed mosquito. It was present in 37 of the 42 experimental pits sampled. *Culex* (*Lophoceraomyia*) spp. was the second most common group of mosquitoes (35 pits) followed by *Uranotaenia* spp. (28 pits), and *Culex* (*Culiciomyia*) (15 pits). These four groups of mosquitoes occurred together in 11 of the 42 pits (Table 2). They appeared to vary sequentially with seasonal changes. When *An. dirus* declined in the dry season, *Culex* spp. and *Uranotaenia* spp. tended to increase (Table 7).

Adult biting and resting collections yielded a total of 2,905 specimens representing 11 species of *Anopheles* (Table 8). Monthly adult *An. dirus* collections were closely related with *An. dirus* pupal collections during the same month (Tables 7 and 8).

Table 1. List of adult mosquitoes emerging from pupae collected from gem pits at Tha-Mai District from November 1986 through June 1988.

1. *Anopheles* (*Cellia*) *dirus*
2. *Aedes* (*Pseudoskusea*) spp.
3. *Anopheles* (*Anopheles*) *barbirostris*
4. *Anopheles* (*Anopheles*) *hyrcanus nigerimus*
5. *Anopheles* (*Anopheles*) *umbrosus*
6. *Anopheles* (*Cellia*) *tessellatus*
7. *Culex* (*Culex*) *fuscocephala*
8. *Culex* (*Culex*) *gelidus*
9. *Culex* (*Culex*) *mimeticus* group
10. *Culex* (*Culex*) *sitiens*
11. *Culex* (*Culex*) *tritaeniorhynchus*
12. *Culex* (*Culex*) *vishnui* subgroup
13. *Culex* (*Culiciomyia*) *nigropunctatus*
13. *Culex* (*Culiciomyia*) *pallidothorax*
14. *Culex* (*Lophoceraomyia*) spp.
15. *Culex* (*Lutzia*) spp.
16. *Uranotaenia* spp.
17. unidentified species

Table 2. Number of *An. dirus* and other mosquito pupae collected in gem pits. * See Table 1 for explanation of numerical code. Pits are group according to their pupal production of *An. dirus*.

Pit	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total
E																		0
T													31	78	10	22	17	158
X														2				2
N′			1			1						2	1	105	1	4	144	259
P′			12	1										5			1	19
G′	1													5				6
E′	2							1				5	24	144	1	2	124	303
K′	2				1								8	15		1	9	36
M′	2		14			1		1			1		8	63	2	9	129	230
K	3	1								6			3	28	1	6	14	62
I′	4						1	5					2	18	2	12	8	52
L′	5		9										1	5			2	22
V	6		1											3			2	12
C′	7												13	220		14	111	365
F′	9												12	36		5	29	91
O′	9													3			1	13
P	10													1		10	4	25
F	14																	14
S	15													11			2	28
Y	15																	15
B′	15												2	22	1	7	6	53
J′	15										1	1		15		8	5	45
I	16					1								134	1	29	82	263
B	17													8		1	2	28
D′	18												4	17		1	98	138
G	19													12		10	8	49
A	20													3		3	1	27
J	21					1								43		8	22	95
Q	21																10	31
H′	21													14		2	6	43
N	22												59	107	3	14	162	367
R	23																1	24
W	25													5		1		31
Z	27													5	1	8	6	47
L	31							3					8	65		53	31	191
U	32		7			1	1	3	4			1	10	40	3		101	203
A′	37		4											39		2	15	97
C	39													1		11	21	72
H	39													23		3	14	79
D	51															3	7	61
M	58													43			16	117
O	70															23	11	104
Total	741	1	48	1	1	5	2	13	4	6	2	9	186	1338	26	272	1223	3877

Table 3. List of aquatic insects and other macroorganisms collected and visually identified *in situ* in gem pits.

	Code
Ephemeroptera	
Ephemeridae	1.
Odonata	
Coenagrionidae	2.
Libellulidae	3.
Hemiptera	
Notonectidae	4.
Gerridae	5.
Hebridae	6.
Hydrometridae	7.
Coleoptera	
Dytiscidae	8.
Diptera	
Chironomidae	9.
Culicidae	
Anopheles	10.
Culex and *Uranotaenia*	11.
Other Macroorganisms	
Copepods	12.
Fish (Guppy)	13.
Tadpoles	14.
Snails	15.
Prawns	16.
Leeches	17.

Table 4. Total number of mosquito larvae and other aquatic insects collected from gem pits from Nov. 1987 through June 1988 (see Table 3 for explanation of code). Grouping of the pits follows Table 2.

PIT	10	11	1	2	3	4	5	6	7	8	9	12	13	14	15	16	17
E	3	4				1	>77	2					24				
T	7	24	6	3		24	118	>113		4		>34		5			
X		1		9		31	27	95	1	8		>96	9	15		22	
N′	15	>330	19	5	4	4	23	>533		2		>410		26	1		
P′	56	1	2			46	37	>136		3		>14	1	>188		1	
G′	1		1	1			30	4					5	1			
E′		3				3	26	21			1	>14	2				7
K′	3	2		5		1		18		1		>44	1	13			
M′	23	>80	3	8		72	5	>252		3	1	>263	1	28			
K		61		1	2		30					>40	54	15			
I′	>47	41	3	5		44	13	>223	5			>32		29		1	
L′	4			8			5	17				>33		1			
V	9	2	1			16	21	>363		3		>10		12			5
C′	5	4				33	6	22	1			>164		>35			
F′	4	9				7	57	97				>72	1	>40			7
P	11	3		8		9	12	35	1		1						
F	8					4	5	5				1	1			1	
S		2	>39			42	10	>82	4	9				11			
Y	7					1	138	57						1			
B′	49	>22	7	1	4	1	87	>158		7		>70	1				
J′	18	18	4	6	1	44	21	>134		2		7	1	12		1	
I	>41	>89	9	11	1	13	68	>130				>11		4			
B	51	2				36	101	25						>50	1		
D′	7	15				10	9	50				>51					
G	7	20				2	37	12				>42		1			
A	7	1		1			52	25						3			
J	30	>65	2	1		3	8	>288				>120		28			
Q	17	1	2				12	>238									
H′	4	>24		2			4	>275	5			>162		>52			
N	2	1		2	1	20	85	2			1	2					
R	2		15			7	1	31							1		
W	16		1	3		4	26	>100									
Z	54	2		13	1	6	7	>177						24			
L	12	>410		14		3	2	>529		2		>138		7			
U	42	>114	15	7	2	2	15	>287		9		>105		>97			
A′	22	2	2	1		6	22	94		10		>280	1				>18
C	39		8			50	30	>114			1	>30		28			
H	>100	>46	1	6		33	14	91		4		>201		>64			
D	4		5			16	20	10				>10	1	9	1		
M	2						1	6						2			
O	35			1		1	65	20				>20					

Table 5. The association of predaceous Notonectidae and fish (Guppy) with *An. dirus* larvae and pupae in selected pits. See Table 2 for selection and grouping of the particular pits by numbers of *An. dirus* pupae.

		Nov 1987	Dec	Jan 1988	Feb	Mar	Apr	May	Jun	Total
Pit x	*An. dirus*(L)									0
	An. dirus(P)									0
	Notonectidae	9	1	4	3	9	4	1		31
	Fish (Guppy)		2	4	3					9
Pit e	*An. dirus*(L)		3							3
	An. dirus(P)									0
	Notonectidae	1								1
	Fish (Guppy)	3	7	4	10					24
Pit t	*An. dirus*(L)		2		5					7
	An. dirus(P)									0
	Notonectidae	1	9		6	3	4	1		24
Pit n′	*An. dirus*(L)		1	1	10		2	1		15
	An. dirus(P)									0
	Notonectidae		3					1		4
Pit p′	*An. dirus*(L)		4	24	2	26				56
	An. dirus(P)									0
	Notonectidae	>21	2	6	14	2			1	>46
Pit c	*An. dirus*(L)		5	25	9		D			39
	An. dirus(P)						D			0
	Notonectidae	11	14	6	4	15	D			50
Pit h	*An. dirus*(L)	19		5	>55		D	21		>100
	An. dirus(P)	5					D	2		7
	Notonectidae		17	3	10	3	D			33*
Pit d	*An. dirus*(L)							4		4
	An. dirus(P)							4		4
	Notonectidae	2	6	4	4					16*
Pit m	*An. dirus*(L)	2	D	D	D	D	D	D		2
	An. dirus(P)		D	D	D	D	D	D	4	4
	Notonectidae		D	D	D	D	D	D		0
Pit o	*An. dirus*(L)	31	4			D	D	D	D	35
	An. dirus(P)	5				D	D	D	D	5
	Notonectidae					D	D	D	D	0*

* none were present during months that *An. dirus* pupae were produced.

Table 6. The association of predaceous Notonectidae and fish (Guppy) with all mosquito larvae and pupae except *Anopheles* in selected pits. See Table 2 for selection and grouping of the particular pits by numbers of *An. dirus* pupae.

		Nov 1987	Dec	Jan 1988	Feb	Mar	Apr	May	Jun	Total
Pit x	Mosquitoes(L)						1			1
	Mosquitoes(P)									0
	Notonectidae	9	1	4	3	9	4	1		31
	Fish (Guppy)		2	4	3					9
Pit e	Mosquitoes(L)							3	1	4
	Mosquitoes(P)									0
	Notonectidae	1								1
	Fish (Guppy)	3	7	4	10					24
Pit t	Mosquitoes(L)		1	18	4	1				24
	Mosquitoes(P)			8					1	9
	Notonectidae	1	9		6	3	4	1		24*
Pit n′	Mosquitoes(L)	8	>37	14	>107	>39	33	>77	>15	>330
	Mosquitoes(P)	2	27	5	17	5	9	21	2	88
	Notonectidae		3					1		4*
Pit p′	Mosquitoes(L)					1				1
	Mosquitoes(P)									0
	Notonectidae	>21	2	6	14	2			1	46
Pit c	Mosquitoes(L)						D			0
	Mosquitoes(P)		1	2			D			3
	Notonectidae	11	14	6	4	15	D			50
Pit h	Mosquitoes(L)		4	1	6	2	D	11	>22	46
	Mosquitoes(P)			1			D	18		19
	Notonectidae		17	3	10	3	D			33*
Pit d	Mosquitoes(L)									0
	Mosquitoes(P)									0
	Notonectidae	2	6	4	4					16
Pit m	Mosquitoes(L)		D	D	D	D	D	D		0
	Mosquitoes(P)	2	D	D	D	D	D	D	2	4
	Notonectidae		D	D	D	D	D	D		0
Pit o	Mosquitoes(L)					D	D	D	D	0
	Mosquitoes(P)		22		1	D	D	D	D	23
	Notonectidae					D	D	D	D	0

* none were present during months that mosquito pupae were produced.

Table 7. Adult mosquitoes emerging from pupae collected in study gem pits each month.

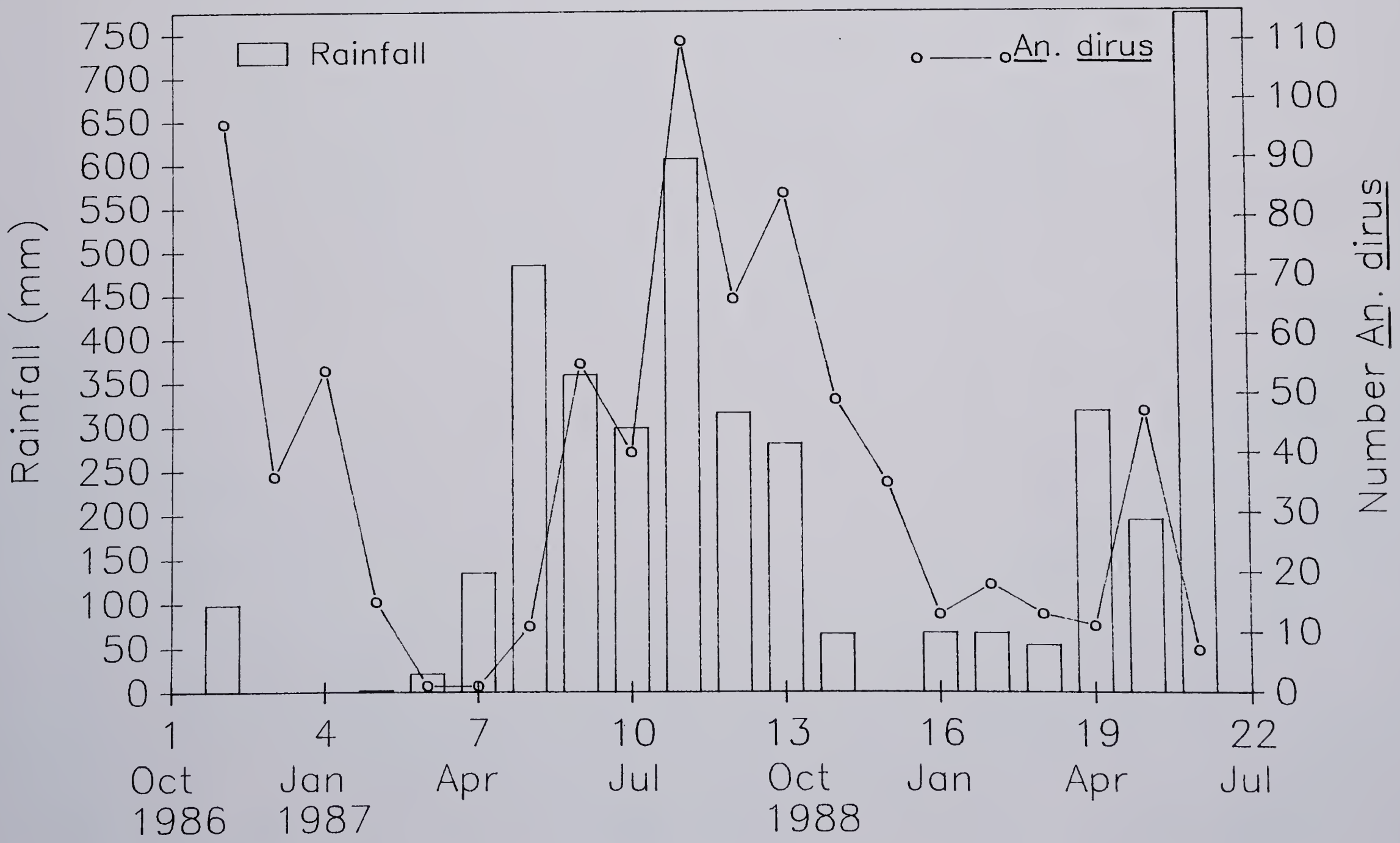
Mosquitoes	1986 Nov	Dec	1987 Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	1988 Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Total
An.dirus	96	36	54	15	1	1	11	55	40	110	66	84	49	35	13	18	*	3**	47	7	741
Other Anopheles	1	7	13	5								3	1	14	8	3					55
Culex spp.	3	36	83	209	139	355	146	161	48	47	49	16	6	54	57	58	19*	17	51	32	1586
Uranotaenia spp.	4	11	25	37	24	31	11	8		8		2		41	29	12	17	11	1		272
Unidentified	11	17	23	50	31	185	515	227	6	25	8	8	10	26	16	27	13	2	20	2	1222
Total	115	107	198	316	195	572	683	452	94	190	123	113	66	170	123	118	49	33	119	41	3877

* +13 An.dirus from extra pits : AA, BB, CC
+4 Culex and 1 unidentified from pit BB
** +8 An.dirus from pit CC

Table 8. Adult *Anopheles* species collected in Sri-Phraya from Nov. 1986 through Mar. 1988.

	1986		1987											1988			
Anopheles sp.	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total
dirus	45	26	51	11	5	5	33	121	130	82	191	107	84	40	15	20	10	976
aconitus	2																	2
annularis														4				4
barbirostris	200	41	40	14	6	7	40	30	36	18	17	43	138	67	31	37	40	805
campestris								1	16	10	1	1	10					39
hyrcanus gr.	96	18	1	4		1	1	3	16	73	56	87	164	5	5	13	14	557
karwari	3	1	1								3							8
philippinensis																1		1
tessellatus	48	16	4					21	12	85	131	19	26	39	2	3		406
umbrosus								1										1
vagus					1	3	35	48		5						1	13	106
Total	394	102	97	29	12	16	109	225	210	273	399	257	422	155	53	75	77	2905

Figure 5. Monthly rainfall and adult *An. dirus* populations.


Rainfall
An. dirus
Rainfall (mm)
Number An. dirus
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
110
100
90
80
70
60
50
40
30
20
10
0
1
Oct
1986
4
Jan
1987
7
Apr
10
Jul
13
Oct
1988
16
Jan
19
Apr
22
Jul

Discussion

Anopheles dirus was historically associated with jungle seepages. The typical breeding habitats were small, clear, shaded pools in clay soil (Colless 1956, Scanlon and Sandhinand 1965). At my study site, *An. dirus* larvae were found in gem pits containing underground water or rainwater under varying degrees of shade. The largest number of both *An. dirus* pupae and biting/resting adults were collected from June to October (see Tables 5 and 6). The marked reduction in numbers from November to April may have been caused by both the habitat drying up and the habitat becoming less suitable. However, during the dry season some reproduction was maintained in the heavily shaded, undisturbed pools which were hidden under vegetation. When the rains returned, *An. dirus* reappeared in most of the study pits. Toward the end of the rainy season, I collected humid soil from suspected oviposition site (i.e. from pit m) and returned it to the laboratory. I found that 1st instar *An. dirus* larvae hatched from artificially flooded soil. This indicates that the eggs of *An. dirus* can tolerate some drying. In the laboratory, I observed that 2.5 % of the eggs were viable after 16-18 days without water but in high humidity (maintained by soaked cotton under the filter paper containing the eggs). Only .003 % remained viable after 33 days. These data suggest that the high natural humidity in my study area could support egg

viability between rains when ground pools temporarily dried up.

Some pits with many mosquito larvae also produced many pupae. However, other pits containing many larvae produced few pupae. The abundance of *An. dirus* larvae and the presence of pupae were negatively correlated with the abundance of aquatic predators, especially Notonectidae (see Appendix 2 for data on preliminary laboratory tests with this predator) and fish (Guppy). *Anopheles dirus* pupae were most numerous in pits o, m and d, possibly due to the low numbers of predators in these pits. The impact of Notonectidae and fish on *An. dirus* larvae appeared to be absolute for pits e, t, x, n′ and p′ since pupae were never found in these pits.

Seasonal changes in the aquatic habitat were correlated with the larval abundance of different kinds of mosquitoes. *Anopheles dirus* dominated from June to October. After October *An. dirus* decreased but *Culex* and *Uranotaenia* began to increase (see Table 7). After the first rains the water quality and nutritional status of the neuston surface layer in the study pits were suitable for *An. dirus*. Their density was maintained throughout the rainy season because clean water conditions were maintained. When the rains stopped, the water in the pits become stagnant and polluted. This condition allowed *Culex* and *Uranotaenia* to replace *An. dirus* during the dry season.

Conclusions

1. The principal larval habitats of *An. dirus* in Tha-Mai District are man-made gem pits containing clear water under full or partial shade.

2. High natural humidity can provide for some egg viability when ground pools temporarily dry up.

3. Notonectidae and fish (Guppy) are natural predators which appear to strongly influence populations of immature *An. dirus* in gem pits.

4. *Anopheles dirus* from adult collection were related to pupal collection.

5. *Anopheles dirus* populations fluctuate with rainfall and peak between June and October. They tend to be positively correlated with other *Anopheles* but negatively correlated with *Culex* and *Uranotaenia*.

CHAPTER IV

EFFECT OF SEASONAL VARIATION IN THE AQUATIC HABITAT OF IMMATURE *ANOPHELES DIRUS* ON THE SIZE AND SURVIVORSHIP OF EMERGING ADULTS

Introduction

The distribution and abundance of mosquito larvae reflect both the oviposition preferences of females as well as the ability of the immature stages to tolerate and develop in the conditions under which they are forced to exist once the eggs hatch. Several investigations have suggested that the distribution of mosquito larvae in naturally occurring habitats is related to water quality (Hagstrum and Gunstream 1971, Vrtiska and Pappas 1984). Knowledge of those environmental factors that limit the occurrence of mosquito species or impact on their growth and development is important in planning successful management programs (Axtell 1979).

Biotic and abiotic differences within aquatic habitats can regulate larval density and the body size of emerging adults (Nayar and Sauerman 1970, Kreb 1978, McCombs 1980). Environmental factors also may influence vectorial capacity (Takahashi 1976, Baqar et al. 1980). Within the range of environmental conditions which a mosquito species can tolerate, some habitats are clearly more advantageous than others (Fish 1985). The interaction

of factors which contribute to the productivity and fitness of mosquitoes is not well understand.

Seasonal changes effect both the food resource available and physical factors (e.g., temperature) which regulate growth, development and survivorship (McCombs 1980, Grimstad and Haramis 1984, Haramis 1985).

Anopheles dirus is one of the primary vectors of malaria in Thailand but its biology, especially of the immature stages, is poorly understood. One objective of this study was to examine the seasonal variation in the adult size and survivorship of *An. dirus* emerging from various larval habitats. Secondly, I examined the effect of water quality on the abundance and presence or absence of *An. dirus*. Dissolved oxygen, pH, turbidity and protein were considered. Rainfall, temperature, and humidity were recorded. Wing length was chosen to represent body size because it is one of the simplest and most reliable estimates (Christophers 1960, McCombs 1980).

Materials and Methods

Field studies

Details of the study site are described in Chap. 1. Collections of pupae were made between 0800 and 1000 hr on ten consecutive days each month from January 1987 through June 1988 (see details of the collections in Chap. 3). Pupal mosquitoes were placed in vials (one per vial) and allowed to emerge. After emergence, adult *An. dirus* were

identified and provided with distilled water only. Mortality was recorded every 12 hr (at 0800 and 2000 hr) until each specimen was dead. Size was determined by wing length measurement as described in Chap. 5. Cohabiting mosquito species also were identified and recorded but were not measured.

During pupal sampling, water temperature, dissolved oxygen (DO), pH, and atmospheric humidity were recorded at each gem pit habitat. Daily maximum and minimum water temperature measurements were also made by a Taylor No. 5458 thermometer. Collection temperature was recorded and dissolved oxygen was measured with a YSI Model 54ARC or 54ABP meter. A portable digital pH meter (Hanna Instruments) was used to measure pH and humidity was measured with a Sling Psychrometer (Taylor Products). Rainfall was measured and recorded daily with a rain gauge made by the Meteorological Department, Bangkok, Thailand.

Water samples were taken once each month (on the final collection day) from the surface zone where larvae of *An. dirus* occur. Each sample was placed in a clean polyethylene screw-cap container and kept on ice during the return trip to the laboratory. Turbidity measurement and protein analysis occurred on the following two days.

Laboratory studies

Protein analysis was conducted following the procedure of Van Handel (1986). Each sample from each habitat was first passed through an 80 mesh sieve to remove large particulates. Then, depending on the nutrient load, between 100 and 500 ml of water was filtered using a microfiltration system. The system was composed of a membrane filter (47 mm dia. and 0.45 um pore size), microfiltration assembly (300 ml funnel, ground glass base with stopper and anodized aluminum spring clamp), a side-arm vacuum filtering flask and a portable vacuum pump. This equipment was assembled according to manufacturer (Whatman) instructions.

When all samples were filtered, protein analysis were prepared as follows:

1. For soluble protein (passed through the membrane filter) and total protein (the original sample), 25 ml of sample solution was mixed with 1 ml sodium deoxycholate (5 g/liter) in 50 ml glass centrifuge tubes, followed by 2 ml of trichloroacetic acid solution (100 %). Tubes were centrifuged at 3,300 g for 30 min, and the supernatants decanted. Precipitates were dissolved in 5 ml of reagent A*. Undissolved materials were removed by centrifugation. Color development for optical density reading was as follows:

   a. Transfer 1 ml of supernatant sample to clean tube.

b. Fill reagent A* to the 5 ml mark.

c. Add 0.2 ml reagent B*.

d. Add 0.2 ml Folin-Ciocalteau reagent.

Blue color develops and reaches a maximum in 10-20 min. It remains stable for 1-2 hr. Optical density was read at 650 nm with a spectrophotometer (HITACHI 150-20) and compared with the standard curve obtained from serial solutions of standard protein. This is the protein assay of Van Handel (1986) which was modified from Lowry et al. (1951).

2. For particle protein analysis, all materials from the membrane filter were removed with reagent A and centrifuged; material not dissolved in the reagent was discarded. Color development was done as described above.

Turbidity was measured in a spectrophotometer with the following nephelometric method:

1. Preparation of the standard.

a. Solution A: One g hydrazine sulfate $(NH_2)_2.H_2SO_4$ dissolved in distilled water and diluted to 100 ml in a volumetric flask.

