LABORATORY COLONIZATION OF *ANOPHELES MINIMUS* THEOBALD

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ABSTRACT. A laboratory colony of *Anopheles minimus* Theobald, 1901 was established from the progeny of wild caught females using forced mating techniques. Females oviposited 3 to 15 days after mating. Larval development was completed within 26 days, with half of the larvae pupating by the 13th day. Larval mortality was low and appeared to be directly related to cleanliness of the rearing water. The pupal stage lasted 2 to 3 days, and nearly all pupae reached the adult stage. Slightly more females (53 percent) emerged than males (47 percent). Impregnated females lived up to 23 days in the insectary.

INTRODUCTION. *Anopheles minimus* Theobald, 1901 is considered an important malaria vector in monsoon areas north of the Malayan peninsula extending from Assam to the Philippines (Reid, 1968). In Thailand, the species was first incriminated in the transmission of human malaria by Vejjasastra in 1933 and was regarded as the only important malaria vector in that country until the early 1960's when the importance of *Anopheles balabacensis* Baisas in forested areas was recognized (Ayurakitkosol & Griffith 1963, Scanlon & Sandhinand 1965). Although the role of *A. minimus* as a malaria vector in Thailand has been greatly reduced in recent years as a result of the kingdom's National Malaria Eradication Program, this species should still be considered a potential vector whenever it is present in malarious areas.

A. minimus is found generally in open areas near forested foothills where it breeds along the margins of unpolluted streams and occasionally in garden drains and shallow wells (Muirhead-Thomson, 1940a, b, c; Covell, 1944). The adults are strongly anthropophilic (Covell, 1944), but the species is also attracted to cattle and water buffalo (Bruce-Chwatt & Gockel, 1960).

The behavior of *A. minimus* in Assam was extensively studied by Muirhead-Thomson (1940a, b, c–1941a, b) but he was unable to establish a colony, despite repeated attempts. He attributed his failure to the refusal of the adults to mate in captivity, although he also had difficulty rearing the larvae in the laboratory. Santiago (1961) attempted to colonize *A. minimus flavirostris* by introducing large numbers of pupae into an outdoor cage which was placed over a stream. His efforts to establish a colony also failed, but the presence of some gravid females with fertile eggs indicated that a certain amount of natural mating had occurred in the cage.

A small colony of *A. minimus* was established at the SEATO Medical Research Laboratory in 1968 for taxonomic and genetic investigations (Harrison; 1970), but the colony was allowed to die off at the conclusion of those studies. In 1970, we undertook a comparison of the infectivity of chloroquine-resistant strains of *Plasmodium falciparum* to *A. minimus* and *A. balabacensis* (Wilkinson *et al.*, 1972) and a colony of *A. minimus* was once again established. The present paper describes the methods of colonization and results obtained with this latter colony during 1971 and 1972.

MATERIALS AND METHODS. In December 1970, 32 engorged female *A. minimus* were collected from water buffaloes in Saraburi Province in Central Thailand and transferred to our insectary for colonization. Each wild-caught female was placed in a 9 dram vial ⅓ filled with water and plugged with cotton. Eggs deposited in these vials and all subsequent eggs laid in the colony were transferred with a small camel's hair brush from the oviposition container to cups of clean water. The eggs were placed inside plastic straw circles to keep them from adhering to the sides of the cup. Within a few hours after hatching, larvae were transferred to plastic pans

(19.5 x 26.5 x 5 cm) containing 800 ml of water over a layer of mud approximately 3 mm thick. The mud, which came from streams in which *A. minimus* larvae had been found, was boiled for approximately one hour and dried prior to use. Approximately 200 larvae were placed in each rearing pan. The insectary temperature was 24–28° C and the relative humidity ranged from 55 to 90 percent. Windows in the insectary allowed for natural lighting. Tap water that was stored for 2–3 days before use was used for rearing all mosquito larvae.