* Reagent A. Dissolve 4 g sodium hydroxide and 20 g anhydrous sodium carbonate in a liter of water.
Reagent B. Dissolve 0.5 g copper sulfate and 1.5 g sodium potassium tartrate in 100 ml distilled water.

b. Solution B: Ten g hexamethylenetetramine $(CH_2)_6N_4$ dissolved in distilled water and diluted to 100 ml in a volumetric flask.

c. Five ml solution A and 5 ml solution B mixed and held for 24 hr at 25 ± 3°C; diluted with distilled water to 100 ml mark and mixed. Turbidity of this suspension is 400 NTU.

2. Preparation of the standard curve.

Zero, 5, 20, 30, and 40 NTU of the standard solution are used. Optical density (OD) is read at 600 nm and compared with the blank (distilled water).

3. Turbidity measurement of samples.

Optical density of the sample is read at 600 nm and converted to the NTU unit by using the standard curve.

Data analysis

Stepwise regression analysis (Proc REG, SAS 1987) was performed for the following relationships: 1) mean adult size and environmental factors in each pit (i.e. collection water temperature, humidity, dissolved oxygen, pH, maximum and minimum water temperature, turbidity, particulate protein, total protein), 2) mean adult size and a variety of mean rainfall measures (i.e. monthly mean of rainfall 0-7 day, 8-14 day and 15-30 day before collection), 3) mean survivorship and environmental factors, 4) mean survivorship and rainfall measures, 5) *An.*

dirus pupal abundance and environmental factors by pit, and 6) *An. dirus* pupal abundance and rainfall measures.

The relationship between individual adult survivorship and size was tested by regression analysis (Proc REG, SAS 1987).

Correlation among all variables and among mosquito genera in each pit and each month were tested by correlation analysis (Proc CORR, SAS 1987).

A cyclic function: wing length = $B_0 + B_1[\sin(B_2 + \text{time})]$ was fitted to the data to evaluate the significance of seasonal changes in male and female wing length using Proc NLIN (SAS Institute 1987), where:

B_0 = average value (mean of wing length)

B_1 = adjustment in Y-axis

B_2 = adjustment in X-axis

Stepwise logistic regression analysis (BMDPLR, BMDP 1987) was performed to predict the presence or absence of *An. dirus* in gem pits. The logistic regression model is:

$$P_i = \frac{e^Y}{1 + e^Y}$$

where: P_i = the predicted value for pit i^{th} observation can be regarded as the probability that *An. dirus* will occur

$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 \ldots\ldots\ldots$

(when a = coefficients)

e = the base of natural logarithms, 2.71828

Results

Stepwise regression analysis suggests that the mean female wing length of adult females from gem pits was related to minimum water temperature. Maximum water temperature, collection water temperature, humidity, dissolved oxygen, pH, turbidity, particulate protein, and total protein appeared to not be factors of direct importance (Table 9). The final model (R^2 = 0.139, $\underline{F}$ = 16.66, df = 1, 103, $\underline{P}$ = 0.0001) is given by mean female wing length in mm = 4.148 - 0.042 (minimum water temperature). Collection water temperature, humidity, dissolved oxygen, maximum water temperature, and turbidity, which were not directly important in the final model, were correlated with the important factor, minimum water temperature (Table 17). Stepwise analysis also suggested that the monthly mean female wing length was related to rainfall during the 15-30 day period before mosquito pupae were collected (Table 10). The final model (R^2 = 0.145, $\underline{F}$ = 2.54, df = 1, 15, $\underline{P}$ = 0.1320) is given by mean female wing length in mm = 3.097 - 0.006 (rain 15-30 d). Rainfall during the 0-7 day period was not included in the stepwise analysis but it was correlated with rainfall during the 15-30 day period (Table 18).

In the final model of stepwise analysis the mean wing length of adult males by pit was related to minimum water temperature and turbidity. Collection water

temperature, humidity, dissolved oxygen, pH, maximum water temperature, particulate protein, and total protein were not directly important factors (Table 11). The final model (R^2 = 0.205, $\underline{F}$ = 12.26, df = 2, 95, $\underline{P}$ = 0.0001) is given by mean male wing length in mm = 3.969 - 0.041 (minimum water temperature) + 0.001(turbidity). Several factors which were not significantly related to wing length, and not included in the final model, were correlated with one or more of the variables included in the stepwise model. Specifically, humidity and maximum water temperature were correlated with minimum water temperature; pH, particulate protein, and total protein were correlated with turbidity; collection water temperature and dissolved oxygen were correlated with both minimum water temperature and turbidity (Table 17).

The relationship between mean male wing length and mean rainfall measures were observed by month. Rainfall during the 0-7 day period before collection showed the strongest relationship in the final model (R^2 = 0.341, $\underline{F}$ = 7.76, df = 1, 15, $\underline{P}$ = 0.0138). The regression equation is given by mean male wing length in mm = 2.978 - 0.005 (rain 0-7 d) (Table 12). Rainfall during the 8-14 day period and 15-30 day period before pupal collection were not included in the final model but were correlated with rainfall during the 0-7 day period (Table 18).

The relationships between mean female survivorship and environmental factors was examined. Minimum water

temperature and pH showed a significant relationship with mean female survivorship in the final model of the stepwise analysis (R^2 = 0.148, $\underline{F}$ = 8.96, df = 2, 103, $\underline{P}$ = 0.0003). Collection water temperature, humidity, dissolved oxygen, maximum water temperature, turbidity, particulate protein and total protein were not correlated (Table 13). The final model is given by mean female survivorship in days = 7.958 - 0.144 (minimum water temperature) - 0.251 (pH). As in the previous analyses, several factors not included in the final model were found to be correlated with one or more of the variables included in the stepwise model. Specifically, dissolved oxygen was correlated with minimum water temperature; particulate protein and total protein were correlated with pH; collection water temperature, humidity, maximum water temperature and turbidity were correlated with both minimum water temperature and pH (Table 17). The stepwise regression analysis also suggested that rainfall during the 8-14 day period before pupal collection was important (Table 14) and the model (R^2 = 0.178, $\underline{F}$ = 3.26, df = 1, 15, $\underline{P}$ = 0.0913) is given by mean female survivorship in days = 2.854 - 0.015 (rain 8-14 d). Rainfall during the 0-7 day period was not included in the model but was found to be correlated with rainfall during the 8-14 day period before pupal collection (Table 18).

The relationship between mean survivorship of males and environmental factors indicates that total protein, minimum water temperature and particulate protein were

important factors while collection water temperature, humidity, dissolved oxygen, pH, maximum water temperature, and turbidity were not (Table 15). In the final model of the stepwise analysis (R^2 = 0.199, $\underline{F}$ = 7.87, df = 3, 95, $\underline{P}$ = 0.0001), the regression equation is given by mean survivorship of males in days = 5.294 + 0.251 (total protein) - 0.107 (minimum water temperature) - 0.478 (particulate protein). Factors which were not directly important were correlated with factors that were important. Specifically, collection water temperature and humidity correlated with minimum water temperature; maximum water temperature correlated with both minimum water temperature and total protein; pH and turbidity correlated with both particulate protein and total protein; dissolved oxygen correlated with total protein, minimum water temperature and particulate protein (Table 17). As in female survivorship analysis, rainfall during the 8-14 day period was important (Table 16). The final model (R^2 = 0.264, $\underline{F}$ = 5.39, df = 1, 15, $\underline{P}$ = 0.0347) is given by mean survivorship of males in days = 2.642 - 0.017 (rain 8-14 d). Again, rainfall during the 0-7 day period was not included in the final model but was correlated with rainfall during 8-14 day period (Table 18).

The relationship between survivorship and wing length of emerging females was statistically significant ($\underline{F}$ = 49.86, df = 1, 341, $\underline{P}$ = 0.0001) but not strongly so (R^2 = 0.1276). The regression equation is given by female

survivorship in days = -1.915 + 1.538 (wing length in mm) (Table 19 and Fig. 6). The relationship between survivorship and wing length of males also was significant ($\underline{F}$ = 13.40, df = 1, 274, $\underline{P}$ = 0.0003) but weakly so (R^2 = 0.0466). The regression equation describing male survivorship in days = - 0.417 + 1.029 (wing length in mm) (Table 20 and Fig. 7).

The regression with a sine function indicates that the seasonal variation in female wing length was significantly different ($\underline{F}$ = 10.268, df = 2, 340, $\underline{P}$ <0.0001). The fitted model was wing length in mm = 3.06 + 0.06 [sin (0.523 x month - 0.096)] (Fig. 8). The seasonal fluctuation in male wing length also was significantly different ($\underline{F}$ = 12.328, df = 2, 273, $\underline{P}$ <0.0001). The fitted model was wing length in mm = 2.927 + .067 [sin (0.523 x month - 0.698)] (Fig. 9).

The relationship between abundance of *An. dirus* in each pit and environmental factors indicates that pH, humidity and dissolved oxygen were important while collection water temperature, maximum water temperature, minimum water temperature, turbidity, particulate protein, and total protein were not (Table 21). The final stepwise analysis model (R^2 = 0.028, $\underline{F}$ = 5.56, df = 3, 580, $\underline{P}$ = 0.0009) is given by *An. dirus* number = 2.437 - 0.645 (pH) + 0.026 (humidity) + 0.166 (DO). Factors which are not important but which were correlated with important factors are: maximum water temperature correlated with pH; minimum

water temperature correlated with both humidity and dissolved oxygen; collection water temperature correlated with both humidity and pH; turbidity, particulate protein and total protein correlated with both pH and dissolved oxygen (Table 17). Results of stepwise regression confirmed that rainfall during the 8-14 day period was significant while rainfall during the 0-7 day and 15-30 day periods were not directly important (Table 22). The final model (R^2 = 0.375, $\underline{F}$ = 10.18, df = 1, 17, $\underline{P}$ = 0.0054) is given by *An. dirus* numbers = 16.597 + 3.235 (rain 8-14 d). Rainfall during the 0-7 day period was not included in final model but was correlated with significant rainfall during the 8-14 day period (Table 18).

Stepwise logistic regression analysis suggests that collection water temperature, humidity, pH and minimum water temperature were significant for the presence or absence of *An. dirus* in gem pits while maximum water temperature, dissolved oxygen, turbidity, particulate protein, and total protein were not (Table 23). The logistic regression model ($P_i = e^Y/1+e^Y$) is given by Y = -6.078 + 0.409 (collection water temperature) + 0.042 (humidity) - 0.380 (pH) - 0.280 (minimum water temperature). The probabilities for the presence and absence of *An. dirus* in gem pits are shown in Figs. 10 and 11. Several factors which were not important were correlated with important factors. Specifically, particulate protein and total protein were correlated with

pH; dissolved oxygen was correlated with both humidity and minimum water temperature; maximum water temperature was correlated with both collection water temperature and pH; turbidity was correlated with collection water temperature, pH and minimum water temperature. Rainfall data did not meet the assumption of stepwise logistic regression analysis since it was not measure for each pit. However, rainfall measures were correlated with certain water quality indicators by month. Specifically, rainfall during the 0-7 day period was correlated with humidity, pH and minimum water temperature; rainfall during the 15-30 day period was correlated with humidity and pH (Table 24).

Correlation of mosquito genera by pit indicated that *An. dirus* is positively correlated with other *Anopheles* but weakly so ($r = 0.074$) whereas *Culex* had a high positive correlation with *Uranotaenia* (Table 25). According to the correlation analysis of mosquito genera by month, both other *Anopheles* and *Culex* were highly correlated with *Uranotaenia* (Table 26).

Discussion

Environmental factors effecting size variation and survivorship were identified from a natural population of newly emerged adult *An. dirus*. Minimum water temperature appeared to be most responsible for the wide range of both male and female wing lengths. Low minimum water temperatures caused an increase in wing length in both

sexes, presumably because feeding and development time were extended compared with higher minimum water temperatures (see comparision of minimum water temperature during hottest and coolest months in Appendix 3). Higher turbidity caused an increase in male wing length but not in female wing length. No explanation can be offered for this finding beyond the fact that turbid waters tended to be cooler. Perhaps males are more sensitive to temperature influences than females. The higher F value for males (Tables 9 and 11) suggests this possibility.

Temperature affects adult size in natural populations either directly through physiological and developmental processes or indirectly by altering the quantity and quality of food in the habitat. Haramis (1985) suggested that increased water temperature accounted for the earlier development of larvae and the corresponding reduced body size of adult *Aedes triseriatus*. Similarly, the size of male and female *An. dirus* seems to depend on temperature.

Protein may be an important factor affecting larval nutrition and wing length but interpretation was complicated by too many other factors. Turbidity was directly correlated with male wing length but not for female wing length. Turbidity was correlated with particulate protein, total protein, dissolved oxygen, pH, collection water temperature, and minimum water

temperature. Therefore, particulate and total protein were indirectly related to male but not to female wing length.

Dissolved oxygen and pH did not prove to be important factors effecting size and survivorship in the analysis of my data. The dissolved oxygen levels of water in the experimental pits were slightly low (generally <3 ppm) indicating the presence of excessive organic matter. Armstrong (1980) postulated that organic debris protects larvae from predation. Guille (1976) reported that organic flocculence provides a medium for bacterial colonies, the major food source of larvae. *Anopheles dirus* can tolerate water with low dissolved oxygen because they usually occur at the water surface and consume oxygen directly from the air through their dorsal spiracles (Merrit and Cummins 1984). However, *Anopheles* tend to not occur in highly polluted water. The pH in the gem pits in my study area was neutral to slightly acid (normally ranged between 5.5 and 7). Most mosquitoes are able to tolerate a wide range of pH (Clements 1963).

The relationship between wing length and rainfall was considered since the water-holding gem pits in this study area are directly dependent on rainfall. It is widely accepted that rainfall and evaporation have a major impact on water quality. According to stepwise regression analysis, rainfall during the 15-30 days prior to pupal collection was related to female wing length while rainfall during the 0-7 days prior to collection was related to male

wing length. However, all three rainfall periods tended to be interrelated. In general, higher rainfall caused a decrease in wing length in both males and females and was apparently responsible for the seasonal variation in wing length shown in Figs. 8 and 9. Adults tended to be larger in the dry season and smaller in the rainy season. It is also possible that in the rainy season, when the density of many types of mosquitoes and aquatic insects are highest and when aquatic food resources are least concentrated, that there is some competition for food and or space which leads to reduce body size.

Decreases in minimum water temperature caused increases in both male and female survivorship Tables 13 and 15. At lower temperature, mosquitoes develop more slowly, so both their size and the energy reserves at emergence are increased. Increased survivorship is the consequence of greater reserves. The relationship between adult size and survivorship was not as strong as in the laboratory (Chap. 7), perhaps because of the variability caused by other factors in the field (see Figs. 6 and 7).

Physical and chemical factors have been used in numerous studies to characterize aquatic habitats (Laird 1988) because of their presumed impact on larval presence and density (Wetzel 1975, Kreb 1978). My study indicated that *An. dirus* densities were highest with slightly acid pH, high atmospheric humidity (characteristic of densely shaded pit locations) and high levels of dissolved oxygen.

Average monthly rainfall appeared to have the greatest influence on *An. dirus* density, especially average monthly rainfall during the 8-14 day period before collection. *Anopheles dirus* tended to increase in number with increased rainfall. This finding offers important clues concerning the factors responsible for seasonal decreases and increases in the density of *An. dirus* larvae. Beside the abiotic factors discussed above, predation was the ecological character whose fluctuations seemed to be most related to variations in *An. dirus* density (Chap. 3).

The character of the environment can limit the occurrence of *An. dirus* by influencing female oviposition, egg hatching and larval development. Collection water temperature and humidity were positively correlated with the presence of *An. dirus* in pits while pH and minimum water temperature were negatively correlated. Although rainfall data could not meet the assumption for stepwise logistic regression analysis, rainfall was correlated with humidity, pH and minimum water temperature (Table 24). The logistic regression model predicting the presence of *An. dirus* in pits where *An. dirus* were actually found was limited to the probability range between 0.068 and 0.415 (Fig. 10). This finding is in agreement with the specific description of favorable *An. dirus* larval habitats in the literature (Peyton and Harrison 1979) which suggested that larvae exhibit limited tolerance for certain physical and chemical factors. The model indicates that the probability

range of predicting pits where *An. dirus* was absent was between 0.051 and 0.466 (Fig. 11). Absence of *An. dirus* is undoubtedly related to factors not considered in this investigation. Through careful observation in the dry season, some pits were found to still contain ground water but *An. dirus* larvae were not found. It is possible that eggs were not laid in these pits because ovipositing females were repelled by undetectable chemical or physical changes in the water.

All *An. dirus* pupae encountered were collected and identified during field sampling. Only the first 20 pupae of other mosquito species were counted. There was a positive correlation between *An. dirus* and other *Anopheles* in the pits; however, there was no correlation between *An. dirus* and other mosquitoes. It is obvious that if other mosquitoes had all been counted, *An. dirus* would have been negatively correlated with *Culex* and *Uranotaenia* on a monthly basis. There is no basis at present to suggest that this displacement was due to competition.

Conclusions

1. Minimum water temperature, turbidity and rainfall are correlated with adult *An. dirus* body size in the field.

2. Minimum water temperature, pH, protein and rainfall are correlated with *An. dirus* survivorship.

3. There is a positive relationship between survivorship and body size in both male and female *An.*

dirus in the field but the relationship is not as strong as that observed in the laboratory (Chap. 7).

4. Dissolved oxygen, pH, atmospheric humidity and rainfall may be useful habitat indicators of *An. dirus* larval density.

5. The presence of *An. dirus* was positively correlated with collection water temperature, atmospheric humidity and rainfall but negatively correlated with pH and minimum water temperature.

6. *Anopheles dirus* and other *Anopheles* were positively correlated by pit whereas there was no correlation between *An. dirus* and other mosquito genera. However, future analysis should pay attention to a possible negative correlation between *An. dirus* and both *Culex* and *Uranotaenia*.

Table 9. Result of stepwise linear regression predicting mean wing length of female *An. dirus* by pits from a variety of environmental measures. Model R^2 = 0.1392, F = 16.66, df = 1, 103, P = 0.0001. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	4.14800004	244.30	0.0001
Min-Temp(H_2O)	-0.04196914	16.66	0.0001
Max-Temp(H_2O)			
Coll-Temp(H_2O)			
Humidity			
DO			
pH			
Turbidity			
Par-Protein			
Tot-Protein			

Table 10. Result of stepwise linear regression predicting mean wing length of female *An. dirus* by month from a variety of mean rainfall measures. Model R^2 = 0.1447, F = 2.54, df = 1, 15, P = 0.1320. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	3.09728419	11976.90	0.0001
Rain(15-30d)	-0.00566930	2.54	0.1320
Rain(0-7d)			
Rain(8-14d)			

Table 11. Result of stepwise linear regression predicting mean wing length of male *An. dirus* by pits from a variety of environmental measures. Model R^2 = 0.2051, F = 12.26, df = 2, 95, P = 0.0001. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	3.96899696	329.05	0.0001
Min-Temp(H_2O)	-0.04136732	23.39	0.0001
Turbidity	0.00097466	3.15	0.0789
Coll-Temp(H_2O)			
Humidity			
DO			
pH			
Max-Temp(H_2O)			
Par-Protein			
Tot-Protein			

Table 12. Result of stepwise linear regression predicting mean wing length of male *An. dirus* by month from a variety of mean rainfall measures. Model R^2 = 0.3411, F = 7.76, df = 1, 15, P = 0.0138. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	2.97763819	17013.30	0.0001
Rain(0-7d)	-0.00475244	7.76	0.0138
Rain(8-14d)			
Rain(15-30d)			

Table 13. Result of stepwise linear regression predicting mean survivorship of female *An. dirus* by pits from a variety of environmental measures. Model R^2 = 0.1482, F = 8.96, df = 2, 103, P = 0.0003. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	7.95754490	36.79	0.0001
Min-Temp(H_2O)	-0.14365534	16.58	0.0001
pH	-0.25124266	4.05	0.0469
Coll-Temp(H_2O)			
Humidity			
DO			
Max-Temp(H_2O)			
Turbidity			
Par-Protein			
Tot-Protein			

Table 14. Result of stepwise linear regression predicting mean survivorship of female *An. dirus* by month from a variety of mean rainfall measures. Model R^2 = 0.1783, F = 3.26, df = 1, 15, P = 0.0913. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	2.85351653	800.61	0.0001
Rain(8-14d)	-0.01529659	3.26	0.0913
Rain(0-7d)			
Rain(15-30d)			

Table 15. Result of stepwise linear regression predicting mean survivorship of male *An. dirus* by pits from a variety of environmental measures. Model R^2 = 0.1991, F = 7.87, df = 3, 95, P = 0.0001. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	5.29395655	39.38	0.0001
Tot-Protein	0.25082054	11.57	0.0010
Min-Temp(H_2O)	-0.10744028	10.35	0.0018
Par-Protein	-0.47779199	8.95	0.0035
Coll-Temp(H_2O)			
Humidity			
DO			
pH			
Max-Temp(H_2O)			
Turbidity			

Table 16. Result of stepwise linear regression predicting mean survivorship of male *An. dirus* by month from a variety of mean rainfall measures. Model R^2 = 0.2643, F = 5.39, df = 1, 15, P = 0.0347. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	2.64226206	908.70	0.0001
Rain(8-14d)	-0.01710661	5.39	0.0347
Rain(0-7d)			
Rain(15-30d)			

Table 17. Pearson correlation coefficients of all variables by pit.

	Coll-Temp	Humidity	DO	pH	Max Temp	Min Temp	Turbidity	Par-Protein	Tot-Protein
Coll-Temp	(608)	(607)	(608)	(607)	(602)	(602)	(588)	(588)	(588)
Humidity	0.657*	(609)	(607)	(608)	(602)	(602)	(588)	(588)	(588)
DO	-0.075	-0.172*	(608)	(607)	(602)	(602)	(588)	(588)	(588)
pH	-0.159*	-0.137*	-0.042	(623)	(601)	(601)	(602)	(602)	(602)
Max-Temp	0.283*	0.058	0.031	0.146*	(602)	(602)	(584)	(584)	(584)
Min-Temp	0.850*	0.794*	-0.088*	-0.074	0.172*	(602)	(584)	(584)	(584)
Turbidity	-0.103*	-0.062	-0.203*	0.146*	0.020	-0.123*	(606)	(604)	(604)
Par-Protein	-0.021	0.039	-0.348*	0.218*	0.077	0.043	0.516*	(604)	(604)
Tot-Protein	0.034	0.008	-0.280*	0.174*	0.148*	0.064	0.440*	0.810*	(604)

Note: * P < 0.050
(N)

Table 18. Pearson correlation coefficients of rainfall variables by month. (N = 19)

	Rain (0-7d)	Rain (8-14d)
Rain(8-14d)	0.498*	
Rain(15-30d)	0.560*	0.219

Note: * is $\underline{P} < 0.05$

Table 19. Results of individual regression analysis of survivorship on female *An. dirus* wing length. There is statistical significance ($R^2 = 0.1276$, $\underline{F} = 49.86$, df = 1, 341, $\underline{P} = 0.0001$).

Varible	Parameter estimate	$\underline{P}$-value
Intercept	-1.914638	0.0042
Wing length	1.537960	0.0001

Table 20. Results of individual regression analysis of survivorship on male *An. dirus* wing length. There is statistical significance ($R^2 = 0.0466$, $\underline{F} = 13.40$, df = 1, 274, $\underline{P} = 0.0003$).

Varible	Parameter estimate	$\underline{P}$-value
Intercept	-0.417463	0.6113
Wing length	1.029370	0.0001

Figure 6. Relationship between survivorship and wing length of female *An. dirus*.

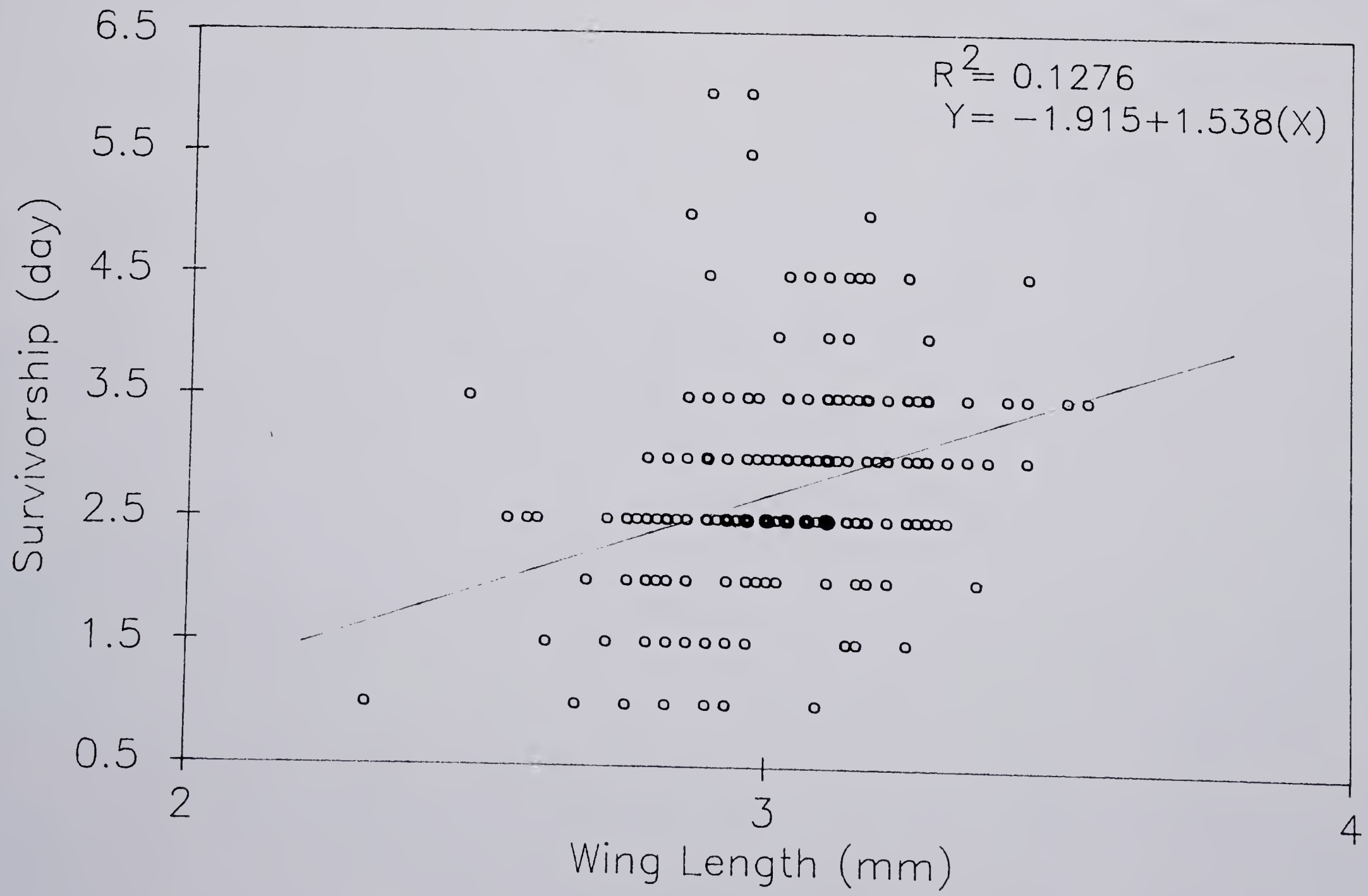

R^2= 0.1276
Y= −1.915+1.538(X)
Survivorship (day)
6.5
5.5
4.5
3.5
2.5
1.5
0.5
2
3
4
Wing Length (mm)

Figure 7. Relationship between survivorship and wing length of male *An. dirus*.

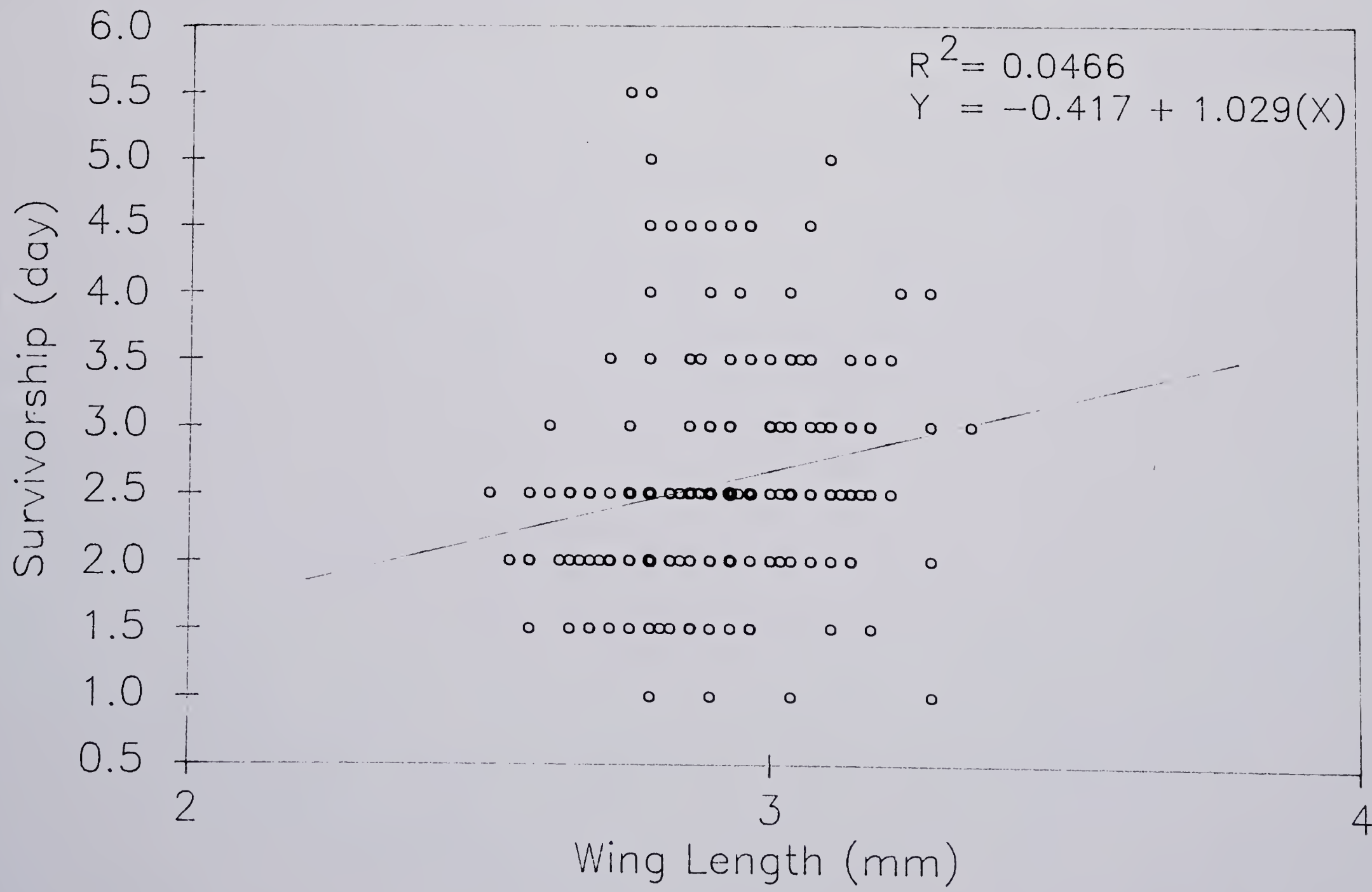

R^2= 0.0466
Y = −0.417 + 1.029(X)
Survivorship (day)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
2
3
4
Wing Length (mm)

Figure 8. Results of observed female wing length fitted to a sine curve.

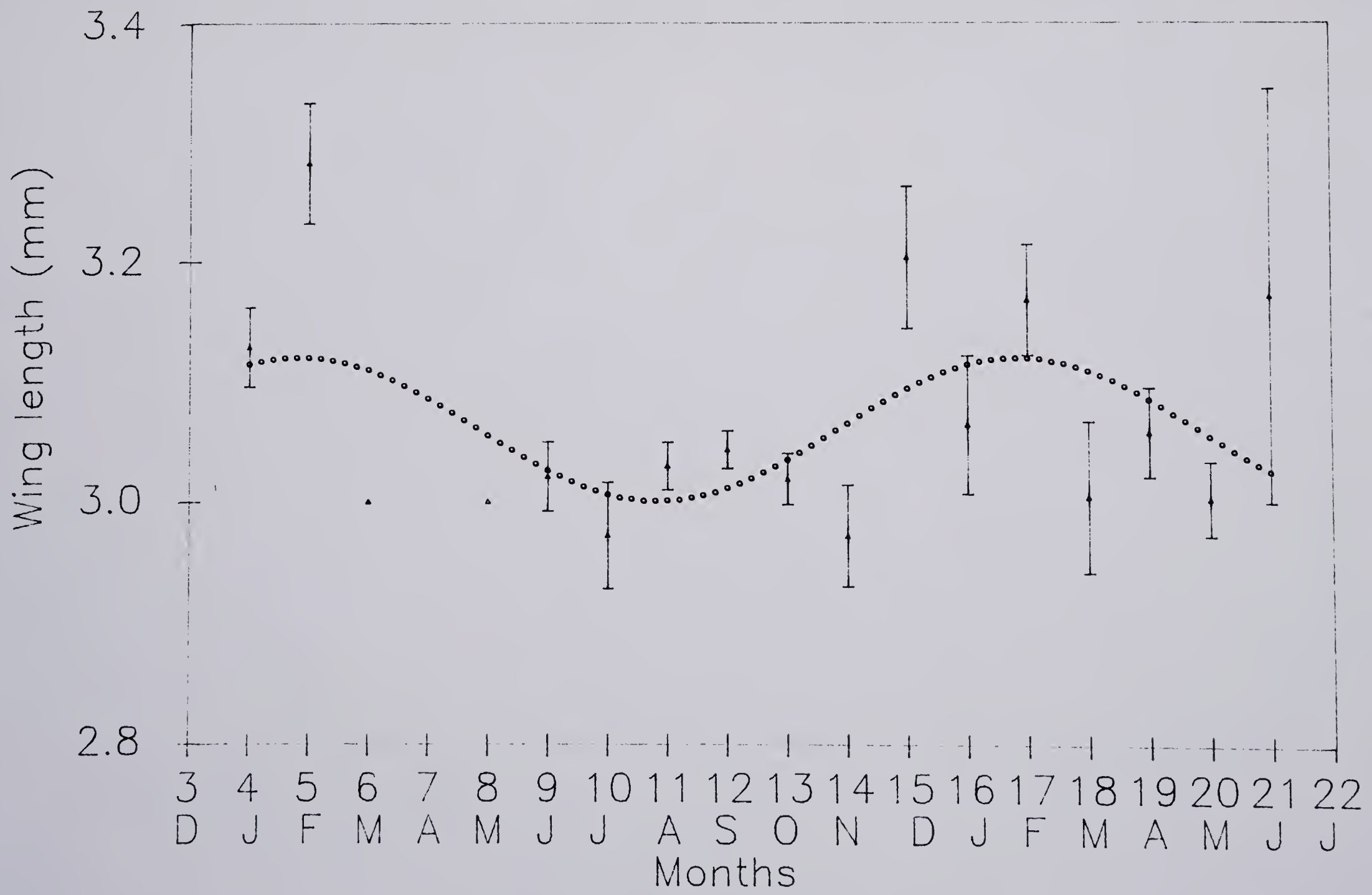

Wing length (mm)
3.4
3.2
3.0
2.8
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
D J F M A M J J A S O N D J F M A M J J
Months

Figure 9. Results of observed male wing length fitted to a sine curve.

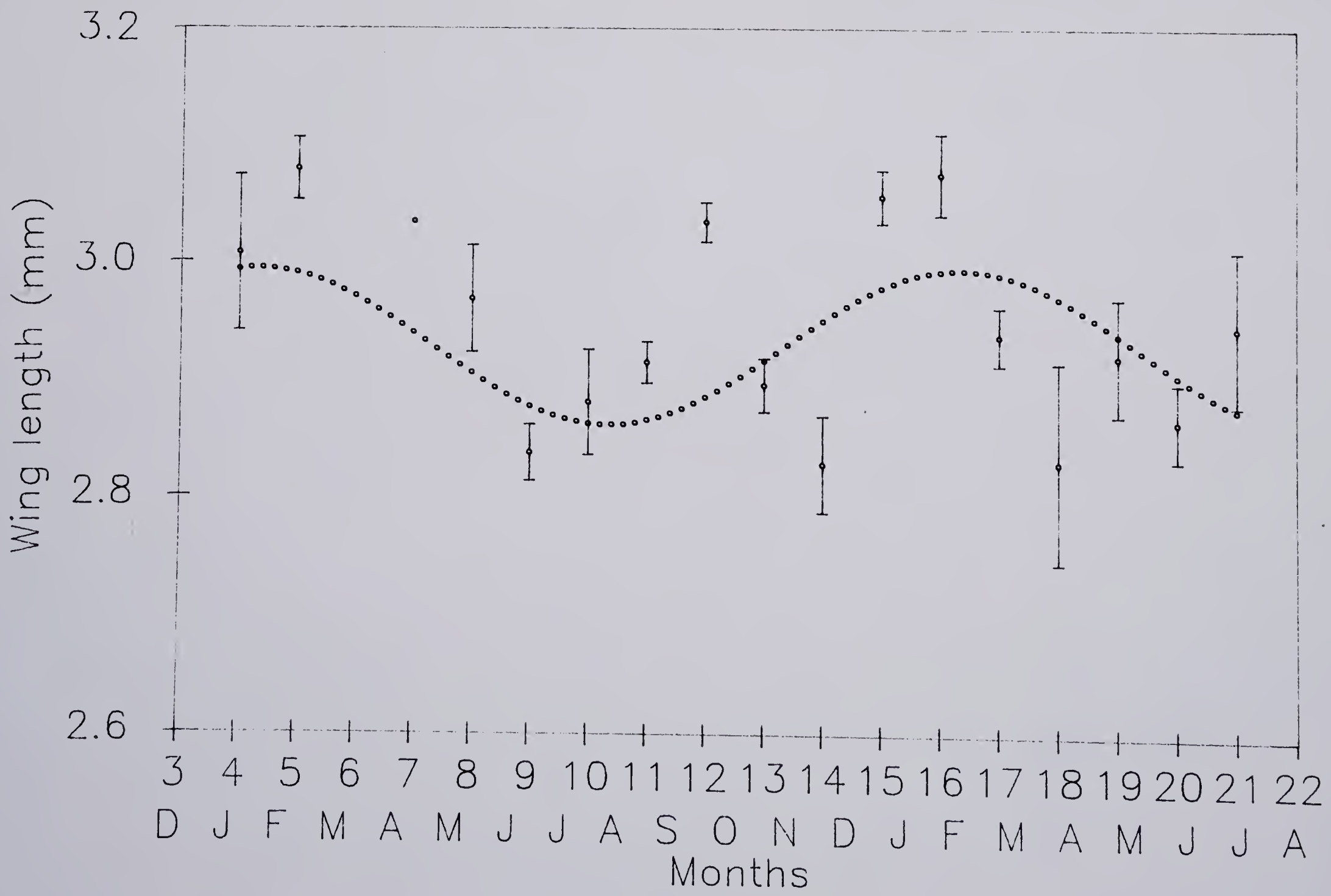

3.2
3.0
2.8
2.6
Wing length (mm)
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
D J F M A M J J A S O N D J F M A M J J A
Months

Table 21. Result of stepwise linear regression predicting *An. dirus* density by pit from a variety of environmental measures. Model R^2 = 0.0280, F = 5.56, df = 3, 580, P = 0.0009. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	2.43672567	1.52	0.2176
pH	-0.64522523	7.58	0.0061
Humidity	0.02644190	4.60	0.0324
DO	0.16566615	3.61	0.0579
Coll-Temp(H_2O)			
Max-Temp(H_2O)			
Min-Temp(H_2O)			
Turbidity			
Par-Protein			
Tot-Protein			

Table 22. Result of stepwise linear regression predicting *An. dirus* density by month from a variety of mean rainfall measures. Model R^2 = 0.3745, F = 10.18, df = 1, 17, P = 0.0054. Factors without parameter estimates did not meet 0.1500 significance level for entry into the model.

Variable	Parameter estimate	F	P-value
Intercept	16.59737711	4.58	0.0472
Rain(8-14d)	3.23545392	10.18	0.0054
Rain(0-7d)			
Rain(15-30d)			

Table 23. Stepwise logistic regression model for predicting occurrence of *An. dirus*.

Variable	Coefficients (±SE)	P-value
Intercept	-6.0783(±3.293)	0.060055
Coll-Temp(H_2O)	0.4092(±0.175)	0.017613
Humidity	0.0418(±0.017)	0.015036
pH	-0.3804(±0.204)	0.063387
Min-Temp(H_2O)	-0.2796(±0.134)	0.038323
Max-Temp(H_2O)		0.201566
DO		0.499150
Turbidity		0.341436
Par-Protein		0.190955
Tot-Protein		0.179730

Note: Rainfall may not directly affect the present and absent *An. dirus* but may affect water quality.

Table 24. Pearson correlation coefficients of all factors (means) by month.

	Coll-temp	Humi-dity	DO	pH	Max-Temp	Min-Temp	Turbi-dity	Par-Protein	Tot-Protein	Rain (0-7d)	Rain (8-14d)	Rain (15-30d)
Coll-temp	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)
Humidity	0.776*	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)
DO	-0.340	-0.246	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)
pH	0.039	-0.265	0.016	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)
Max-Temp(H2O)	0.611*	0.094	-0.443	0.258	(18)	(18)	(18)	(18)	(18)	(18)	(18)	(18)
Min-Temp(H2O)	0.908*	0.892*	-0.274	0.068	0.404	(18)	(18)	(18)	(18)	(18)	(18)	(18)
Turbidity	-0.003	-0.255	-0.336	0.099	0.153	-0.226	(18)	(18)	(18)	(18)	(18)	(18)
Par-Protein	0.295	0.022	-0.536*	0.475*	0.532*	0.248	0.543*	(18)	(18)	(18)	(18)	(18)
Tot-Protein	0.296	-0.032	-0.450	0.383	0.613*	0.215	0.644*	0.891*	(18)	(18)	(18)	(18)
Rain(0-7d)	0.446	0.679*	-0.275	-0.498*	0.032	0.476*	-0.269	-0.080	-0.159	(19)	(19)	(19)
Rain(8-14d)	0.417	0.440	-0.233	-0.253	0.322	0.420	-0.223	-0.111	-0.086	0.498*	(19)	(19)
Rain(15-30d)	0.316	0.560*	-0.190	-0.644*	0.037	0.399	-0.424	-0.115	-0.206	0.560*	0.219	(19)

Note: * P < 0.050
(N)

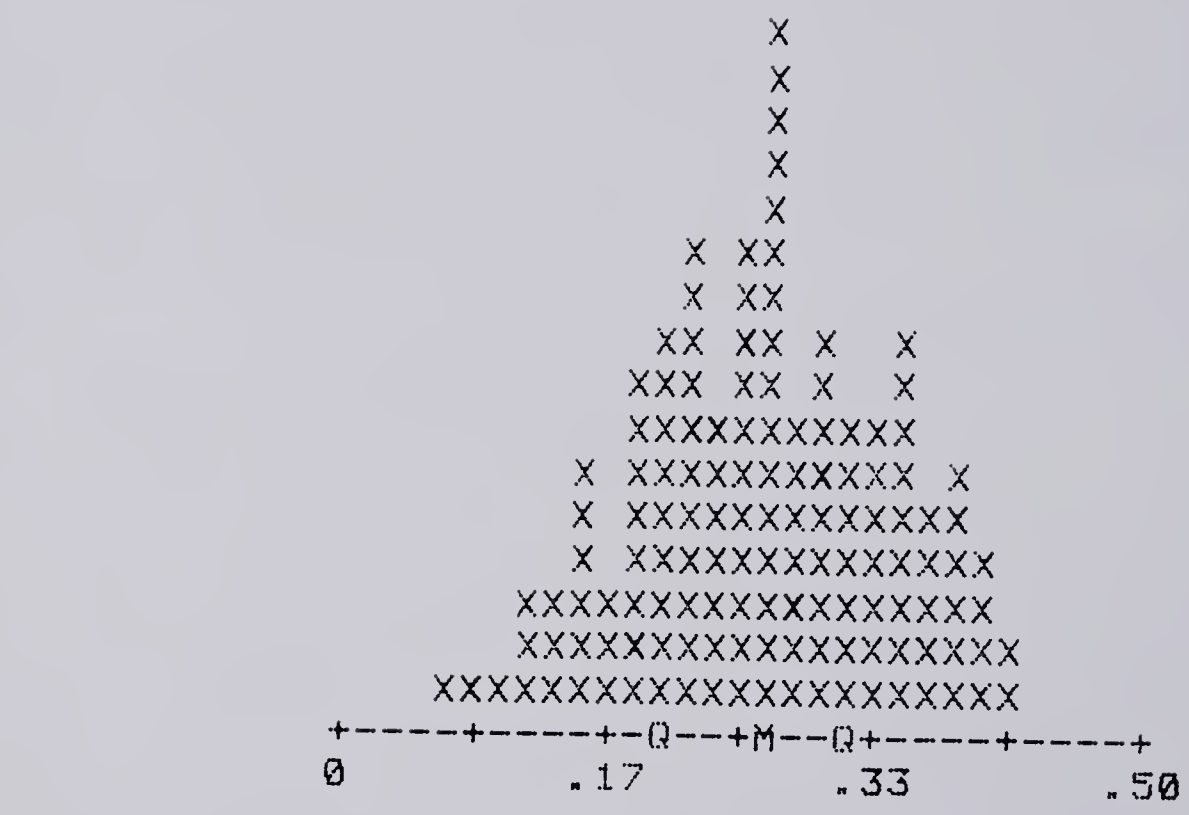


Figure 10. Histrogram of predicted probabilities of *An. dirus* presence. Each 'x' represents 1 response. 'M' marks the median; 'Q' marks the quartiles.

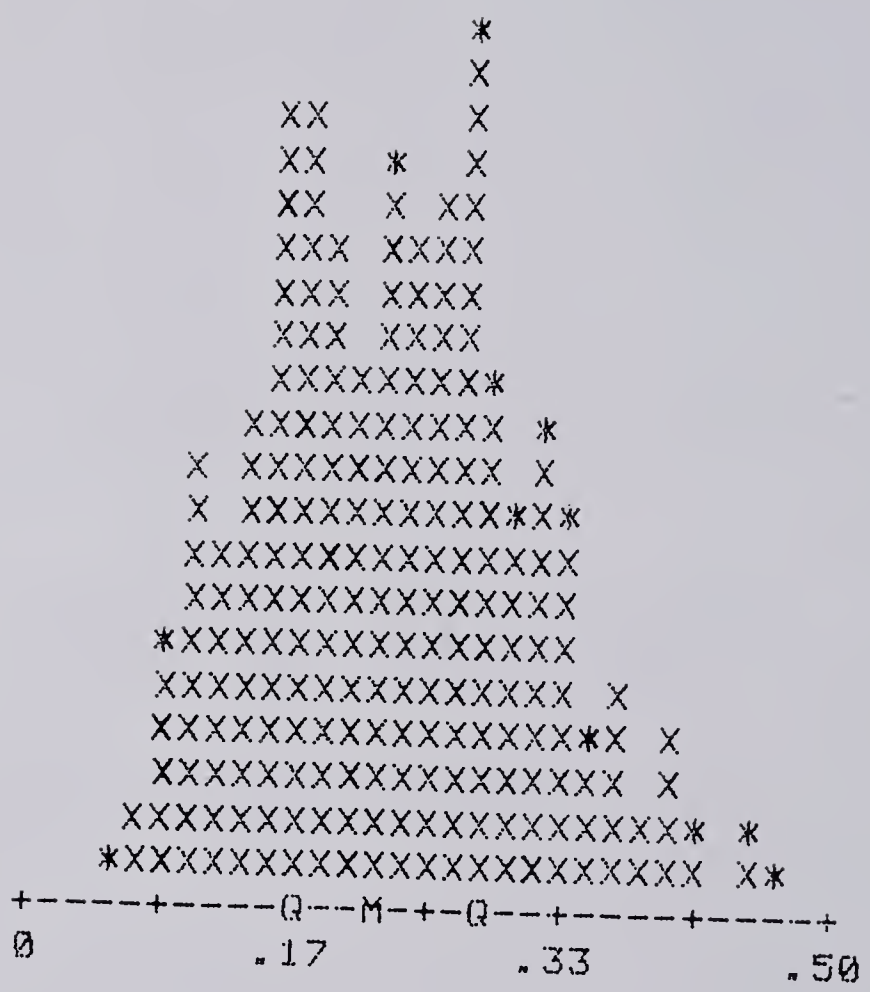


Figure 11. Histrogram of predicted probabilities of *An. dirus* absence. Each 'x' represents 2 responses, '*' represents less than 2 responses. 'M' marks the median; 'Q' marks the quartiles.

Table 25. Pearson correlation coefficients of mosquitoes by pit. (N = 758)

	An. dirus	Other *Anopheles*	*Culex*
Other *Anopheles*	0.074*		
Culex	0.009	0.003	
Urnotaenia	0.002	0.009	0.360*

Table 26. Pearson correlation coefficients of mosquitoes by month. (N = 19)

	An. dirus	Other *Anopheles*	*Culex*
Other *Anopheles*	0.068		
Culex	-0.290	-0.039	
Urnotaenia	-0.333	0.657*	0.540*

Note: * P < 0.05

CHAPTER V

RELATIONSHIP BETWEEN FEMALE *ANOPHELES DIRUS* BODY SIZE AND PARITY IN A FIELD POPULATION

Introduction

The body size of individual mosquitoes in nature has received increasing attention by researchers. Several recent papers have dealt with females of *Aedes* and *Culex* species but few studies have included *Anopheles*. Adult size may be influenced by several factors during development but the primary factors appear to be food availability and temperature. Variation occurs in the size of individual mosquitoes emerging from different larval habitats and during different seasons (Fish 1985, Hawley 1985). I questioned whether the size of adult *Anopheles dirus* influences vectorial capacity (VC) by affecting the probability of malaria infection and transmission. The ecological efficiency of *An. dirus* as a malaria vector depends on how long it lives, the host on which it feeds, and on how frequently it feeds. Longevity is an important component of VC (Macdonald 1957). Larger mosquitoes generally appear to live longer and therefore may have more host contacts than small-bodied individuals.