A 1:1 combination of ground commercial guinea pig-rabbit food [1] and mouse food [2] was fed to the first few generations of larvae. Later, an equal portion of Bacto liver powder was added to the above mixture. The larval food was placed in a vial covered with a fine mesh nylon screen (34 x 43 threads per cm^2) and sprinkled on the water in the larval pans until the particles ceased to move rapidly across the surface. First and second stage larvae were fed twice daily, and this schedule was increased to 3–4 times daily after most of the larvae reached third instar. Before each feeding, floating clumps of excess food were removed by dragging a paper towel across the water surface. Any larvae trapped on the towel during the cleaning process were dislodged by rinsing the towel in a pan of clean water and returned to the appropriate rearing pan.

Pupae were removed from the rearing pans twice daily and transferred to containers of water in emergence cages. Initially, adults were allowed to emerge in small cages (21 x 21 x 21 cm), but as the colony increased in size, larger cages (46 x 46 x 46 cm) were employed. Each morning, adults were counted and the sexes separated. Males were placed in paper cups covered with nylon screen for ease in handling, and females were returned to cages. Both sexes were continuously provided a multi-vitamin syrup [3] diluted to 40 percent with tap water. Two days after emergence, females were permitted to feed on a hamster overnight. The next day, engorged females were mated with 2–3 day old males using the insemination system described by Yang *et al.* (1963). After mating, the females were placed in a cage in which cups of tap water had been placed for oviposition. After the first oviposition, mated females were offered a hamster nightly. Eggs were collected and counted each morning.

RESULTS AND DISCUSSION. The original 32 wild females deposited 1,572 eggs (average 49 eggs per female), of which 90 percent hatched. A hatch of approximately 75 percent was maintained in successive generations. At first, females were reluctant to feed on hamsters or guinea pigs. After several generations were fed on humans, in subdued light during the day, hamsters were successfully reintroduced as the routine source of blood meals for the colony. Females preferred to oviposit on the surface of the water in cups rather than on moist filter paper or sand. During the period described, an average of 90 eggs per female were deposited 3 to 15 days after mating. Inseminated females survived for up to 23 days under our insectary conditions.

Observations on the development of 4400 *A. minimus* larvae (200 larvae per pan) are summarized in Table 1. Larval

TABLE 1.—Summary of observations on development of 4400 *A. minimus* larvae.

Stage	Day present	Day 50 percent attain given stage	Percent mortality
1st Instar	0–7	..	1.9
2nd "	2–11	3	0.7
3rd "	5–18	7	0.1
4th "	6–25	10	0.7
Pupae	8–26	13	N.D.*

* Not determined.

[1] Manufactured by G. L. Baking Co., P.O. Box 430, Frederick, Md.

[2] Manufactured by Ralston Purina Co., St. Louis, Mo. 63168.

[3] Manufactured by Silom Medical Co., Ltd., 27/2 Phayathai Rd., Bangkok, Thailand.

development was completed between 8–26 days, with half of the larvae pupating by day 13. The mean duration of the 1st, 3rd, and 4th instars was approximately 3 days while the 2nd instar lasted about 4 days. Pupation was first observed on the 8th day and by day 20 nearly 90 percent of the larvae had pupated. A total of 150 (3.4 percent) of the original 4400 larvae died before pupation (Table 1). In routine rearing, when over 100 larval rearing pans were being maintained, we observed an average of 20 percent (range 2–50 percent) larval mortality per pan.

Observations made on an additional 2,500 larvae from the colony indicated that 64 percent of the pupae formed between 1800 and 0600 hrs and that the duration of the pupal stage was 2 to 3 days. Approximately 96 percent of the pupae reached the adult stage, but 5.8 percent of these adults died at, or shortly after, emergence. The majority of adults (59 percent) emerged between 1800 and 0600 hrs.

The low larval mortality (3.4 percent) observed during the period of this study indicates that the larval diet and rearing procedures employed were satisfactory enough to sustain a laboratory colony. The higher mortalities observed when maintaining the colony at a high level of production occurred in pans in which the water became cloudy when small clumps of food inadvertently were not removed. The lowest larval mortalities and the shortest duration of larval instars were observed when a mud substrate was used. However, the use of a sand substrate gave better results than when nothing covered the bottom of the pan. When water from a nearby running stream was tested for possible use in the colony, slightly higher larval mortalities were observed than when seasoned tap water was utilized.