Large body size has been experimentally related to increased survival in adult *Aedes triseriatus* (Say) (McCombs 1980) and *Culex tarsalis* Coquillett (Reisen et al.

1984). Parity studies have been used as a measure of the survival rate of field populations (Davidson 1954, Macdonald 1973, Service 1976). Populations with a large proportion of parous females have increased VC. Haramis (1983) observed an increased parity rate among large *Ae. triseriatus* compared with smaller individuals. This suggested that larger individuals either had a higher probability of surviving or increased blood-feeding success. Recently, several researchers have observed that larger size classes of several species have higher parity rates (Hawley 1985, Nasci 1986a, b, Nasci 1987). Therefore, variation in adult female body size may be directly related to the VC of females in the population.

Landry et al. (1988) were unable to demonstrate consistent year to year variation in the body size of *Ae. triseriatus* which would support the idea that increased size is correlated with the transmission season of Lac Crosse (LAC) virus, a locally important cause of human disease in the hardwood forests of the upper Midwest. Moreover, there is laboratory evidence that small *Ae. triseriatus* may be more susceptible to infection by (LAC) virus than larger individuals. However, this observation would be epidemiologically meaningless if small individuals do not survive the extrinsic incubation period of the virus (Grimstad and Haramis 1984).

The objectives of my study were to:

1. Compare the relationship between female body size and parity in field populations of *An. dirus* from Chanthaburi Province, Thailand.

2. Investigate the seasonal variation in body size among female *An. dirus* in relation to the seasonal pattern of malaria transmission.

3. Examine the relationship between adult body size, rainfall and air temperature.

4. Predict the daily survival rate of *An. dirus* populations from parity correlations and calculate the expectation of survival after first feed and after first infection.

Materials and Methods

Mosquito collections

The study site in Tha-Mai District and collection methods are described in Chap. 1 and 3. Indoor and outdoor human-biting and indoor resting *Anopheles* were collected from 1800-2400 hr for 6 consecutive nights each month for 15 months (Jan 1987 through Mar 1988). In addition, on 4 nights each month outdoor biting collections were extended until 0600 hr. Preliminary biting collections made for 6 nights (1800-2400 hr) during November and December of 1986 are also included in the analysis.

Maximum and minimum air temperature and humidity at this study site were recorded daily with an electronic thermohygrograph meter (ISUZU).

Identification

Anopheles dirus in Thailand represents at least 5 different forms: A, B, C, D and F. However, form A greatly predominated in my study area. This was demonstrated by testing specimens with DNA probes (Panyim et al. 1988). All of 58 field specimens collected at different season and tested with these probes were identified as form A.

The morning after collection, all specimens were identified (Peyton and Scanlon 1966, Swadiwongporn 1985) with a 10X hand lens, assigned an ID number, and *An. dirus* separated out for dissection and wing measurement. Other *Anopheles* were sun-dried in individual specimen vials and kept at room temperature for future ELISA testing.

Wing measurement

The wings of each female *An. dirus* were removed with forceps, placed on a glass slide and covered with a cover slip. The length of the right wing was measured from the humeral cross vein to the apical margin, excluding the fringe of scales (Fig. 12). In the event of damage to the right wing, the left wing was measured. Measurements were done under a binocular dissecting microscope equipped with an ocular micrometer.

Dissection

Dissections of *An. dirus* were carried out by the Thai Malaria Division of the Ministry of Public Health. Parity of *An. dirus* was determined by removing ovaries in a drop of mosquito saline and observing the condition of the tracheoles under the compound microscope. Parity is easily determined in *An. dirus* because ovarian tracheoles are obviously uncoiled in parous females and remain tightly coiled in nulliparous ones. Salivary glands and midguts of both parous and nulliparous females were dissected in separate large drops of saline before parity examination of ovaries. Glands and midguts of parous females were examined under the compound microscope. Glands and midguts of both parous and nulliparous females were then preserved in blocking buffer in individually labeled, vials and frozen for future ELISA testing.

Statistical methods

Size differences between parous and nulliparous females were analysed with a 2-way analysis of variance including time as the second variable (Proc GLM, SAS Institute 1987). A cyclic function of the form

$$\text{wing length} = B0 + B1\,[\sin(B2 + \text{time})]$$

was fitted to the data to evaluate the significance of seasonal changes in wing length using Proc NLIN (SAS Institute 1987), where:

B0 = average value (mean of wing length)

B1 = adjustment in Y-axis

B2 = adjustment in X-axis

The probability of daily survival was calculated from the following Davidson (1954) equation:

$$P = {}^{n}/_{\overline{m}}$$

where: P = probability of daily survival

n = gonotrophic cycle (the gonotrophic cycle of *An. dirus* in this study was designated as 4 days)

m = proportion parous

Then, life expectancy in days after first feed was determined using the Macdonald (1973) formula:

$$1/(-\text{Ln } P)$$

where: P^t= probability that the population is expected to live long enough to become infective

Therefore, life expectancy in days after surviving t days is:

$$P^t/(-\text{Ln } P)$$

Results

A total of 974 adult female *An. dirus* were collected. The wing lengths of 964 females were measured; 10 were lost in processing. The mean wing length was 3.038 ± 0.006 mm (X ± SE); the coefficient of variation (CV) of wing length measurements was 6.13 and range was 2.17 - 3.72

mm (Table 27). It is difficult to collect male *An. dirus* in the field; only two resting males were collected from Sri-Phraya village. Their mean wing length was 2.655 ± 0.135 mm which is considerately smaller than the females (Tables 27 and 28). The mean wing lengths for parous and nulliparous females were 3.039 and 3.035 mm respectively. Differences in wing length between parous and nulliparous females were not significant in 2-way ANOVA ($\underline{F}$ = 0.16, df = 1, 883, $\underline{P}$ = 0.6861). The seasonal differences in both size and parity suggest that significant wing length differences may have occurred but in my sample, the relationship could not be demonstrated with confidence ($\underline{F}$ = 1.60, d.f. = 16, 883, $\underline{P}$ = 0.0625) (Table 28). Monthly parous and nulliparous wing lengths are compared in Table 29 and Figure 13. These data indicate that wing lengths for parous and nulliparous were at times significantly different but without any consistent pattern.

Regression with a sine function shows that the seasonal fluctuation in wing length was highly significant ($\underline{F}$ = 71.5025, df = 2, 963, $\underline{P}$ = 0.000). The fitted model was wing length = 3.079 + 0.177 [sin (0.523 x month - 1.065)]. Figure 14 illustrates the expected values from the model with the observed means (± S.E.). Regression analysis suggests that the wing length of biting females was related to minimum air temperature during the period 15-30 days before collection (Table 30). In the final model of the stepwise analysis, the relationship with

minimum air temperature (15-30 days) was confirmed as well as the relationship with rainfall 8-14 days before collection (Table 31). The final model (R^2 = 0.5648, $\underline{F}$ = 7.14, df = 2, 11 and $\underline{P}$ = 0.0103) is given by wing length = 4.285 - 0.059 (minimum air temperature 15-30 days before) + 0.004 (rain 8-14 days before). Expected wing lengths are plotted against observed wing lengths in Figure 15.

The daily probability of survival (P) was calculated ($P=\sqrt[n]{\overline{m}}$) for each month (Table 32). The life expectancy of the population was 1/(-Ln P) (Macdonald 1973). Survival to the first (infective) blood meal was highest in February 1987 and March 1988. In February 1987, 81.82 % of females caught biting were parous. Of these, 63 % would be expected to live the 9 days necessary for *Plasmodium vivax* development and 54 % of these females would be expected to live the 12 days required for *Plasmodium falciparum* development. The mean life expectancy after surviving the 9 and 12 day extrinsic incubation periods was 12.29 days and 10.54 days respectively.

Figure 12. *Anopheles dirus* wing measurement from the humeral cross vein to the apical margin.

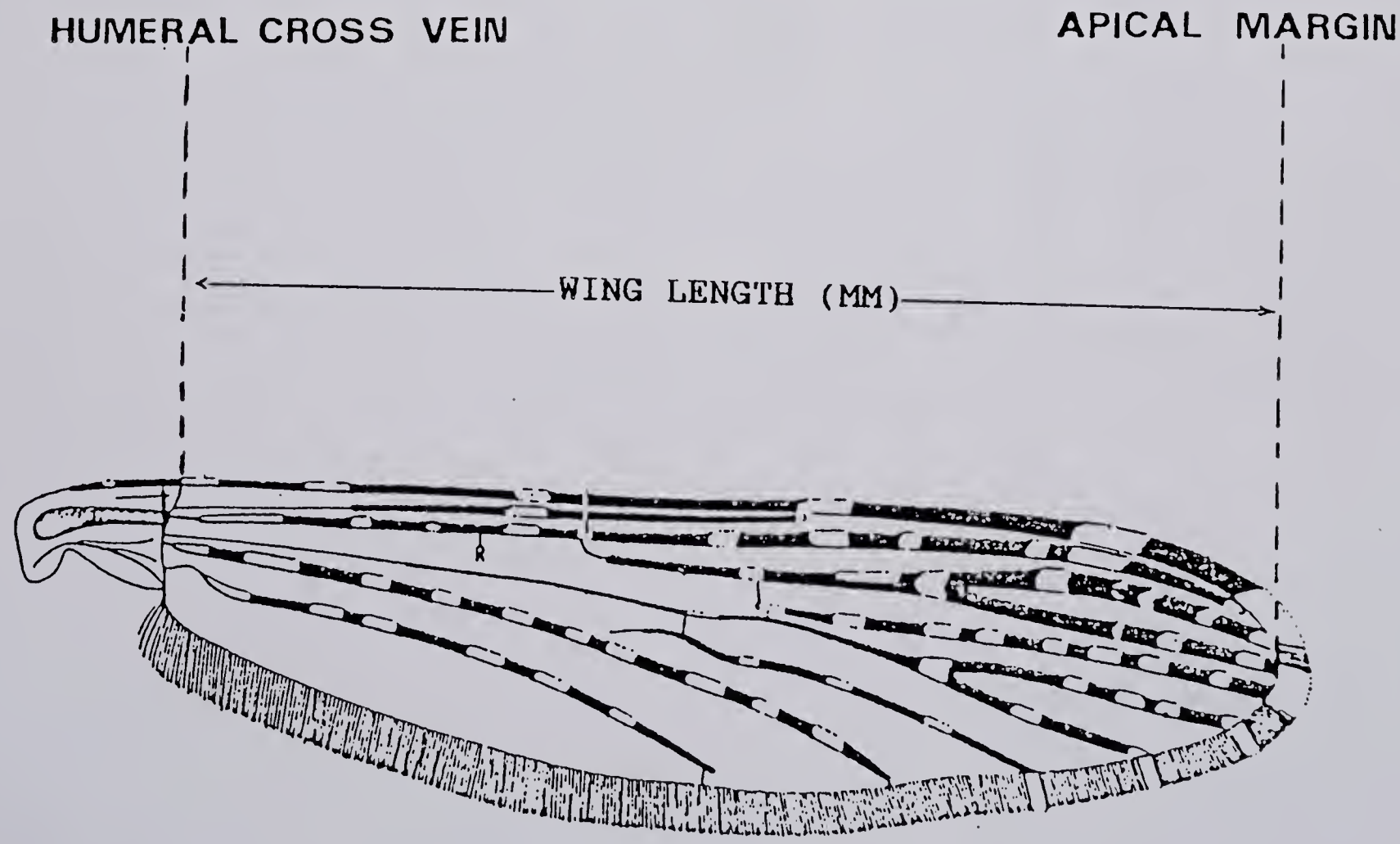

HUMERAL CROSS VEIN
APICAL MARGIN
WING LENGTH (MM)
R

Table 27. Monthly wing lengths of female* *An. dirus*.

Month (Year)	No. observed	No. measured	Minimum (mm)	Maximum (mm)	Mean (mm)	SE	CV
Nov(1986)	45	45	2.72	3.45	3.084	0.026	5.66
Dec	25	25	2.69	3.52	3.191	0.051	7.99
Jan(1987)	51	51	2.79	3.72	3.256	0.029	6.36
Feb	11	11	2.93	3.45	3.128	0.061	6.47
Mar	5	5	2.83	3.07	2.966	0.042	3.17
Apr	5	5	2.93	3.41	3.174	0.093	6.55
May	33	32	2.79	3.45	3.117	0.032	5.81
Jun	121	120	2.59	3.48	3.043	0.014	5.04
Jul	130	127	2.52	3.41	2.945	0.035	13.39
Aug	82	81	2.17	3.40	2.952	0.022	6.71
Sep	190	189	2.50	3.45	3.017	0.013	5.92
Oct	107	106	2.59	3.31	2.965	0.015	5.21
Nov	84	84	2.41	3.45	3.046	0.023	6.92
Dec	40	38	2.6	3.48	3.132	0.033	6.49
Jan(1988)	15	15	2.83	3.45	3.168	0.056	6.85
Feb	20	20	2.98	3.52	3.216	0.033	4.59
Mar	10	10	2.76	3.14	2.957	0.043	4.60
Total	974	964	2.17	3.72	3.038	0.006	6.13

Note: The coefficient of variation (CV)

$$CV = \frac{\text{Standard Deviation} \times 100}{\text{Mean}}$$

* Two males were caught in Dec. 1986 and Sept. 1987; and wing lengths were 2.52 and 2.79 mm respectively. Their mean wing length $\pm$ (SE) was 2.655 $\pm$ (0.135).

Table 28. Wing length of *An. dirus* collected in Sri-Phraya Village.

Parity	No. observed	No. measured	Mean (mm)	Maximum (mm)	Minimum (mm)
Parous*	474	473	3.039	3.62	2.17
Nulliparous*	445	444	3.035	3.72	2.50
Male	2	2	2.655	2.79	2.52

* No significant difference between parous and nulliparous female wing lengths ($\underline{F}$ = 0.16, df = 1, 883, $\underline{P}$ = 0.6861). Interactions between season and parity suggest differences occur but they are not consistently significant by 2-way ANOVA ($\underline{F}$ = 1.60, df = 16, 883, $\underline{P}$ = 0.0625).

Table 29. Monthly wing lengths of parous and nulliparous *An. dirus*.

	Parous		Nulliparous	
Month (Year)	No. measured	Mean (SE) (mm)	No. measured	Mean (SE) (mm)
Nov(1986)	30	3.049 (0.031)	14	3.149 (0.049)
Dec	13	3.205 (0.066)	9	3.218 (0.086)
Jan(1987)	27	3.231 (0.042)	22	3.281 (0.043)
Feb	9	3.168 (0.068)	2	2.950 (0.020)
Mar&Apr	3	3.000 (0.070)	7	3.100 (0.079)
May	19	3.119 (0.035)	11	3.119 (0.068)
Jun	58	3.056 (0.021)	58	3.028 (0.019)
Jul	72	2.980 (0.021)	50	2.894 (0.025)
Aug	53	2.923 (0.027)	22	2.999 (0.041)
Sep	70	3.037 (0.021)	103	2.994 (0.018)
Oct	45	2.981 (0.023)	59	2.950 (0.020)
Nov	31	3.008 (0.039)	51	3.081 (0.028)
Dec	20	3.096 (0.044)	15	3.200 (0.045)
Jan(1988)	9	3.192 (0.058)	6	3.132 (0.117)
Feb	6	3.232 (0.037)	13	3.227 (0.045)
Mar	8	2.973 (0.046)	2	2.895 (0.135)
Total	473	3.039 (0.009)	444	3.035 (0.010)

Figure 13. Monthly wing length of parous and nulliparous *An. dirus*.

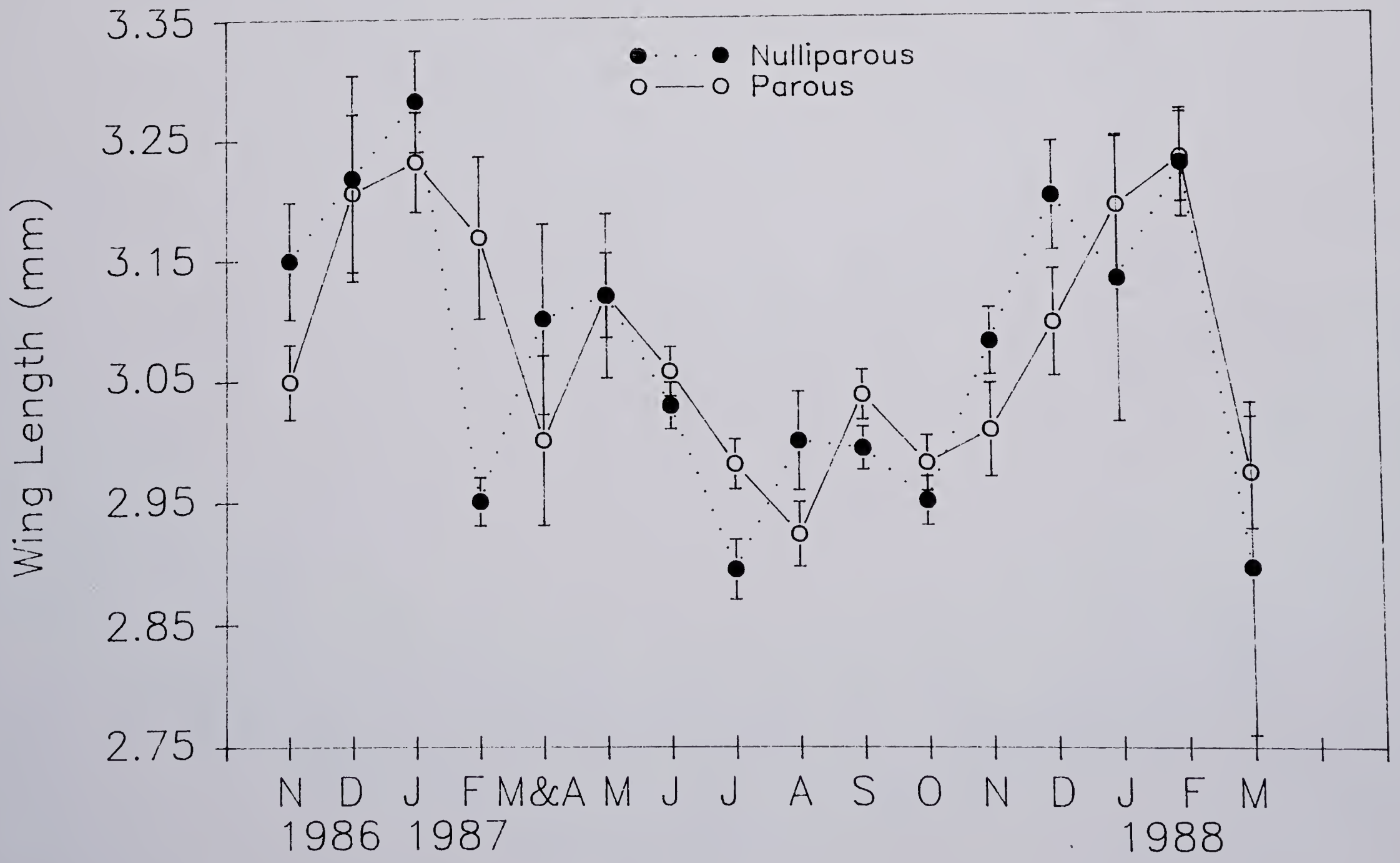

Nulliparous
Parous
Wing Length (mm)
3.35
3.25
3.15
3.05
2.95
2.85
2.75
N D J F M&A M J J A S O N D J F M
1986 1987
1988

Figure 14. Results of observed values fits with a sine curve.

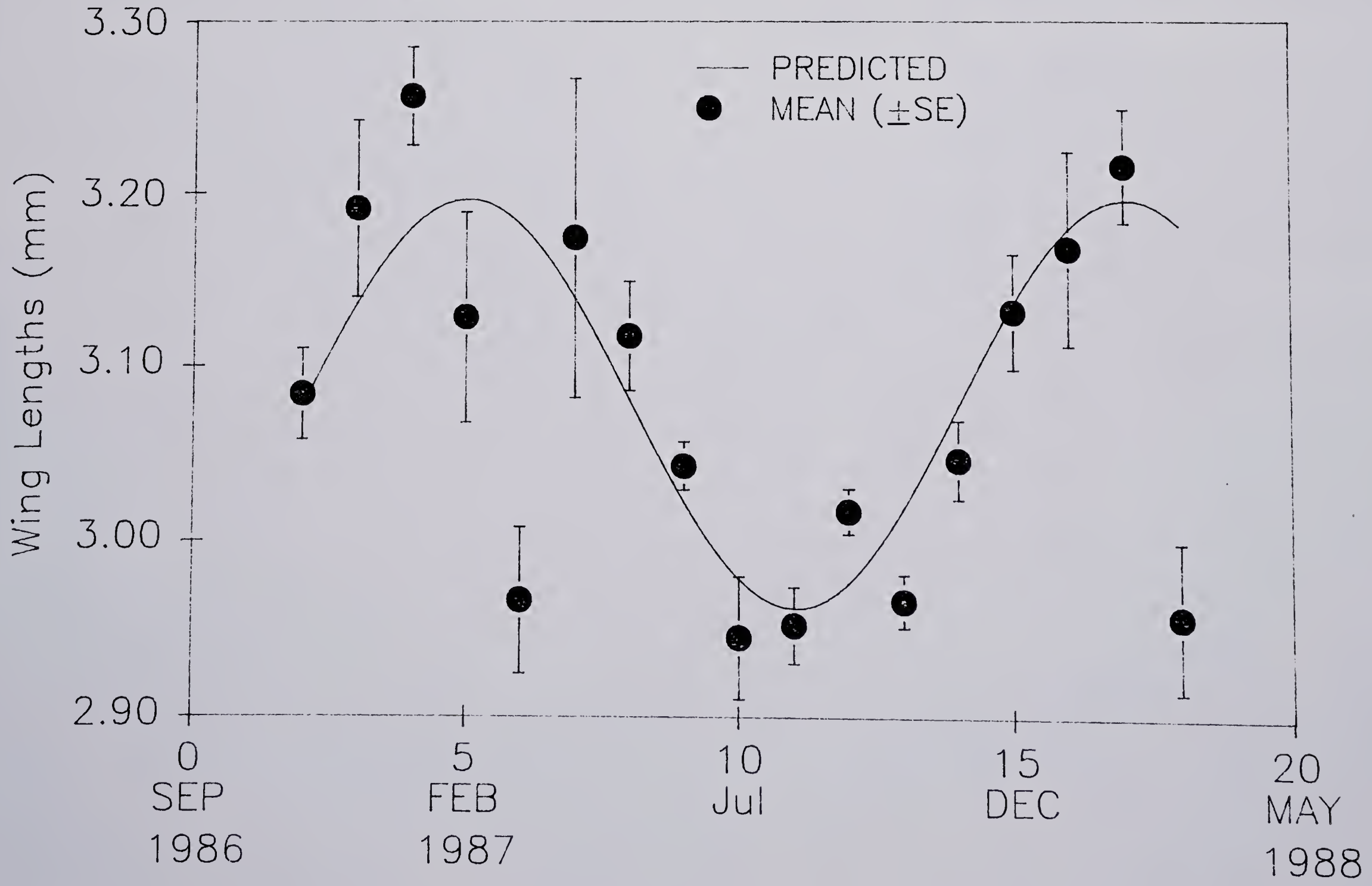

3.30
3.20
3.10
3.00
2.90
Wing Lengths (mm)
PREDICTED
MEAN (±SE)
0
SEP
1986
5
FEB
1987
10
Jul
15
DEC
20
MAY
1988

Table 30. Regression analysis of wing length with rainfall, maximum air temperature and minimum air temperature (df = 1, 12).

Variable	R^2	F	P -value
Day-Rain(0-7)	0.0646	0.8289	0.3805
Night-Rain(0-7)	0.0092	0.1112	0.7445
Day-Rain(8-14)	0.0071	0.0858	0.7746
Night-Rain(8-14)	0.0031	0.0379	0.8489
Day-Rain(15-30)	0.1358	1.8862	0.1947
Night-Rain(15-30)	0.0658	0.8454	0.3760
Max air temperature(0-7)	0.0196	0.2395	0.6334
Min air temperature(0-7)	0.1099	1.4820	0.2469
Max air temperature(8-14)	0.0410	0.5129	0.4876
Min air temperature(8-14)	0.0898	1.1836	0.2980
Max air temperature(15-30)	0.0001	0.0011	0.9739
Min air temperature(15-30)	0.3779	7.2882	0.0193*
Total rain(0-7)	0.0278	0.3432	0.5689
Total rain(8-14)	0.0056	0.0676	0.7992
Total rain(15-30)	0.1030	1.3786	0.2631
Mean air temperature(0-7)	0.0860	1.1287	0.3090
Mean air temperature(8-14)	0.0127	0.1541	0.7016
Mean air temperature(15-30)	0.1518	2.1477	0.1685

* The regression relationship between the wing length of adult biting females and minimum air temperature (measured 15-30 days before collection).

Table 31. Results of stepwise linear regression predicting wing length of adult females from daily rain and air temperature. Model R^2 = 0.5648, F = 7.14, df = 2, 11 and P = 0.0103. Factors without parameter estimates were excluded from the model.

Variable	Parameter estimate	F	P-value	Partial R^2
Intercept	4.28467681	166.25	0.0001	
Min air temp(15-30)	-0.05859864	14.14	0.0032	0.5592
Total rain(8-14)	0.00444399	4.73	0.0524	0.1870
Day-Rain(0-7)		0.2225	0.6473	
Night-Rain(0-7)		0.0615	0.8092	
Day-Rain(8-14)		0.2150	0.6528	
Night-Rain(8-14)		0.2150	0.6528	
Day-Rain(15-30)		0.1437	0.7125	
Night-Rain(15-30)		0.0139	0.9084	
Max air temperature(0-7)		0.0603	0.8110	
Min air temperature(0-7)		0.0660	0.8025	
Max air temperature(8-14)		1.3133	0.2785	
Min air temperature(8-14)		0.0080	0.9304	
Max air temperature(15-30)		0.3542	0.5649	
Total rain(0-7)		0.0009	0.9763	
Total rain(15-30)		0.0152	0.9044	
Mean air temperature(0-7)		0.0004	0.9854	
Mean air temperature(8-14)		0.3606	0.5616	
Mean air temperature(15-30)		0.3542	0.5649	

Figure 15. Result of observed wing lengths related to expected wing lengths based on minimum air temperature 15-30 days before collection and rainfall 8-14 days before collection.

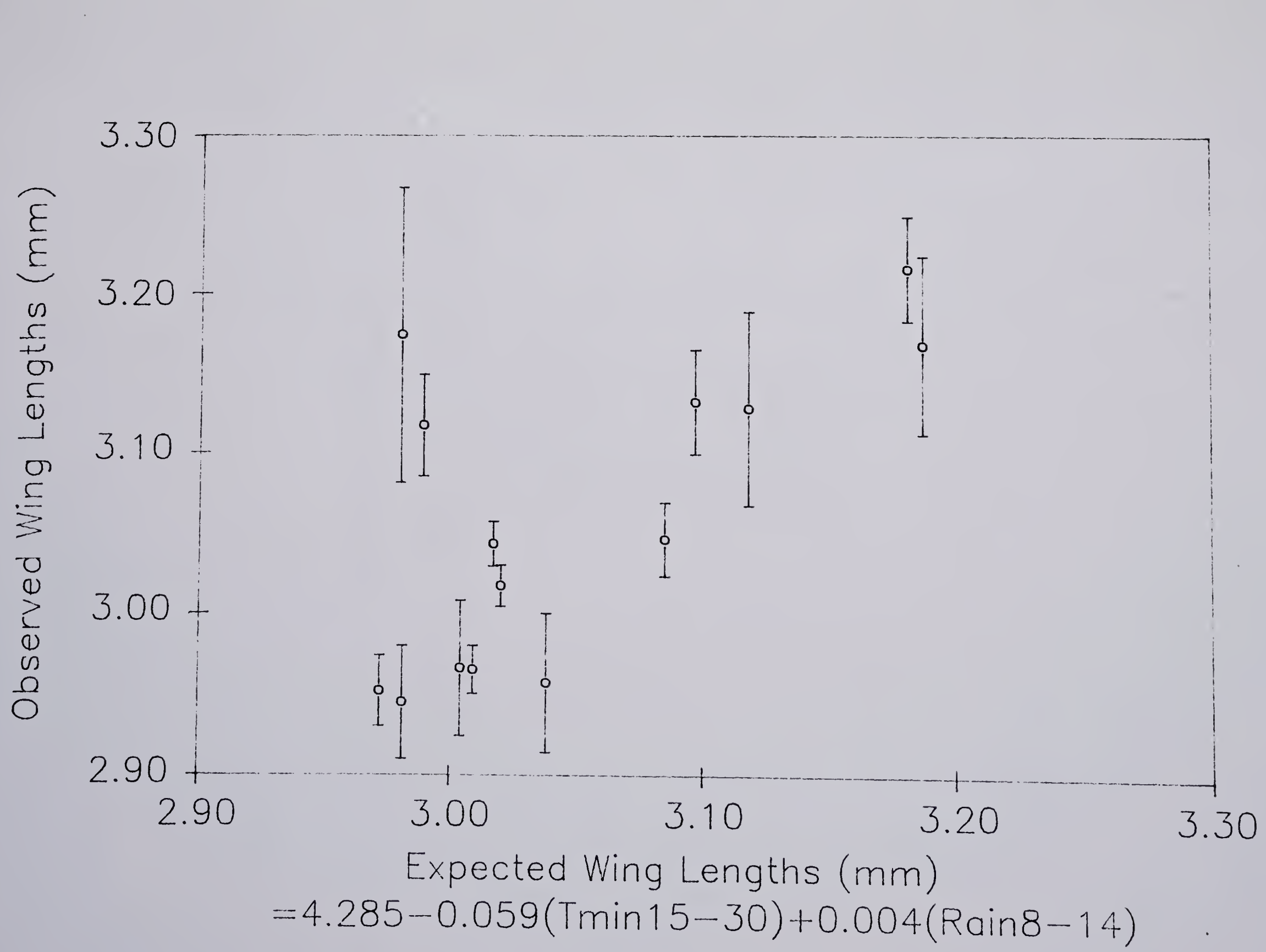
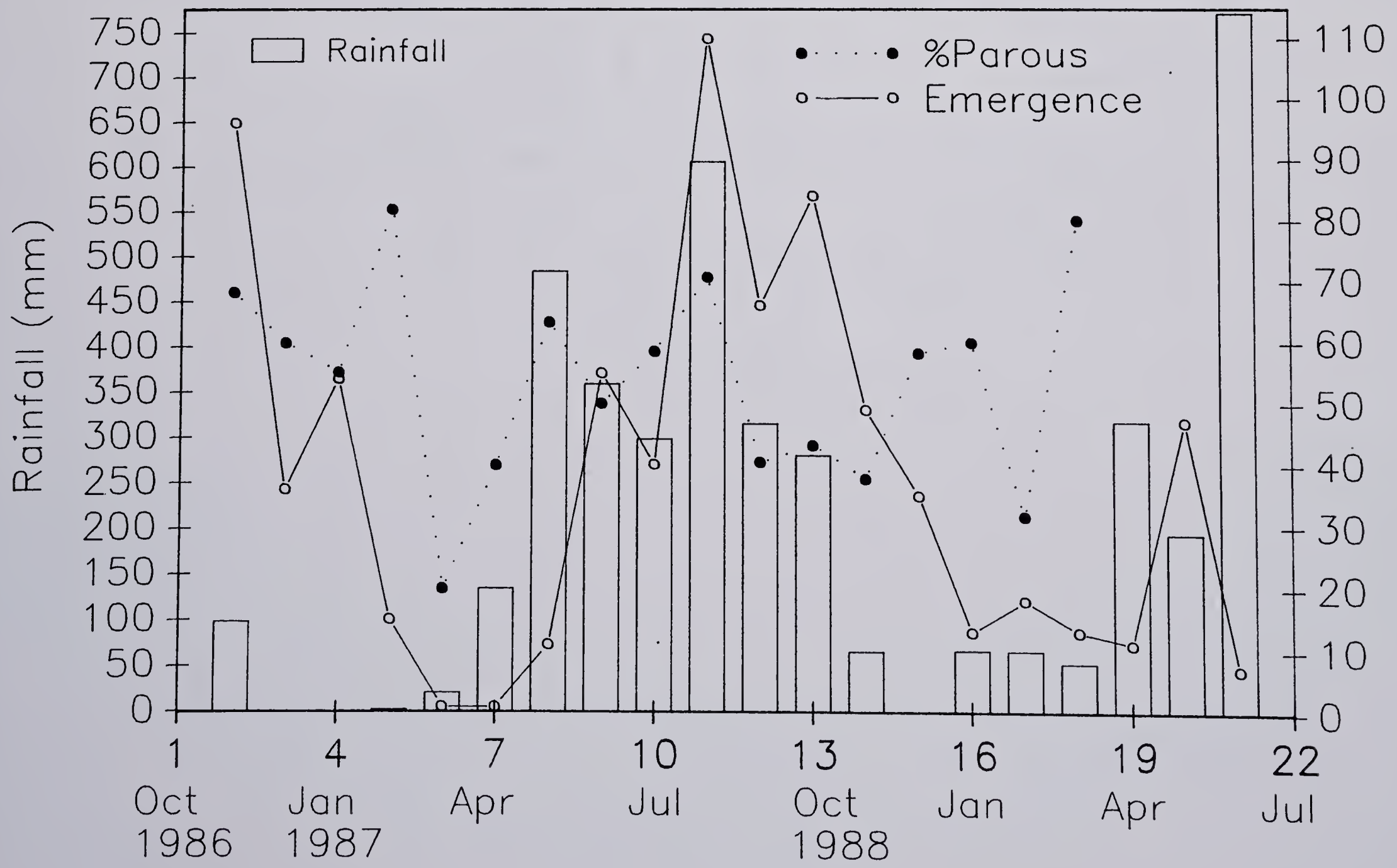

Observed Wing Lengths (mm)
3.30
3.20
3.10
3.00
2.90
2.90
3.00
3.10
3.20
3.30
Expected Wing Lengths (mm)
=4.285−0.059(Tmin15−30)+0.004(Rain8−14)

Table 32. Probability of *An. dirus* survival to infectivity at Sri-Phraya, Tha-Mai District.

Month (Year)	No. observed	No. Parous (%)	P^{**}	1/-Ln P	*P.falciparum* P^t	*P.falciparum* P^t/-LnP	*P.vivax* P^t	*P.vivax* P^t/-LnP
Nov (1986)	44	30 (68.18)	0.91	10.60	0.32	3.42	0.43	4.54
Dec	22	13 (59.91)	0.88	7.82	0.22	1.69	0.32	2.48
Jan (1987)	49	27 (55.10)	0.86	6.63	0.16	1.08	0.26	1.71
Feb	11	9 (81.82)	0.95	19.50	0.54	10.54	0.63	12.29
Mar	5	1 (20.00)	0.67	2.50	0.01	0.02	0.03	0.07
Apr	5	2 (40.00)	0.80	4.48	0.07	0.31	0.13	0.60
May	30	19 (63.33)	0.89	8.58	0.25	2.12	0.35	3.01
Jun	116	58 (50.00)	0.84	5.74	0.12	0.70	0.21	1.19
Jul	123	72 (58.54)	0.87	7.18	0.19	1.35	0.29	2.05
Aug	75	53 (70.67)	0.92	11.99	0.37	4.41	0.47	5.66
Sep	173	70 (40.46)	0.80	4.48	0.07	0.31	0.13	0.60
Oct	104	45 (43.27)	0.81	4.75	0.08	0.38	0.15	0.71
Nov	82	31 (37.80)	0.78	4.03	0.05	0.20	0.11	0.43
Dec	36	21 (58.33)	0.87	7.18	0.19	1.35	0.29	2.05
Jan (1988)	15	9 (60.00)	0.88	7.82	0.22	1.69	0.32	2.48
Feb	19	6 (31.58)	0.75	3.48	0.03	0.11	0.08	0.26
Mar	10	8 (80.00)	0.95	19.50	0.54	10.54	0.63	12.29
Total	919	474						

Note: P^{**} Probability of survival for 1 day
1/-Ln P = Life expectancy for the day after first feed
P^t = Probability of survival to infective stage (t for *P. falciparum* = 12 days; for *P. vivax* = 9 days)
P^t/-Ln P = Life expectancy in days after surviving t days

Figure 16. Variation in rainfall, new adult emergence and percent parous.


Rainfall
%Parous
Emergence
Rainfall (mm)
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
110
100
90
80
70
60
50
40
30
20
10
0
1
Oct
1986
4
Jan
1987
7
Apr
10
Jul
13
Oct
1988
16
Jan
19
Apr
22
Jul

Discussion

Body size varies in field populations of *An. dirus*, as it does in populations of all other mosquito species studied to date (McCombs 1980, Bock and Milby 1981, Haramis 1983, Nasci 1986a, b). Although size variation has a genetic component (Greenough et al. 1971), all *An. dirus* in this study were collected from a single village (Sri-Phraya Village) and were overwhelmingly of the same cytospecies (i.e. form A). This suggest that most size variation was likely a response to environmental variation.

The lack of significant difference in wing length between parous and nulliparous cohorts is at variance with most other studies. The coefficient of variation (CV) for the wing length of *An. dirus* is equal to 6.13 (Table 27) which suggest that this species is less variable in size than *Ae. triseriatus*, a species which is highly variable (Fish 1985) and exhibits a strong relationship between adult size and parity (Nasci 1988). However, differences between parous and nulliparous wing lengths occurred at the 93.75 % confidence level when seasonal variation was taken into account in the analysis. Seasonal changes in the size of nulliparous females were followed by similar changes in the size of parous females. Wing length and parous rate both varied over time (Fig. 14 and Table 32) and *An. dirus* tended to have both a higher parity and a larger size in the dry season compared to the rainy season. Early adult

mortality may result from poor larval food quality, temperature-induced accelerated larval development, high larval density or other adverse biotic and abiotic factors. For example, high larval density may result in a larger number of adults but they may be smaller and have decreased survivorship. Also, larger numbers of adults may lead to reduced feeding success because host defenses are correlated with biting density (Edman et al. 1972). Furthermore, adult mortality may result from adverse weather conditions (e.g., low humidity) which may have a greater impact on small individuals.

My laboratory experiments with *An. dirus* indicated that larger females have greater survivorship potential (Chap. 7). Thus, the hypothesis that large field *An. dirus* survive longer and have more host contacts (= greater VC) seems reasonable given the fact that field populations showed size differences comparable to those I produced in the laboratory.

The body size of *An. dirus* changed over time with maximum wing lengths occurring in February and minimum wing lengths in August (Fig. 14). In Tha-Mai District, the rainy season is from April to November and most mosquitoes are produced during this period. Mosquitoes collected outside of this season mainly represented surviving individuals from earlier cohorts. Presumably larger mosquitoes have increased vigor and nutritional reserves to carry them through the dry season. Thus, differential

survivorship of various size mosquitoes may be the result of physical and chemical parameters experienced during development (Chap. 4). Rainfall and temperature may impact on the quality and quantity of food available and on the metabolic rate. Both can increase or decrease adult size and survivorship. Cooler developmental temperatures cause slower growth and subsequent larger adult mosquitoes. Rainfall may promote larger mosquitoes by bringing more particulate food into the aquatic surface layer (neuston) where *Anopheles* feed. Coincidentaly, the highest temperatures at Tha-Mai also occur in the driest season. Expected values were related to observed values every months except during April and May (Fig. 15). Adult biting collections in April and May were likely a mixture of very old and very new mosquitoes and this would complicate any examination of the relationship between the observed and expected values.

Mosquito survivorship can be directly measured in nature only by mark-recapture methods. In this study, I estimated seasonal survivorship from the percent parous (Macdonald 1973) even though this method has limitations when overlapping generations occur. In the laboratory, *An. dirus* usually oviposited on the third, fourth and fifth nights after a blood meal at 26^{o}C. Thus, a 4 day feeding cycle (n=4) was used. During 4 (Nov. 1986, Feb. 1987, Aug. 1987 and Mar. 1988) of the 17 months of study, >40 % of the females captured were expected to live long enough to

develop mature *P. vivax* sporozoites. During 2 months (Feb. 1986 and Mar 1988), >40 % of females were expected to survive long enough to produce mature *P. falciparum* sporozoites. Monthly calculations of survival showed that seasonal parous rates were quite stable from year to year, e.g., in February 1987 and March 1988 daily survivorship was at its peak (= 95 %). Variation in the percent parous among biting populations was probably influenced by variation in the size of newly emerged individuals which was related to rainfall (Fig. 16). In the rainy season at Tha-Mai, aquatic developmental sites for *Anopheles* covered a wide area giving rise to a large emergence of adult mosquitoes. This resulted in the lowest proportion of parous of any time during the year.

Conclusions

1. There was no significant difference in the body size of parous and nulliparous *An. dirus* from the combined adult collections at Tha-Mai District. There was a difference at the 93.75 % confidence level when seasonal variation was considered.

2. The largest females and the highest parity rates occurred in the dry season.

3. Size variation is correlated with minimum air temperature during the previous 15-30 days and rainfall during the previous 8-14 days.

4. Estimation of the survival rate of *An. dirus* females based on the percent parous, indicated that *An. dirus* collected in February 1987 and March 1988 lived the longest and therefore had the greatest vectorial capacity for malaria.

CHAPTER VI

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TESTS FOR SPOROZOITES IN FIELD POPULATIONS OF *ANOPHELES*

Introduction

The ability to identify malaria sporozoites and determine the sporozoite rate in mosquitoes is important in epidemiological studies (Macdonald 1952). Several methods have been used to detect sporozoites: microscopic examination, immunoradiometric assay (IRMA), immunofluorescent assay (IFA) and the enzyme-linked immunosorbent assay (ELISA). The most widely used procedure today is the ELISA. Introduction of monoclonal antibodies significantly improved the specificity of the ELISA as a diagnostic test. Recently, it has been developed to distinguish between *Plasmodium falciparum* (Welch) and *Plasmodium vivax* (Grassi & Feletti) sporozoites in infected mosquitoes. This technique offers several advantages: sporozoites can be identified to species, specimens can be dried or frozen for several months before analysis, numbers of sporozoites can be estimated, and a large number of mosquitoes can be examined in a short period of time.

The double antibody sandwich ELISA test was developed by Burkot et al. (1984a). Subsequently, the

basic two-site "Sandwich" ELISA method for detecting *P. falciparum* and *P. vivax* was developed by Burkot et al. (1984b) and Wirtz et al. (1985), and later modified by Wirtz (1987). This method uses stable reagents, can be read visually or with a spectrophotometer, and is capable of highly specific and accurate detection of sporozoites.

The dissection technique requires personnel who are highly skilled in microscopic examination. This technique can only be used with freshly caught specimens and does not identify sporozoites to species. The IRMA will identify the malaria species in sporozoite-infected mosquitoes, but this method requires the use of radioactive reagents which creates handling and disposal problems. The IRMA has been successfully field tested for the identification of *P. falciparum* (Collins et al. 1984). The IFA method for identifying *Plasmodium* sporozoites offers good sensitivity and also is suitable for use with dried specimens (Ramsey et al. 1983). However, both the IRMA and the IFA require the use of expensive equipment and power supplies which are difficult to transport and maintain under tropical field conditions.

In Thailand, malaria is common in many areas of the country. According to Prasittisuk (1985), *Anopheles dirus* Peyton & Harrison, *Anopheles minimus* Theobald, and *Anopheles maculatus* Theobald are the primary vectors. *Anopheles sundaicus* (Redenwaldt) and *Anopheles aconitus* Donitz are also considered to be vectors but other

Anopheles species also may be responsible for some malaria transmission.

This study was conducted in Sri-Phraya village in Tha-Mai district, where *An. dirus* has been considered the primary vector.

Materials and Methods

Anopheles collection

Human-biting and resting mosquitoes were collected monthly from November 1986 through March 1988 in Sri-Phraya Village. Specimens were transported to the field lab, sorted, and identified to species based on the taxonomic criteria of Peyton and Scanlon (1966) and Swadiwongporn (1985). Only *An. dirus* were dissected. Other species were sun-dried in individual specimen vials and kept at room temperature until tested for the presence of sporozoites by ELISA at the AFRIMS laboratory in Bangkok.

Dissection of *Anopheles dirus*

Anopheles dirus wings were removed and mounted under a coverslip on a glass slide for wing length measurement (Chap. 5). Mosquito dissection and parity examination was carried out by the entomology team of the Malaria Division. Salivary glands and midguts of all parous females were examined microscopically for oocysts and sporozoites. Then, salivary glands and midguts of both parous and nulliparous females were preserved in 50 ul of blocking

buffer (BB)* and frozen individually in labeled vials for later ELISA testing.

Preparation of specimens for ELISA testing

Intact females of other *Anopheles* species were divided into 2 parts by cutting between the thorax and abdomen with a sharp blade under a dissecting microscope. The head-thorax and abdomen portions were placed into separate labeled microcentrifuge tubes (1.5 ml) containing 50 ul of blocking buffer (BB). All freshly divided or thawed (i.e. previously dissected *An. dirus*) samples were then homogenized with a tissue grinder. Three hundred microliters of BB was added to each homogenate to bring the final volume to 350 ul. The homogenate was tested by ELISA in 96-well polyvinylchloride microtiter plates with "U"-bottom wells.

ELISA method

After samples were prepared for ELISA testing the following methods were employed:

1. Work sheets were prepared and plates were marked with a waterproof pen.

* one liter BB = 10 g BSA; 5.0 g Casein; 0.1 g Thimerosal; 0.01 g Phenol red; 1,000 ml Phosphate Buffer Saline (PBS) pH 7.4 with Nonidet P-40 (NP-40) (5 ul NP-40/ml BB)

2. Fifty ul of monoclonal antibody (MAB) 2A10 for *P. falciparum* (0.10 ug/50 ul PBS/well) or MAB NSV3 for *P. vivax* (0.025 ug/50 ul PBS/well) was added to each well and plates were incubated overnight at room temperature.
3. The following morning, monoclonal solution was aspirated from the wells and wells were refilled with BB for 1 hr.
4. Plates were again aspirated and 50 ul of each homogenized mosquito extract was added per well. Positive and negative controls also were placed in designated wells.
5. After 2 hr incubation at room temperature, wells were washed twice with 0.05 % PBS-TW 20 (0.5 ml Tween-20/ 1 liter PBS, pH 7.4).
6. Horseradish peroxidase (HRPO) conjugated monoclonal antibody was added to each well (0.05 ug/50 ul BB/well) and incubated for 1 hr.
7. Wells were aspirated and washed 3 times with PBS-TW 20.
8. One hundred ul of peroxidase substrate (substrate ABTS:H_2O_2, 1:1) was added to each well.
9. Optical density was read after 15, 30 and 60 min using an ELISA plate reader (Titertek Multican, Flow laboratories Inc., McLean, VA) set at 414 nm.

10. The mean and standard deviation (SD) for negative controls were calculated.
11. Mosquitoes were considered infected when the ELISA absorbance values exceeded the mean plus 3 SD of the negative control mosquitoes on the same plate.
12. Positive mosquitoes were retested to confirm the results and to determine the estimated number of sporozoite-equivalents per mosquito.
13. Absorbance values were plotted against antigen dilutions (concentration of sporozoites) to generate a standard curve. Sporozoite equivalent values were estimated by determining the absorbance of the samples and locating it on the ordinate. A straight line was then drawn parallel to the abscissa until it intersected the standard curve. A perpendicular line was then dropped to the abscissa and the value at the point of intersection was the estimated number of sporozoites in the samples.

Results

Results of *Anopheles* species collected and tested each month are given in Table 33. Specimens representing 10 of the 11 species of *Anopheles* caught were tested by ELISA. Two *Anopheles aconitus* collected in November 1986 were not tested because only *An. dirus* were initially examined. A total of 2,905 *Anopheles* were collected from November 1986 through March 1988; 2,199 of these were

tested and the overall infection rate was 0.32 %. The infection rates for individual species were: 0.49 % for *An. dirus*, 0.23 % for *An. hyrcanus* group, 0.30 % for *An. tessellatus*, and 1.0 % for *An. vagus*.

Seven females representing 4 different species tested positive for malaria sporozoites (Table 34). These were 4 *An. dirus* (57.16 %) and one each of *An. hyrcanus* group, *An. tessellatus*, and *An. vagus* (14.28 %). Two mosquitoes were ELISA positive for *P. falciparum* and 5 for *P. vivax*. Both parous and nulliparous *An. dirus* were found to be sporozoite-antigen positive.

All 4 *An. dirus*, 1 *An. tessellatus* and 1 *An. vagus* were positive in the abdomen portion only. *Anopheles hyrcanus* group was positive in the head-thorax portion.

Table 33. *Anopheles* species collected and tested by ELISA. Numbers tested are shown in paranthesis.

	1986		1987								
Anopheles sp.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Sep.
dirus	45(31)	26(16)	51(28)	11(8)	5(5)	5(5)	33(32)	121(120)	130(128)	82(81)	191(86)
aconitus	2										
annularis											
barbirostris	200	41	40	14	6	7	40(36)	30(29)	36(31)	18(18)	17(17)
campestris								1(1)	16(12)	10(10)	1(1)
hyrcanus gr.	96	18	1	4		1	1	3(3)	16(11)	73(72)	56(55)
karwari	3	1	1								3(3)
philippinensis											
tessellatus	48	16	4					21(21)	12(7)	85(85)	131(129)
umbrosus								1(1)			
vagus					1	3	35(33)	48(48)		5(5)	
Total	394(31)	102(16)	97(27)	29(8)	12(5)	16(5)	109(101)	225(223)	210(189)	273(271)	399(291)

Table 1. Continued.

Anopheles sp.	Oct.	Nov.	Dec.	1988 Jan.	Feb.	Mar.	Total	% Positive n = 7	Infection rate
dirus	107(106)	84(84)	40(39)	15(15)	20(19)	10(10)	976(813)	4 (57.16%)	0.49%
aconitus							2		
annularis			4(4)				4(4)		
barbirostris	43(43)	138(138)	67(66)	31(31)	37(37)	40(39)	805(485)		
campestris	1(1)	10(10)					39(35)		
hyrcanus gr.	87(86)	164(162)	5(5)	5(5)	13(13)	14(14)	557(426)	1 (14.28%)	0.23%
karwari							8(3)		
philippinensis				1(1)			1(1)		
tessellatus	19(19)	26(26)	39(39)	2(2)	3(3)		406(331)	1 (14.28%)	0.30%
umbrosus							1(1)		
vagus					1(1)	13(13)	106(100)	1 (14.28%)	1.00%
Total	257(255)	422(420)	155(153)	53(53)	75(74)	77(76)	2905(2199)	7 (100%)	0.32%

Table 34. Summary of 7 malaria-infected *Anopheles* determinated by ElISA. All specimens collected in Sriphraya village from November 1986 through March 1988.

Month Year	Species (P/N)[a]	Wglgth (mm)	Coll. Type[b]	Head[c] Pv	Head[c] Pf	Abdomen[c] Pv	Abdomen[c] Pf
June 1987	*An.vagus*		IR			2100	
	An.dirus (N)	3.00	ODB			840	
	An.dirus (P)	2.86	ODB			1190	
	An.dirus (N)	2.91	ODB			840	
	An.dirus (P)	3.17	IDB			840	
Sept. 1987	*An.tessellatus*		ODB				1624
Nov. 1987	*An.hyrcanus* gr.		IDB		11200		

a P = parous, N = nulliparous
b ODB = outdoor biting
IDB = indoor biting
IR = indoor resting
c Estimated number of sporozoites of *P. falciparum* and *P. vivax* in body region

Discussion

Only *An. dirus* were dissected for microscopic detection of sporozoites and oocysts before being tested in the ELISA. Dissected *An. dirus* were all scored negative for both midgut oocysts and sporozoites in the salivary glands. Nonetheless, ELISA tests revealed that 4 *An. dirus* had abdominal infections with *P. vivax*. These results indicate that the ELISA test is more sensitive in detecting malaria sporozoites than field examination of mosquitoes dissected under the microscope. The skill of the examiner, the quality of the optics and the intensity of natural light (no artificial light was used) are all potential limitations to the accuracy of the dissection method as used in this study. In their numerous investigations at Sri-Phraya village, the Thai Malaria Division has reported no sporozoite-positive *Anopheles* from microscopic examinations despite the fact that villagers develop malaria each year (pers. observ.). This was the first time that specimens from this village were tested by ELISA and it suggests that improvements in the efficiency of the dissection method are needed if results are to accurately reflect natural infection rates.

Both parous and nulliparous *An. dirus* were positive in ELISA tests and the estimated number of sporozoites did not differ greatly between the two groups. Blood meals that are insufficient for egg development can conceivably

still produce a transmissible infection in mosquitoes (Mitchell et al. 1979). The relative size of the blood meal may have less effect on the number of sporozoites produced than the density of gametocytes in the blood ingested (Jeffery and Eyles 1955). Therefore, nulliparous female can be infected and may carry a large number of sporozoites. Parity in *An. dirus* is quite obvious under the microscope. If the ovary shows tightly coiled tracheoles, it is nulliparous. If it shows stretched and uncoiled tracheoles, it is parous. Therefore, the accuracy of the parity examination in this study should have been quite high.

Among the ten species tested, *An. dirus* is considered the major vector of human malaria in Thailand (Prasittisuk 1985). This species represented 57.16 % (4 of 7) of positive *Anopheles*. No *P. falciparum* infections were detected in *An. dirus* even though *P. falciparum* was often found in human blood surveys in neighboring villages (unpublished data from the Tha-Mai District Malaria Center). All 4 malaria positive *An. dirus* gave negative results from the head-thorax portion. No explanation for the lack of head positives can be offered at this time since the intact glands were very carefully dissected from the females.

It is possible that *An. hyrcanus* group, *An. tessellatus* and *An. vagus* also serve as vectors in this study area. *Anopheles dirus* was common in this village and

seemed to bite people outdoors more than indoors (ODB : IDB = 3 : 1). *Plasmodium falciparum* was detected in *An. hyrcanus* group. ELISA tests detected sporozoites in the head-thorax portions and the largest numbers of sporozoites were observed in this species. These data strongly implicate the *An. hyrcanus* group as a vector in this area.

The single ELISA-positive *An. tessellatus* and *An. vagus* were only positive in the abdomen portion so the question of their infectivity is less clear. Some anopheline species are capable of supporting development of sporozoites that fail to enter the salivary glands (Coatney et al. 1971).

My ELISA data indicated that some species that have never been considered important vectors of malaria in Thailand have infection rates equal to or exceeding those of the putative primary vectors. Further studies in this village are need to better resolve the vector status of these species.

Sporozoites were only found during 3 of the 17 months sampled. *Plasmodium vivax* seemed to be more likely to infect humans in June (1987) whereas *P. falciparum* infected females were most common in September and November (see Table 34). There were no significant differences in wing length between infected and uninfected *An. dirus* and between parous and nulliparous positive *An. dirus*. However, detection of size differences was limited by the small sample.

Conclusions

1. *Plasmodium falciparum* and *P. vivax* were detected by ELISA in both the head-thorax and abdomen portions of 4 species of *Anopheles* collected at Sri-Phraya.

2. Both parous and nulliparous *An. dirus* contained sporozoites but no differences in the size of *An. dirus* were noted between infected parous and infected nulliparous females.

3. Vectors other than *An. dirus*, especially *An. vagus, tessellatus* and *hyrcanus* group may play an important role in malaria transmission at Sri-Phraya village in Tha-Mai District.

CHAPTER VII

EVALUATION OF SURVIVAL POTENTIAL AND MALARIA SUSCEPTIBILITY AMONG DIFFERENT SIZE CLASSES OF LAB-REARED *ANOPHELES DIRUS*

Introduction

Interest in malaria in Thailand has been stimulated by the emergence of strains of *Plasmodium falciparum* that are resistant to chloroquine and other widely-used antimalarial drugs. Drug resistance has caused increasing public health problems due to malaria. The majority of malaria cases are detected in areas where the *Anopheles balabacensis* group is the putative principal vector. *Anopheles dirus*, the only member of the *An. balabacensis* group in Thailand, is a highly efficient vector of *P. falciparum*. It is more susceptible to infection than *Anopheles minimus* which is thought to be another primary vectors in Thailand (Wilkinson et al. 1972). Scanlon and Sandhinand (1965) demonstrated that *An. dirus* is the main vector in forested, hilly regions. Early interest in this species as a malaria vector led to the establishment of a laboratory colony by forced mating (Yang et al. 1963, Esah and Scanlon 1966). Colonies provided for controlled studies of malaria infection in this mosquito. They also make possible studies on the interrelationships between

body size, blood meal size, survivorship and malaria susceptibility.

In the laboratory, different size classes can be produced by manipulating food availability or population density (Reisen and Emory 1977). There is some evidence that laboratory-reared and nutritionally-stressed, small mosquitoes may be more susceptible to infection by certain viruses (but not others) than larger individuals (Bagar et al. 1980, Grimstad and Haramis 1984, Reisen et al. 1984, Patrican and DeFoliart 1985, Kay et al. in press). However, field studies suggest that small mosquitoes may not survive sufficiently long to serve as vectors (Haramis 1983, Hawley 1985, Nasci 1986a, b). Wing et al. (1985) noted that the body size of *Anopheles quadrimaculatus* had no effect on susceptibility to *Plasmodium yoelii*.

The objective of this study was to compare the survivorship, blood meal size and infectivity of different size classes of female *An. dirus* reared under control conditions in the laboratory and to relate these results to field observations on body size and survivorship (Chap. 4). A better understanding of malaria transmission in order to optimize control strategies is the ultimate objective.

Materials and Methods

General maintenance of *An. dirus* colony

A colony of *An. dirus* (form A) was obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, and maintained in the insectary of the Department of Biology at Mahidol University.

The insectary is an air-conditioned room maintained at 24-28^{o}C and 45-75 % relative humidity. Higher humidity was maintained in cages with adult mosquitoes by hanging damp cloths over the top and one side of each cage (20 x 20 x 20 cm). Cages were provided with a bottle of distilled water, a bottle of multi-vitamin syrup (manufacture by the Government Pharmaceutical Organization, Bangkok, Thailand) as food and a petri dish (9 cm dia.) containing a soaked cotton pad covered with filter paper for female oviposition. Eggs were removed from the filter paper with the aid of a small camel's hair brush and placed inside plastic straw circles floating on the surface of the distilled water in rearing bowls. The straw circles prevented the stranding of eggs along the edges of the bowl and, later, provided floating objects for the larvae.

After hatching, larvae were fed a finely ground fish food (Bangkok Feedmill Co., Ltd.) sprinkled on the water surface. Food was given in small quantities three times a day to prevent fouling of the water causing larval mortality.

Pupae were removed daily with a wide mouthed pipette and placed in bowls of water covered with mosquito net. After emergence, the water was pumped from the bowls and adults were fed a 7 % multi-vitamin syrup (7 cc multi-vitamin syrup and one teaspoon of sugar sucrose per 100 cc of distilled water) soaked in cotton and placed on the covered bowls. Three days after emergence, females were mated artificially by the forced mating technique (Yang et al. 1963). The next day, females were permitted to feed on a restrained hamster. Fully engorged females were transferred to cages for ovarian development and egg-laying.

Rearing procedures for different size classes

Environmental conditions were maintained at 22 ± 2°C, 78 ± 12 % relative humidity and photoperiod of 12 hr light : 12 hr dark. Eggs were hatched in the oviposition bowls containing distilled water. Larvae produced within a 3-hr period were combined in 1.5 liters of distilled water in plastic rearing pans (24 x 30.5 x 4 cm). Water in each pan provides a depth of 24 mm and a surface area of approximately 732 cm^2. Pans were covered with mosquito screen to eliminate dust and other contamination. Water was added daily to replace loss due to evaporation.

Larvae were reared at 4 different densities (100, 200, 300 and 400/ pan) to produce 4 different size classes of adults. Fish food ground to pass through an 80 mesh

sieve (180 um U.S. standard no.) was fed to larvae at the following daily rates/pan regardless of larval density:

day 1 (hatch)	5 mg	day 7	25 mg	day 13	30 mg
		day 8	30 mg	day 14	25 mg
day 2	0 mg				
		day 9	35 mg	day 15	20 mg
day 3	5 mg				
		day 10	40 mg	day 16	15 mg
day 4	10 mg				
		day 11 (1st pupae)	40 mg	day 17	10 mg
day 5	15 mg				
day 6	20 mg	day 12	35 mg		

Survivorship experiment

Adults of each size class were transferred individually to vials (one per vial). Each numbered vial (2.5 x 7 cm) was marked as to emergence time, date and size class. Female were provided with distilled water only and were checked every 12 hr (at 8 a.m. and 8 p.m.) for survival.

Infectivity experiment

Infectivity was evaluated by feeding blood infected with cultured *P. falciparum* gametocytes through an artificial membrane. Only the largest and smallest (from 100 and 400/pan densities) size classes were compared. Females of both size classes were starved for 5-7 hr before blood feeding. Groups (50-75 females/cup) of each size were blood fed simultaneously for 5-7 min through lamb skin membranes (condoms). Heated, defibrinated human blood containing a standard quantity of mature gametocytes (i.e.

0.14-1.1 % of RBC's were infected) from cultured parasites maintained at AFRIMS was used. Fully engorged females from each size cohort were held on 7 % multi-vitamin syrup until dissected. Five-ten fully engorged females from each group were used to determine the blood meal volume by the HiCN method of Breigel et al. (1979). The right wing of each of these control females was also removed and measured to establish the body size of each test group. On day 7-9 post-feeding, females were dissected to observe gut oocysts. Midguts were dissected from females and placed on a microscope slide in a drop of mercurochrome solution (Eyles 1950). The preparation was allowed to stand for a few minutes and then a coverslip was placed on the drop and the number of oocysts in each female was counted under the compound microscope (400X) up to a maximum of 100 oocysts. Seventeen test dates were conducted.

Data analysis

The relationships between: 1) body size and larval density, 2) survivorship and larval density, 3) body size and survivorship, and 4) body size and blood meal size were evaluated by regression analysis. A natural log (Ln) transformation was performed on larval density data to linearize the relationship with body size and survivorship. Normality was verified with Wilk-Shapiro/Rankit plots. Variances were compared by Bartlett's test for equal variance. Cases were weighted by the inverse of the

variance of body size and survivorship within the two categories of larval density to correct for unequal variances. All analyses were performed with "Statistix" (NH Analytical Software).

The relationship between body size and oocyst number was examined by analysis of covariance (Proc GLM, SAS 1987). Wing length was included as a covariate, larval density and % RBC's with gametocytes as fixed (i.e. independent) variables, and test date as a random variable.

Comparison of regression lines for survivorship and body size from laboratory and field populations also employed linear regression analysis (Proc REG, SAS 1987). The linear equation is: survivorship = intercept + a(group) + b(wing length) + c(group x wing length). When field group = 0 and laboratory group = 1, then survivorship (for field) = intercept + b(wing length) and survivorship (for laboratory) = (intercept + a) + (b + c)wing length, where

intercept = intercept for field

b = slope for field population

a = difference between field and lab intercepts

c = difference between field and lab slopes

Differences in the mean longevity of field and laboratory females were evaluated with a one-way ANOVA, where cases were weighted by the inverse of the variance within each group (PROC GLM, SAS Institute 1987).

Results

Adult size was affected by larval density (Fig. 17). The relationship was highly significant (F = 3258, df = 1, 264, P = 0.000 and R^2 = 0.9253). It is described in Fig. 17 by wing length in mm = 3.434 - 0.482 x Ln(density) where 1, 2, 3, and 4 are 100, 200, 300, and 400 larvae/pan. Survivorship was also related to larval density (F = 335.9, df = 1, 264, P = 0.000 and R^2 = 0.5609). The fitted regression line (Fig. 18) is described by survivorship in day = 5.087 - 1.345 x Ln(density) where 1, 2, 3, and 4 are 100, 200, 300 and 400 larvae/pan.

Survivorship and wing length were highly correlated (F = 281.5, df = 1, 264, P = 0.000 and R^2 = 0.517) (Fig. 19). The regression equation of survivorship and wing length is survivorship = a + b(wing length). Therefore, survivorship = -3.851 + 2.578(wing length in mm).

Barlett's Test indicated that the variances of blood meal size were different (X^2 = 305.90, df = 1, P = 0.000) among larvae reared at 100 vs 400 larvae/pan (Fig. 20). The mean meal size of females reared at 400/pan was 4.03 ul (± 1.04); for those reared at 100/pan it was 2.16 ul (± 0.87). Therefore, observations were weighted by the inverse of the variance within each density, and multiple regression was performed. Density of larvae was only important in so far as it influenced size; it was not directly important for blood meal size (P = 0.91).

Therefore, the larval density variable was eliminated from the model. The relationship between blood meal size and wing length (i.e. body size) was significant ($\underline{F}$ = 66.94, df = 1, 63, $\underline{P}$ = 0.00 and R^2 = 0.5192) and is presented in Fig. 20 as: blood meal size ul = -6.409 + 3.037(wing length in mm).

Infectivity experiments with the two different size classes are shown in Table 35. Larval density was not directly significant for oocyst number ($\underline{F}$ = 0.362, df = 1, 684, $\underline{P}$ = 0.5474), therefore it was eliminated from the model. Percent RBC's with gametocytes was not a significant factor ($\underline{F}$ = 0.400, df = 1, 685, $\underline{P}$ = 0.5273), and was eliminated as well. The final model indicated that the number of oocysts per midgut varied significantly with body size (i.e. wing length) and with test dates ($\underline{F}$= 12.964, df = 1, 686, $\underline{P}$ = 0.0003 and $\underline{F}$ = 49.534, df = 16, 686, $\underline{P}$ = 0.0001 respectively). The regression equation of oocyst number and wing length is described by $\sqrt{\text{oocyst no.}}$ = 4.454 + 0.967(wing length in mm) + $\underline{f}$(test date) which means that the intercept term varied among test dates (Table 36). For example, the regression lines of test date 13 and test date 16 are described in Figure 21 by $\sqrt{\text{oocyst no.}}$ = 4.454 + 0.967(wing length in mm) - 4.937 and $\sqrt{\text{oocyst no.}}$ = 4.454 + 0.967(wing length in mm) - 4.489 respectively.

The regression lines describing the relationship between survivorship and wing length were different for

adult mosquitoes from the field and those from the laboratory (F = 224.731, df = 2, 604, P = 0.000). Differences in the intercept (F = 5.54, df = 1, 604, P = 0.0189) and slope (F = 14.96, df = 1, 604, P = 0.0001) were significant. Equations for the regressions for the laboratory and field are: survivorship = -3.85 + 2.578(wing length in mm) and survivorship = -1.915 + 1.682(wing length in mm). A stronger relationship was observed in the laboratory (R^2 = 0.517) than in the field (R^2 = 0.1276) and mean survivorship values also were different (mean = 4.07 in the laboratory versus mean = 2.77 in the field data: F = 310, df = 1, 607, P = 0.0001) (Fig. 22).

Figure 17. Relationship between wing length and larval density.

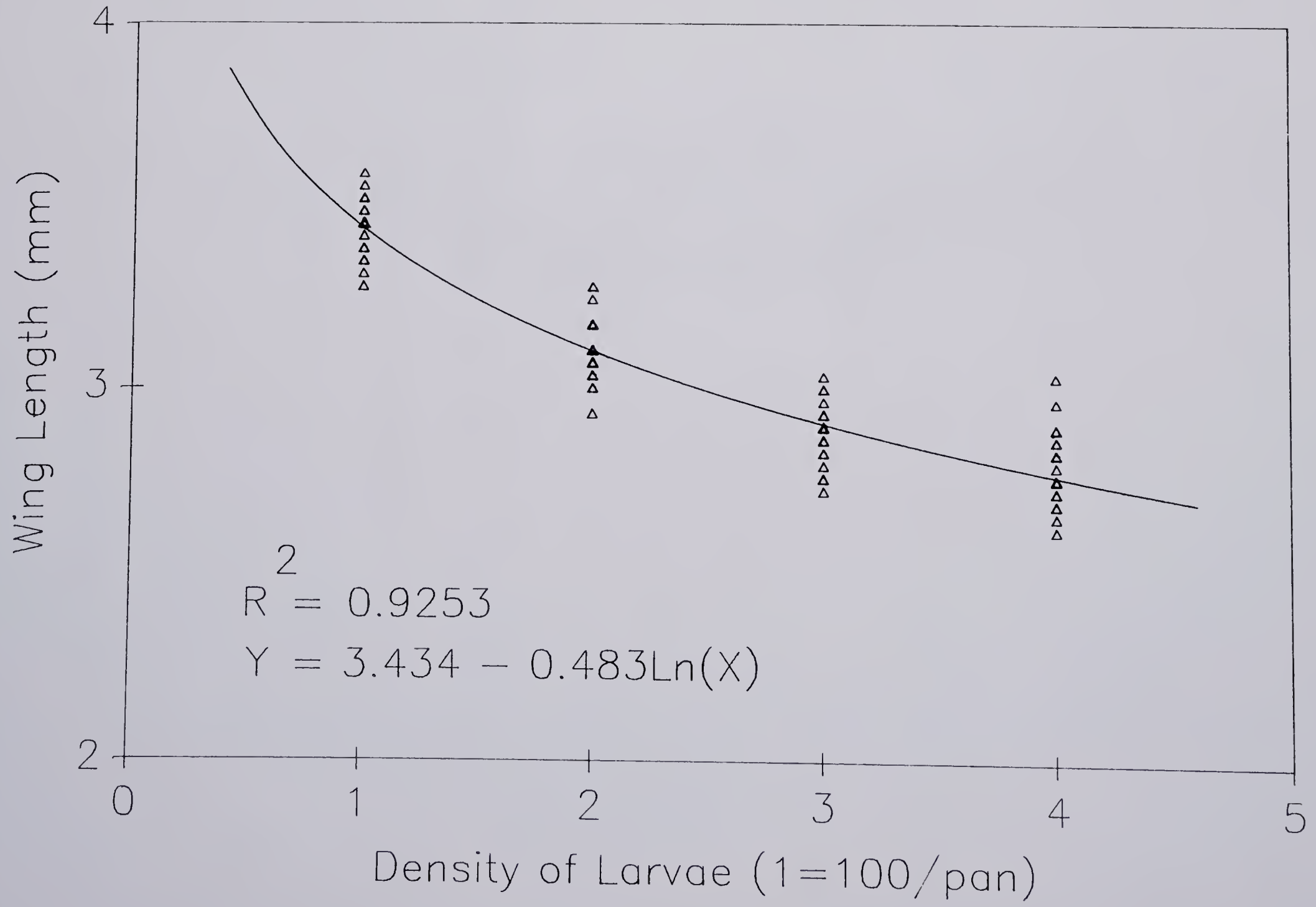

Wing Length (mm)
4
3
2
0
1
2
3
4
5
Density of Larvae (1=100/pan)
R 2 = 0.9253
Y = 3.434 – 0.483Ln(X)

Figure 18. Relationship between survivorship and larval density.

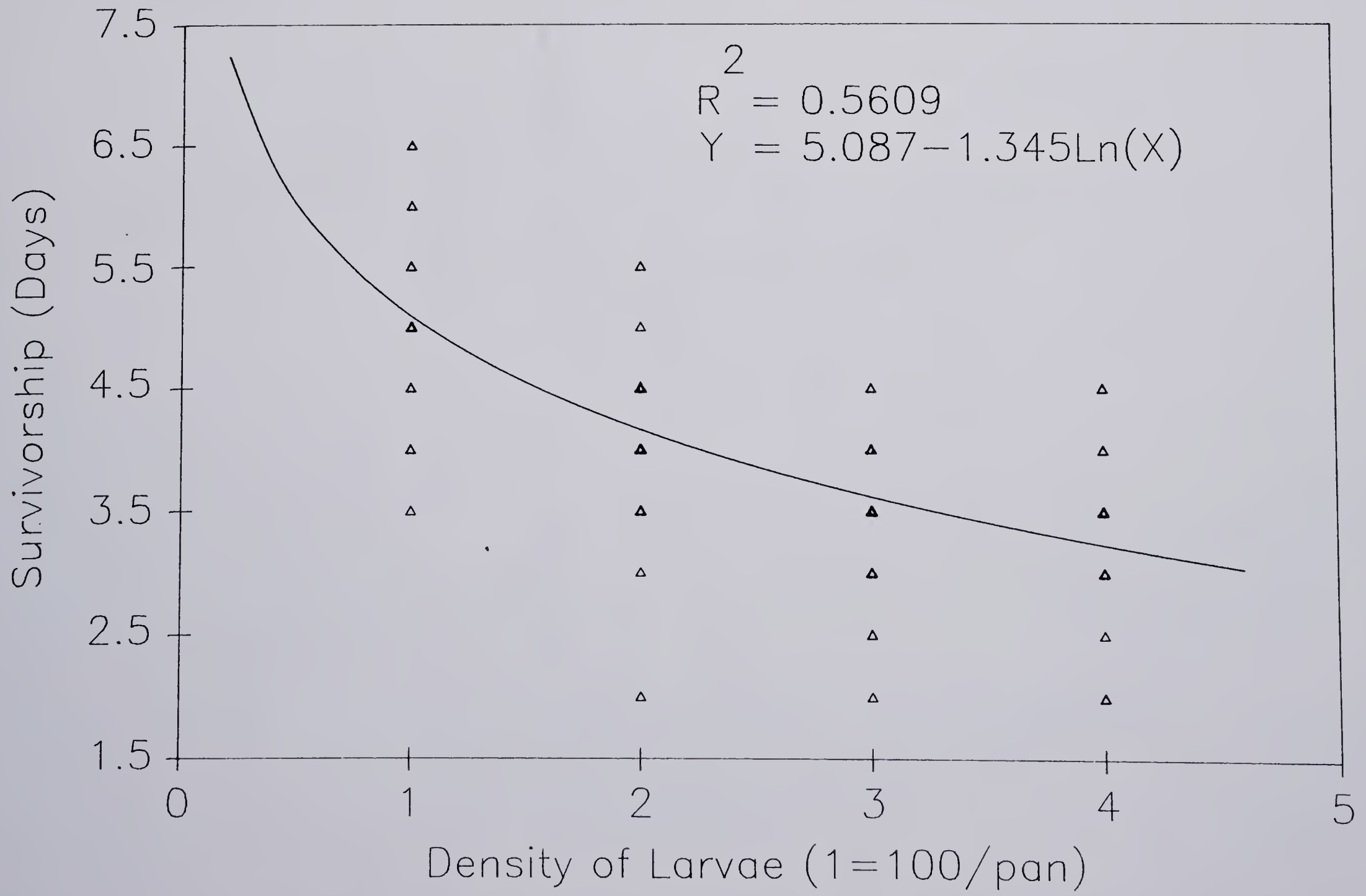

7.5
6.5
5.5
4.5
3.5
2.5
1.5
Survivorship (Days)
0
1
2
3
4
5
Density of Larvae (1=100/pan)
$R^2 = 0.5609$
$Y = 5.087 - 1.345Ln(X)$

Figure 19. Relationship between survivorship and wing length.

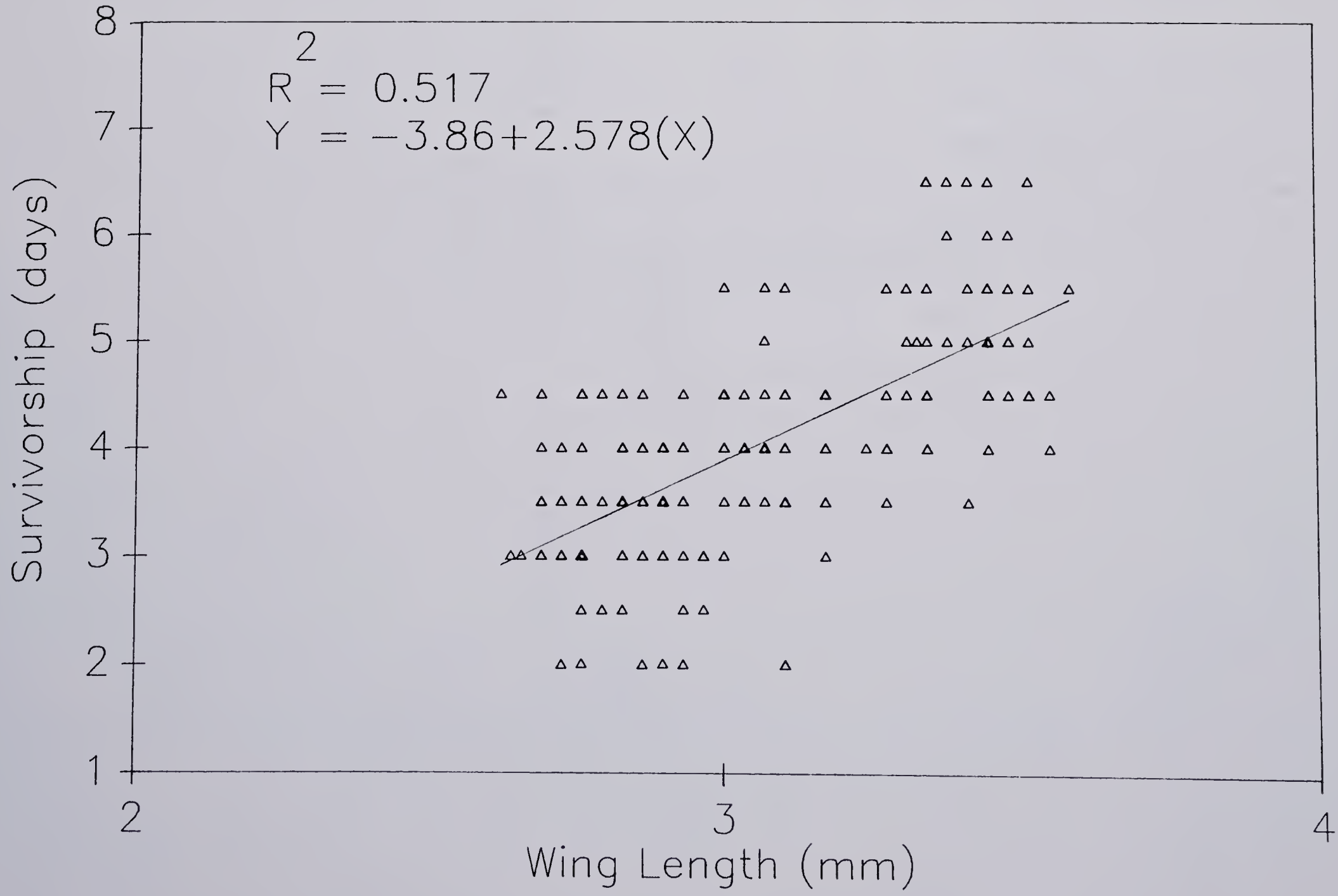

$R^2 = 0.517$
Y = −3.86+2.578(X)
Survivorship (days)
8
7
6
5
4
3
2
1
2
3
4
Wing Length (mm)

Figure 20. Relationship between blood meal size and wing length.

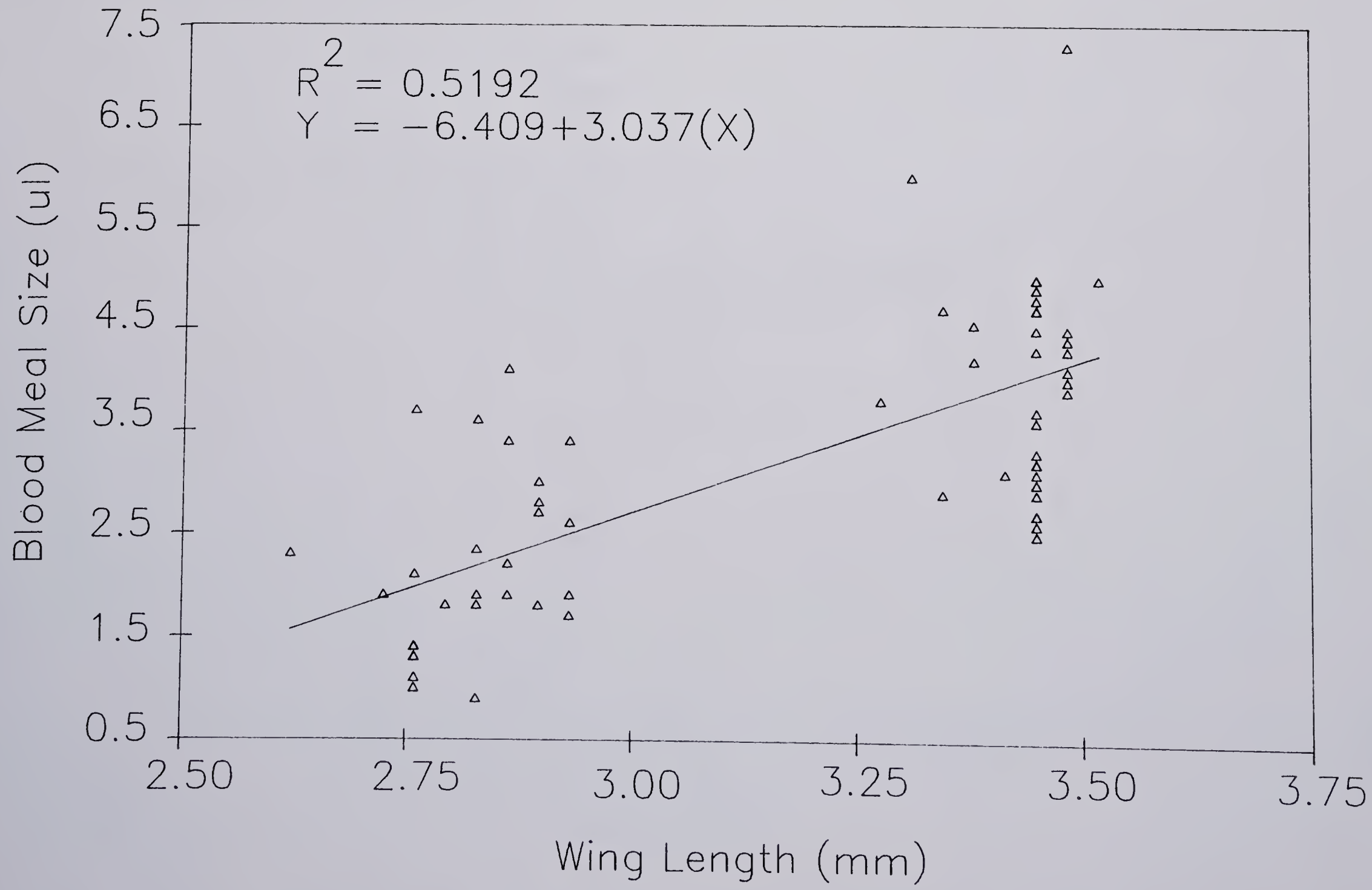

$R^2 = 0.5192$
Y = −6.409+3.037(X)
Blood Meal Size (ul)
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
2.50
2.75
3.00
3.25
3.50
3.75
Wing Length (mm)

Table 35. Comparison of susceptibility between large and small size classes of *An. dirus* to *P. falciparum*. Each test group of large and small females were membrane fed on blood with the same parasitemia. Parasitemia varied from test to test (0.14-1.1% RBC's with gametocytes).

Test date	No. observed	No. positive(%)	Mean oocyst	Mean wing length(±SE)
Large size				
1	8	7 (87.5)	35.3	3.41 (±0.02)
2	10	9 (90.0)	55.8	3.39 (±0.02)
3	15	8 (53.3)	82.9	3.44 (±0.02)
4	6	1 (16.7)	100.0	3.33 (±0.01)
5	25	17 (68.0)	78.4	3.42 (±0.01)
6	33	17 (51.5)	27.8	3.44 (±0.01)
7	56	56 (100.0)	93.2	3.42 (±0.01)
8	12	9 (75.0)	59.4	3.43 (±0.01)
9	16	16 (100.0)	96.1	3.39 (±0.01)
10	39	37 (94.9)	47.2	3.43 (±0.01)
11	32	26 (81.3)	59.0	3.49 (±0.01)
12	56	48 (85.7)	16.8	3.47 (±0.01)
13	59	45 (76.3)	7.4	3.49 (±0.01)
14	31	31 (100.0)	48.3	3.48 (±0.01)
15	30	27 (90.0)	83.1	3.47 (±0.01)
16	73	52 (71.2)	9.3	3.40 (±0.01)
17	72	62 (86.1)	58.2	3.35 (±0.01)
Small size				
1	5	5 (100.0)	16.5	2.84 (±0.04)
2	20	16 (80.0)	49.6	2.90 (±0.02)
3	6	5 (83.3)	56.1	2.76 (±0.04)
4	11	4 (36.4)	26.5	2.86 (±0.02)
5	15	9 (60.0)	55.5	2.84 (±0.02)
6	10	6 (60.0)	11.9	2.70 (±0.02)
7	56	49 (87.5)	84.7	2.80 (±0.01)
8	6	3 (50.0)	62.7	2.76 (±0.05)
9	12	11 (91.7)	94.2	2.82 (±0.02)
10	11	9 (81.8)	44.2	2.72 (±0.03)
11	4	3 (75.0)	45.5	2.72 (±0.03)
12	9	5 (55.6)	8.9	2.84 (±0.03)
13	34	21 (61.8)	6.9	2.82 (±0.02)
14	41	40 (97.6)	49.6	2.79 (±0.01)
15	19	17 (89.5)	55.2	2.79 (±0.02)
16	39	29 (74.4)	8.9	2.75 (±0.02)
17	45	38 (84.4)	51.6	2.71 (±0.02)

Table 36. Results of analysis of covariance predicting the variation of oocyst number from wing length (size). The final model was given by $\widehat{\text{oocyst no.}} = 4.454 + 0.967(\text{wing length in mm}) + f(\text{test date})$. Intercept term varies from test date to test date.

Variable		Parameter estimate	P-value
Intercept		4.453732870	0.0001
Wing length(mm)		0.967151015	0.0003
Test date	1	-2.365699260	0.0004
	2	-0.239344483	0.6246
	3	0.943568557	0.1438
	4	-1.141404205	0.2557
	5	0.807665319	0.0942
	6	-2.796399762	0.0001
	7	1.901941417	0.0001
	8	0.135285153	0.8399
	9	2.251279775	0.0001
	10	-0.814384420	0.0383
	11	-0.175463090	0.7079
	12	-3.763016850	0.0001
	13	-4.937352143	0.0001
	14	-0.117762168	0.7320
	15	0.909180484	0.0221
	16	-4.489203432	0.0001
	17	0.000000000	.

Figure 21. Relationship between oocyst number and wing length on different test dates.

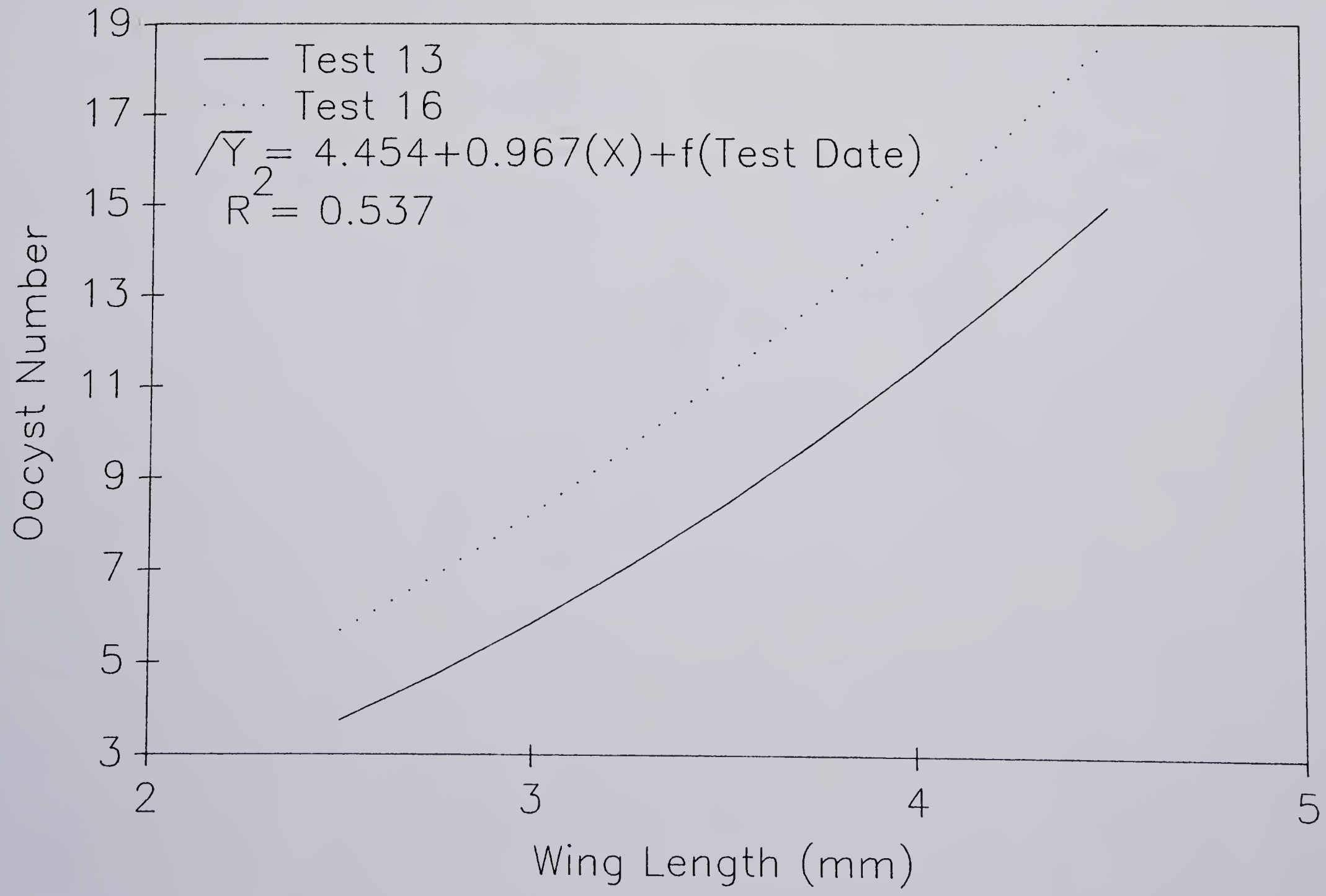
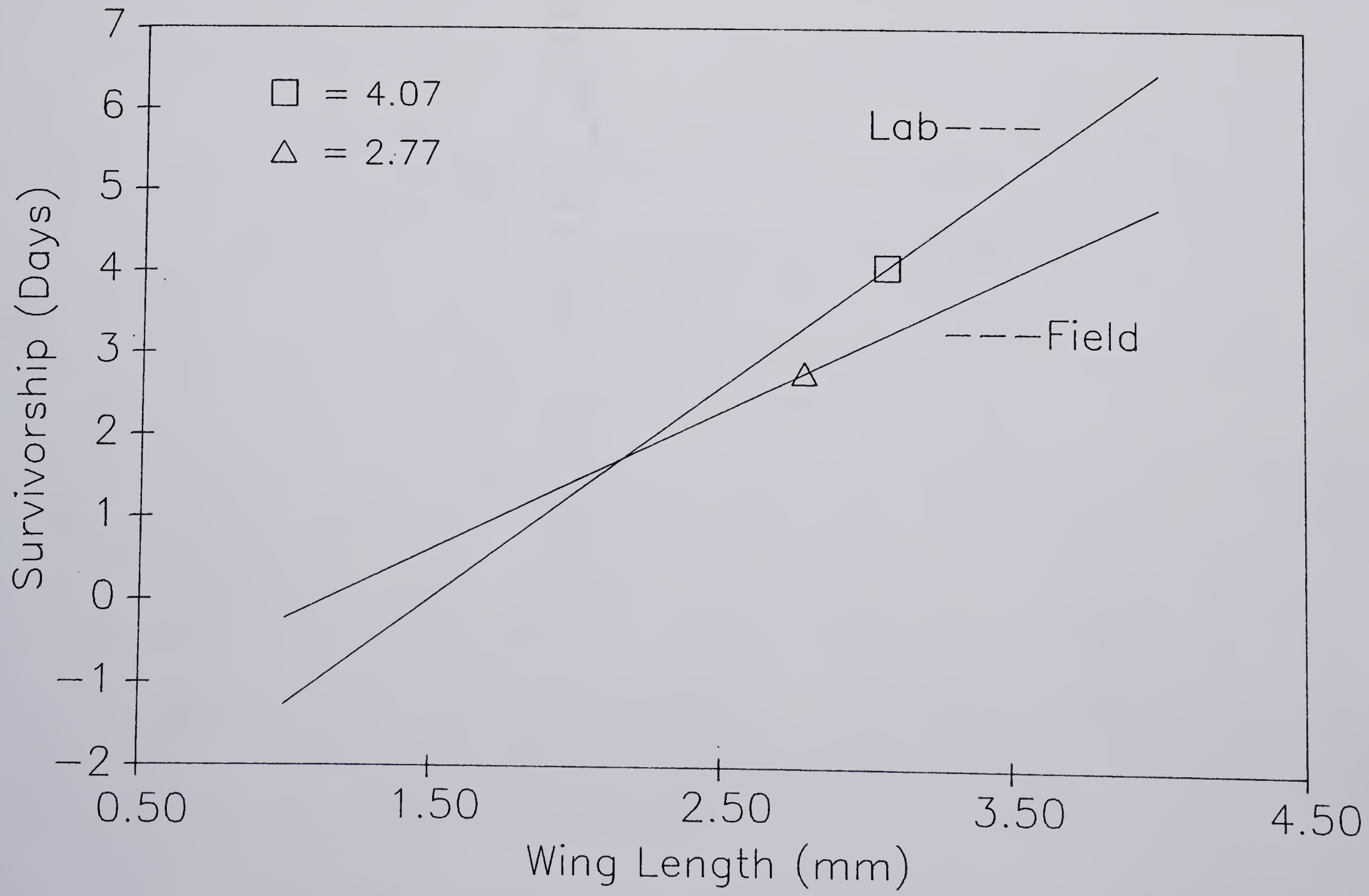

Test 13
Test 16
$\overline{Y}_2$ = 4.454+0.967(X)+f(Test Date)
R^2 = 0.537
Oocyst Number
19
17
15
13
11
9
7
5
3
2
3
4
5
Wing Length (mm)

Figure 22. Comparison of slope for field and laboratory populations.


□ = 4.07
△ = 2.77
Lab---
---Field
Survivorship (Days)
7
6
5
4
3
2
1
0
−1
−2
0.50
1.50
2.50
3.50
4.50
Wing Length (mm)

Discussion

In the laboratory, larval density had a strong impact on adult size. Since the temperature was constant, this was likely caused by larval competition for food and/or space. At lower larval densities, larvae may feed at a higher rate or with greater efficiency than at higher densities (see Appendix 4 for accumulated protein data). In nature large mosquitoes resulting from better nutrition or cooler temperature are likely to live longer and take larger blood meals. McCombs (1980) found that adult *Aedes triseriatus* derived from nutritionally deprived larvae had reduced dry weight, longevity and fecundity. Reisen et al. (1984) determined that large size in *Culex tarsalis* was linked to increased survival rates. Hawley (1985) reported that, in general, larger *Aedes sierrensis* females lived longer than smaller ones. However, size-related survivorship may be confounded by seasonal temperature differences in the field, e.g., if large females are held when temperatures are high they may not survive as well.

In my laboratory study, increased size of female *An. dirus* resulted in increased survivorship (Fig. 19). In my field study, this relationship was weaker but still existed (Chap. 4). There was a significant difference between laboratory and field data. Pupae from the field were collected and held in uncontrolled and perhaps less suitable habitats than those in the laboratory. They also were collected from more than 40 different pits which

introduced additional sources of variation. Other factors in the larval environment such as toxic chemicals may affect adult survivorship without influencing larval development and adult size. Walker et al. (1987) found no relationship between adult size and survivorship in either male or female *Ae. triseriatus* in a mark-release recapture experiment. However, the smaller size classes were not represented in their experiment due to difficulty in synchronously rearing small adults.

Haramis (1983) and Nasci (1986a) noted that large-bodied mosquitoes are more successful in obtaining a blood meal. Patrican and DeFoliart (1985) observed that small females can have significantly higher transmission capabilities in the laboratory. My laboratory results showed that there were significant relationships between female size and survivorship, and between female size and blood meal size. Obviously, larger females take larger blood meals because of a correspondingly larger midgut. Bigger blood meals might increase the number of ingested pathogens, and longer survivorship should increase the probability of transmission.

Analysis of my experimental data revealed a positive relationship between *An. dirus* female size and *P. falciparum* oocyst number. Large *An. dirus* also tended to have higher infection rates (Table 35). However, infection rates varied among the seventeen test dates. It may be that the efficiency of parasites varied in each culture;

however, % RBC's with gametocytes was not a direct factor. Ward (1963) and Wing et al. (1985) found that the size of the female had no influence on susceptibility to malaria infection. In the field, I found no significant difference in female size between the few infected and the many uninfected females (Chap. 6). Grimstad and Haramis (1984) concluded that females reared on optimal larval diets in the laboratory do not reflect the vectorial capacity found in the field population. In drawing inferences about the significance of the observed difference in susceptibility to malaria between size classes one must integrate both laboratory and field results.

Comparison of the regression lines for laboratory and field survivorship data revealed a significant difference. The relationship between survivorship and female size was significant both in the laboratory and in the field but the relationship for the laboratory data was much stronger than for the field data.

Conclusions

1. Strong significant relationships exists between: 1) larval density and adult *An. dirus* female size, 2) larval density and adult survivorship, and 3) adult female size and survivorship. High larval density results in small adults with reduced survivorship potential.

2. A significant relationship exists between adult female *An. dirus* size and blood meal size. Larger, longer lived females take larger blood meals.

3. Adult size has an impact on the malaria susceptibility of *An. dirus* in the laboratory; larger mosquitoes have greater susceptibility to infection with cultured *P. falciparum* parasites.

4. My laboratory and field data both indicate that there is a significant relationship between the size and the survivorship of female *An. dirus*; larger female live longer. This relationship was stronger in laboratory populations than among field populations.

APPENDIX A

BITING ACTIVITY OF *ANOPHELES* MOSQUITOES IN SRI-PHRAYA VILLAGE

Table 37 is a summary of sunset to midnight catches of outdoor biting, indoor biting and indoor resting *Anopheles*. Eleven species of *Anopheles* were caught in this village. *Anopheles dirus*, *barbirostris*, *tessellatus* and *vagus* all tend to feed outdoors while the *An. hyrcanus* group was more commonly caught indoors. Houses in Sri-Phraya Village and most other rural area are constructed with incomplete walls and are on pillars so there is an open space (often used for cooking and parking vehicles) beneath the living quarters. The upstairs area without walls is normally surrounded by a railed balcony. Mosquitoes can enter and exit these houses freely. Thus, there was not a great deal of difference between indoor and outdoor catches.

The outdoor biting cycle from sunset to sunrise is illustrated in Table 38 and Fig 23. Biting activity of *An. dirus* continued throughout the night, but the highest densities were observed between 2400 and 0200 hr. *Anopheles barbirostris* and *campestris* exhibited a similar pattern. Peak biting for the *An. hyrcanus* group occurred early in the evening between 1800 and 2200 hr and after midnight between 2400 and 0200 hr. *Anopheles tessellatus*

and *vagus* exhibited a similar pattern throughout the night (Table 38 and Fig. 23). In the summer, villagers often gather beneath the house or on the balcony, stay up late, and often sleep in these covered but open areas as well. In the dry season, they prefer to gather outdoors around fires to keep warmth. This outdoor activity increases the risk of being bitten by *An. hyrcanus* group, *tessellatus* and *vagus* in early evening. *Anopheles dirus*, *barbirostris* and *campestris* are more likely to bite humans when they stay up late or sleep in open areas.

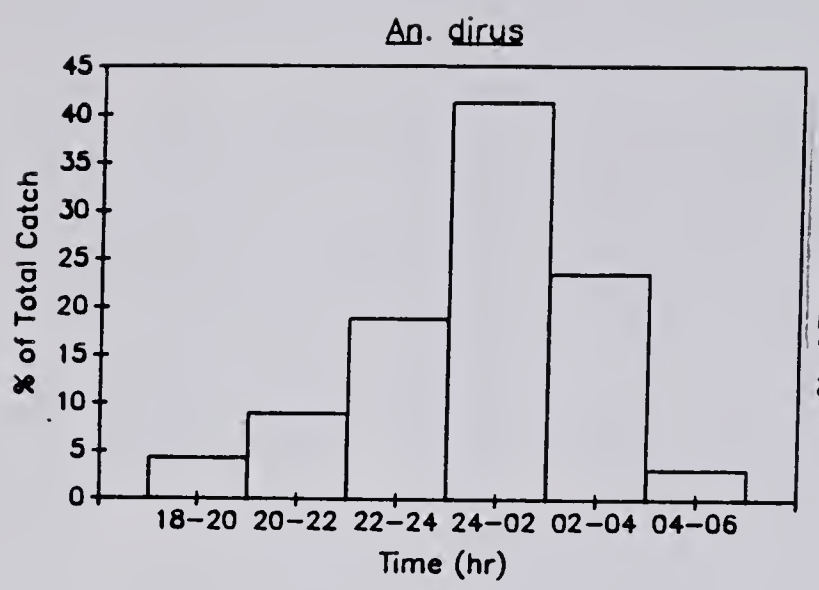
Table 37. Biting/resting collections from 1800-2400 hr on 6 nights each month from Oct. 1986 through Mar. 1988.

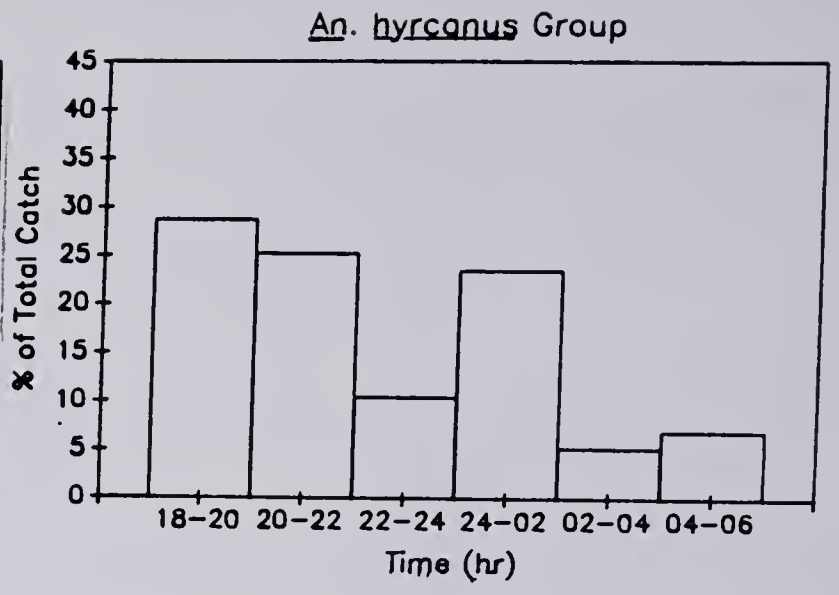
Mosquitoes	Outdoor Biting	Indoor Biting	Indoor Resting	Total
An.aconitus	1	1	0	2
An.annularis	2	0	2	4
An.barbirostris	321	163	187	671
An.campestris	10	6	7	23
An.dirus	277	171	198	646
An.hyrcanus group	293	272	303	868
An.karwari	4	0	3	7
An.philipppinensis	1	0	1	2
An.tessellatus	166	107	96	396
An.umbrosus	1	0	0	1
An.vagus	41	23	29	93
Total	1117	743	826	2686

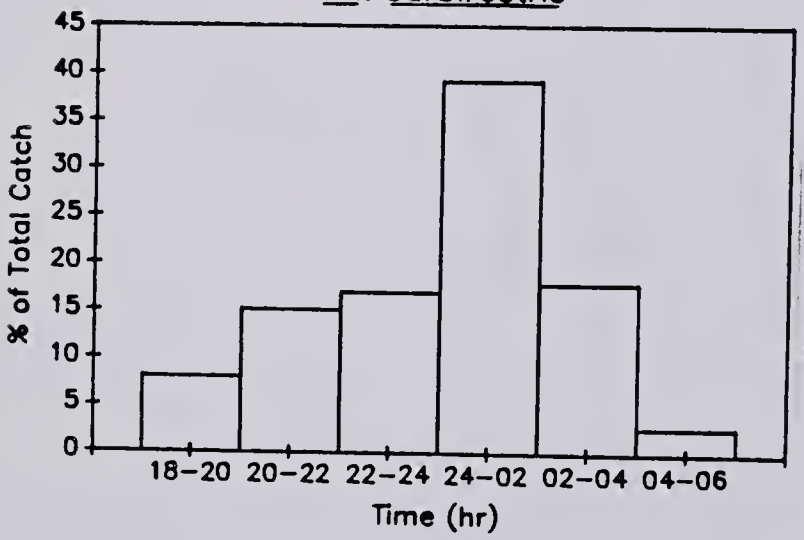
Table 38. All night outdoor biting collection for 4 nights each month from Jan. 1987 through Mar. 1988.

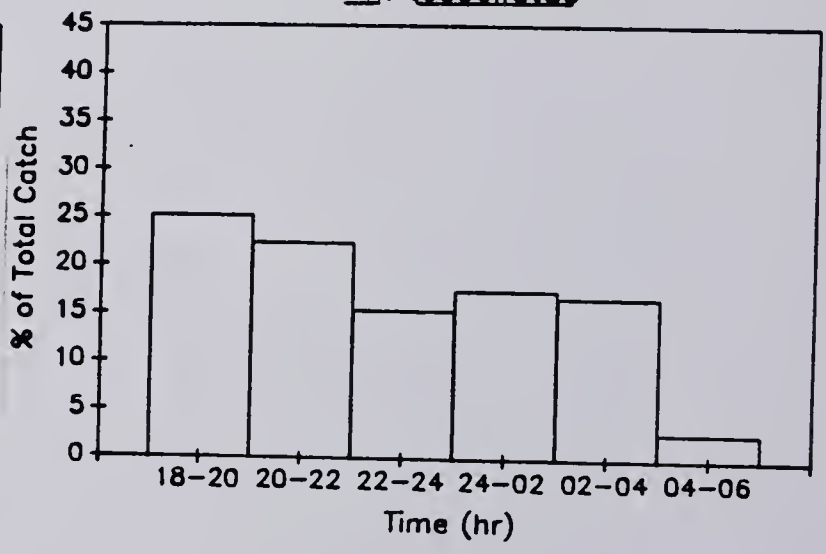
	Collection Time												
Species	18	19	20	21	22	23	24	01	02	03	04	05	Total
annularis	1												1
barbirostris	9	17	20	29	34	21	66	61	39	19	8	1	324
campestris			2		2	1	5	8	2	1			21
dirus	12	11	18	30	57	44	104	117	80	46	17		536
hyrcanus group	15	18	14	15	6	6	11	16	2	4	8		115
karwari										1			1
philippinensis					1								1
tessellatus	13	23	16	16	12	10	15	10	10	14	4		143
vagus	3	8	4	3	5	1	2	4	5	1	1		37
Total	53	77	74	93	117	83	203	216	138	86	38	1	1179

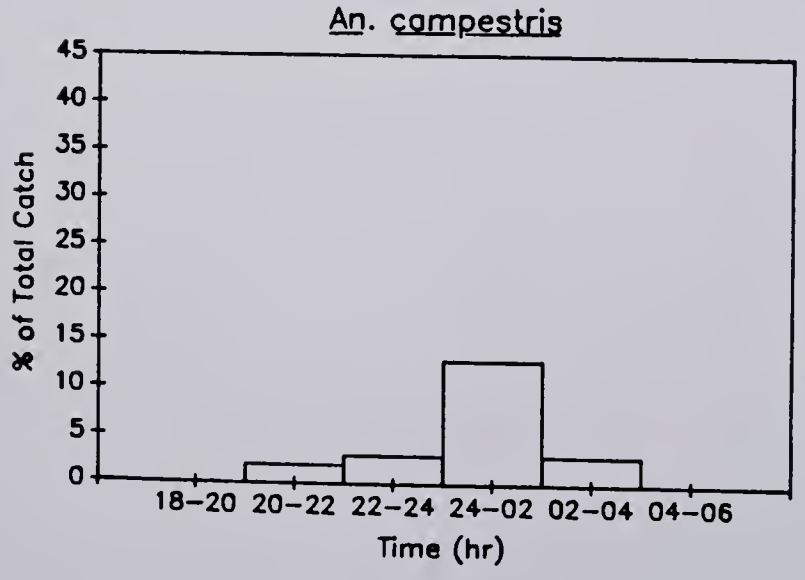
Figure 23. Outdoor diel biting activity of *An. dirus*, *barbirostris*, *campestris*, *hyrcanus* group, *tessellatus* and *vagus* collected in Sri-Phraya Village.

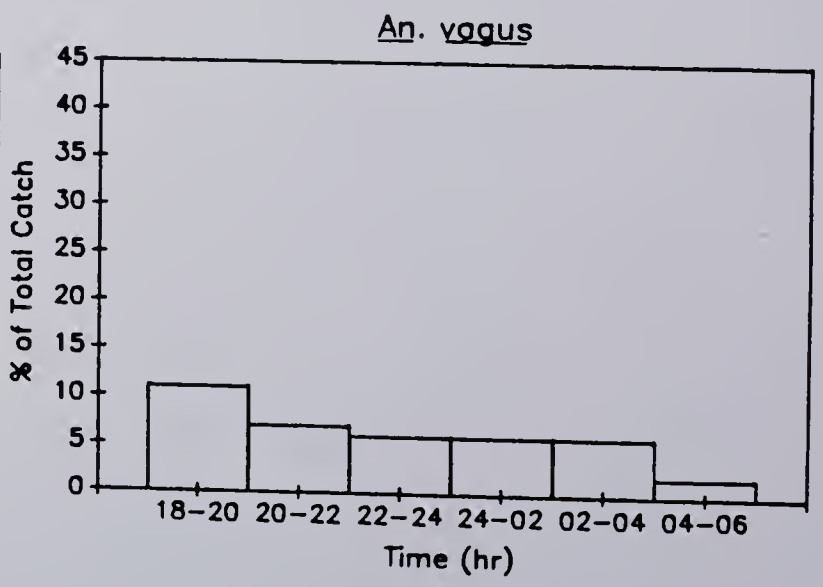

An. dirus
% of Total Catch
0
5
10
15
20
25
30
35
40
45
18–20 20–22 22–24 24–02 02–04 04–06
Time (hr)


An. hyrcanus Group
% of Total Catch
0
5
10
15
20
25
30
35
40
45
18–20 20–22 22–24 24–02 02–04 04–06
Time (hr)


An. barbirostris
% of Total Catch
0
5
10
15
20
25
30
35
40
45
18–20 20–22 22–24 24–02 02–04 04–06
Time (hr)


An. tessellatus
% of Total Catch
0
5
10
15
20
25
30
35
40
45
18–20 20–22 22–24 24–02 02–04 04–06
Time (hr)


An. campestris
% of Total Catch
0
5
10
15
20
25
30
35
40
45
18–20 20–22 22–24 24–02 02–04 04–06
Time (hr)


An. vagus
% of Total Catch
0
5
10
15
20
25
30
35
40
45
18–20 20–22 22–24 24–02 02–04 04–06
Time (hr)

APPENDIX B

EFFECT OF NOTONECTIDAE (HEMIPTERA) ON *ANOPHELES DIRUS*

Based on field observations, predation by Notonectidae seemed to significantly regulate larvae populations. Preliminary laboratory experiments were carried out to test whether Notonectidae are potentially efficient predators of *An. dirus* larvae and pupae. Different sized notonectids collected from the field were transferred separately to round plastic bowls, filled with 1.5 liters of water. Different numbers and stages of *An. dirus* were added to the bowls with notonectids and small amount of fish food were added daily for the larvae. Results (Table 39) indicate that the potential mortality effect of large notonectids on different *An. dirus* stages is high, especially on 1^{st} instar larvae and pupae. It is possible that notonectids consume more 1^{st} instars because they are smaller (i.e. less food value) or easier to capture. Pupae are the easiest stage for Notonectidae to capture and mortality was highest for this stage.

At my study site, Notonectidae live in fresh rain pools, clean streams and gem pits where *An. dirus* larvae also predominate. These "backswimmers" are general predators and tend to attack their prey at the water surface where *An. dirus* larvae also feed and spend most of

their time. Notonectids might be usefully introduced to control malaria vectors in a biological control program and further evaluations under field conditions should be undertaken.

Table 39. *Anopheles dirus* larvae and pupae consumed by Notonectidae in laboratory tests.

An. dirus Stage	Notonectidae Size (mm in length)	No. Eaten per Day per Predator
1st Instar	1 mm	0.38
	8-9 mm	18.25
2nd Instar	3-4 mm	3.84
3rd Instar	1 mm	0.60
	5 mm	4.05
	9-10 mm	6.25
4th Instar	1 mm	0.16
	5 mm	3.23
	9 mm	12.00
Pupae	1 mm	0.26
	5 mm	1.70
	9-10 mm	25.00

APPENDIX C

WATER TEMPERATURES FROM GEM PITS

Table 40. Average maximum and minimum water temperatures from gem pits during hottest and coolest months. Pits which were dried up either month are excluded from this table.

Pit	May 1987 Maximum	May 1987 Minimum	December 1987 Maximum	December 1987 Minimum
A	35.56	27.33	25.89	24.22
C	29.50	26.25	26.33	24.33
D	37.00	26.89	28.11	21.89
E	36.89	26.33	25.00	21.56
F	40.78	27.00	24.00	22.00
G	39.33	27.22	24.78	21.78
H	39.67	26.44	25.00	20.33
I	34.67	26.89	34.78	20.78
J	33.00	27.33	23.44	21.11
K	32.33	26.89	24.00	20.89
l	29.44	26.72	23.33	21.89
N	35.86	27.00	25.78	23.89
O	30.38	28.50	27.78	24.78
Q	30.00	27.33	26.33	22.22
T	33.22	26.22	25.44	23.78
U	31.78	26.56	35.00	21.56
V	30.11	26.67	35.11	21.78
X	36.88	26.00	26.56	23.89
Y	32.00	26.56	27.44	23.11
A′	36.00	26.78	28.56	23.00
B′	39.89	27.00	25.11	20.44
C′	29.44	25.89	31.22	20.44
D′	31.25	26.63	38.00	21.33
E′	31.33	26.67	25.22	20.67
F′	32.89	26.56	25.67	21.22
G′	36.33	27.00	29.50	23.50
H′	35.78	26.00	30.44	22.72
I′	35.89	26.56	25.33	23.56
J′	32.89	26.67	26.00	22.50
M′	32.67	27.00	24.78	22.11
N′	38.33	26.33	25.44	21.78
Total	34.23	26.75	27.40	22.23

APPENDIX D

RELATIONSHIP BETWEEN LARVAL DENSITY AND PROTEIN ACCUMULATION

Larvae were reared at 2 different densities (100 and 400/pan) to produce 2 different size classes of adults. The feeding schedule is described in Chap. 7. The protein content of the food was 77.52 ± 2.97 ug/mg (mean ± SE). The accumulation of protein during larval development is shown in Fig. 24. Data are averages for 3 treatments. Larval development is divided into 4 distinct instars. Larvae in low density pans (100/pan) reached the 4^{th} instar after 8 days and started to pupate after 10 days. High density larvae (400/pan) reached the 4th instar after 10 days and started to pupate after 12 days. Protein accumulation was faster and greater among low density larvae. Hence, the longer survival among larger adults (Chap. 7) is related to protein accumulation during immature life.

Larvae in low density pans consumed almost all the food in the water before they reached the pupal stage while a substantial amount of protein remained in the crowded pans when larvae pupated (Fig. 25). It appears that the smaller adult size associated with larval development in crowded pans was caused by space stress affecting feeding efficiency rather than a shortage of food *per se*.

Figure 24. Total protein assimilated by low and high density larvae.

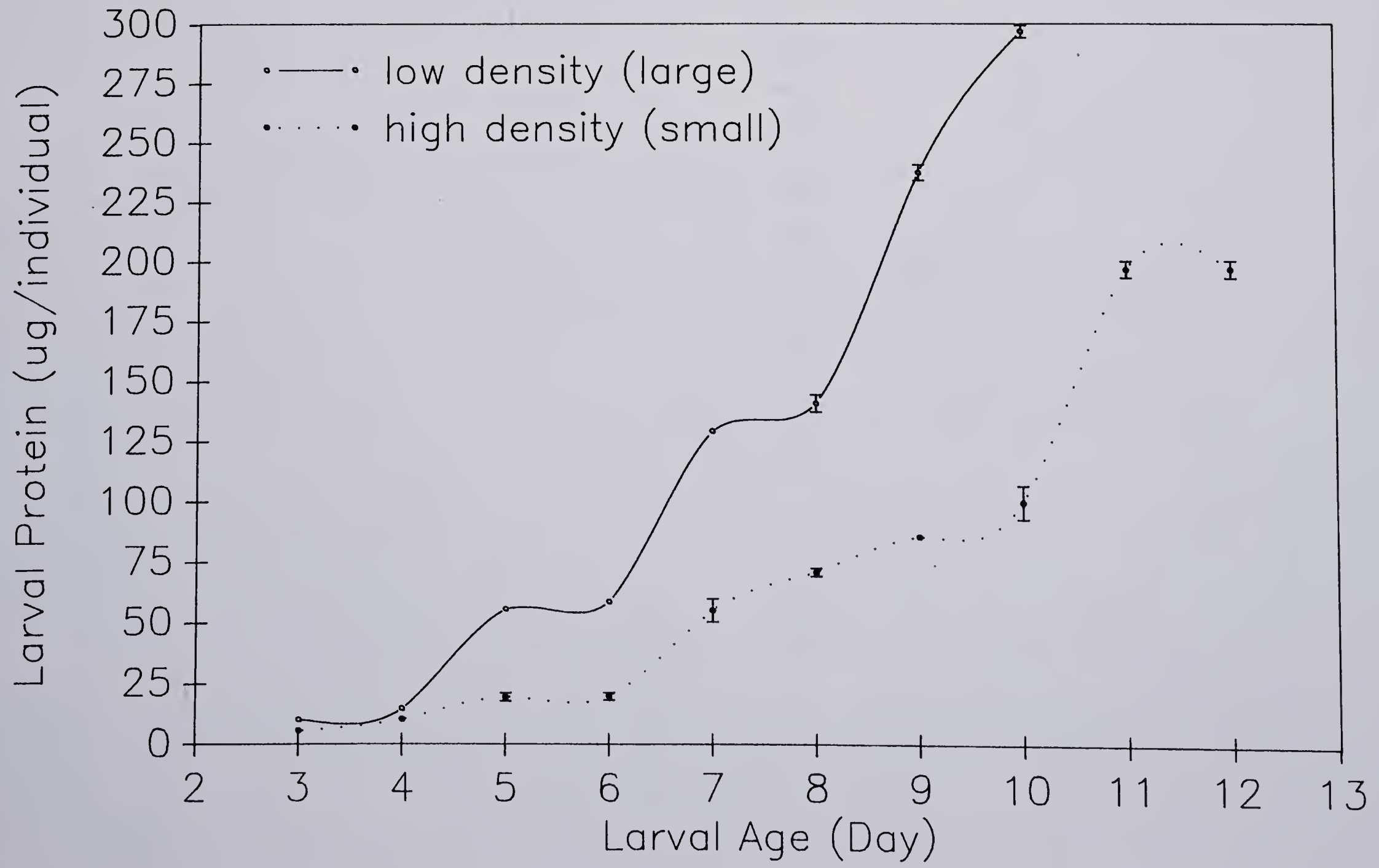
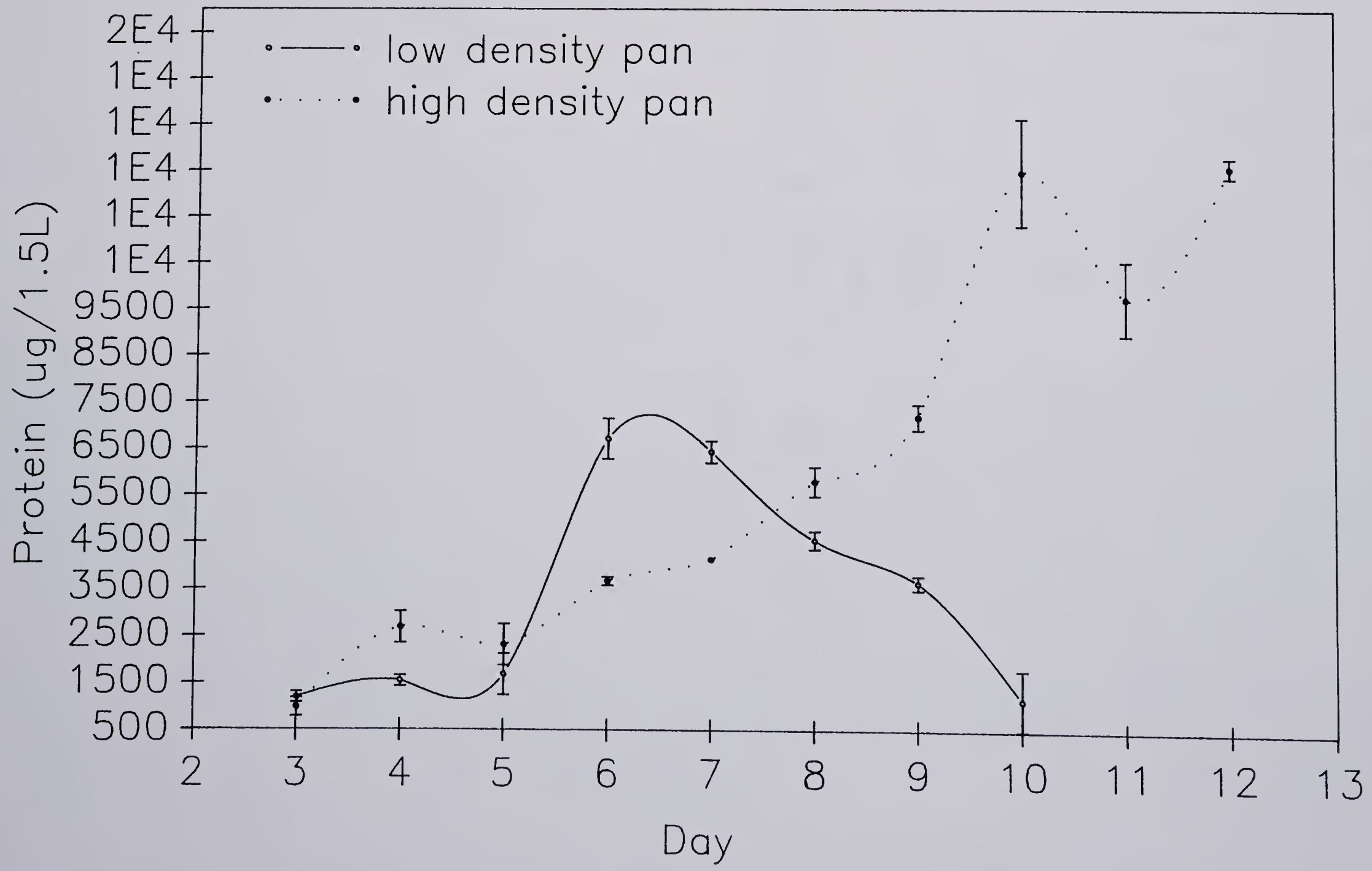

low density (large)
high density (small)
Larval Protein (ug/individual)
300
275
250
225
200
175
150
125
100
75
50
25
0
2
3
4
5
6
7
8
9
10
11
12
13
Larval Age (Day)

Figure 25. Unassimilated protein remaining in low and high density pans.


low density pan
high density pan
Protein (ug/1.5L)
2E4
1E4
1E4
1E4
1E4
1E4
9500
8500
7500
6500
5500
4500
3500
2500
1500
500
2
3
4
5
6
7
8
9
10
11
12
13
Day

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