The duration of the larval stages in our colony was much longer than reported for field populations. Muirhead-Thomson (1940c) found that caged larvae in a natural habitat developed from hatch to pupation in 9–12 days. The maximum and minimum water temperatures in his experiment were 34° C and 27° C, respectively, as compared with a nearly constant 24° C in our colony. Although water temperature is but one factor influencing larval development, the time required for the larvae in our colony to reach pupation could probably have been shortened if the water in larval rearing pans had been maintained at higher temperatures. In addition, the slower rate of development in our colony may indicate the need for some refinement with regard to type of larval food, number larvae per pan, frequency of feeding or other, as yet, unknown factors.

The *A. minimus* colony described here has been maintained for over two years; from June to December 1972 the average monthly production from this colony was 10,518 females (53 percent) and 9,420 males (47 percent).

Although it was established in order to compare the vector efficiency of *A. minimus* with that of *A. balabacensis,* this colony provides a means to conduct standardized studies on *A. minimus* which hopefully will yield greater knowledge of the behavior and vector characteristics of this species.

References Cited

Ayurakitkosol, Luang and Griffith, M. E. 1963. Progress toward malaria eradication in Thailand. Proc. Pacif. Sci. Congr. IX, Bangkok, 1957 17:122–136.

Bruce-Chwatt, L. J. and Gockell, C. W. 1960. A study of the blood-feeding patterns of *Anopheles* mosquitoes through precipitin tests. Bull. Wld. Hlth. Org. 22:685–720.

Covell, G. 1944. Notes on the distribution, breeding places, adults habits and relation to malaria of the anopheline mosquitoes of India and the Far East. J. Malar. Inst. India 5:397–434.

Harrison, B. A. 1970. Mosquito Fauna of Thailand. Ann. Prog. Rep., SEATO Med. Res. Lab., 1969–70.

Muirhead-Thomson, R. C. 1940a. Studies on the behavior of *Anopheles minimus.* I. The selection of the breeding place and the influence of light and shade. J. Malar. Inst. India 3: 265–294.

———. 1940b. Studies on the behavior of *Anopheles minimus.* II. The influence of water movement on the selection of the breeding place. Idem:295–322.

———. 1940c. Studies on the behavior of *Anopheles minimus*. III. The influence of water temperature on the choice and suitability of the breeding place. Idem:323–348.

Muirhead-Thomson, R. C. 1941a. Studies on the behavior of *Anopheles minimus*. IV. The composition of the water and the influence of organic pollution and silt. Ibid. 4:63–102.

———. 1941b. I. Studies on the behavior of *Anopheles minimus*. V. The behavior of adults in relation to feeding and resting in houses. Idem:217–245.

Reid, J. A. 1968. Anopheline Mosquitoes of Malaya and Borneo. Studies from the Institute for Medical Research Malaysia No. 31. 520 pp.

Santiago, D. 1961. Notes on mosquito bionomics. I. Colonization of *A. minimus flavirostris* and *A. vagus limosus* in an outdoor insectary. Philippines J. of Science 90(4):431–549.

Scanlon, J. E. and Sandhinand, U. 1965. The distribution and biology of *Anopheles balabacensis* in Thailand (Diptera:Culicidae). J. Med. Ent. 2(1):61–69.

Vejjasastra, Payung L. 1933. (Malaria survey in Tung Song, Nakhon Si Thammarat) Department of Health (Thailand) Public Health Bulletin, No. 11 (In Thai).

Wilkinson, R. N., Gould, D. J. and Boonyakanist, A. 1972. Comparative susceptibility of *Anopheles balabacensis* and *Anopheles minimus* to naturally occurring *Plasmodium falciparum* in Central Thailand. Proc. Helmin. Soc. Wash. 39, 423–27.

Yang, C. K. O., Sta. Maria, F. L. and Wharton, R. H. 1963. Maintenance of a laboratory colony of *Anopheles maculatus* Theobald by artificial mating. Mosq. News 23(1):34–35.

TOXICITY OF THE REPELLENT DEET (N,N-DIETHYL-META-TOLUAMIDE) TO *GAMBUSIA AFFINIS* (BAIRD AND GIRARD) [1]

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ABSTRACT. The experimental use of DEET (N,N-diethyl-meta-toluamide) as an area space repellent led to an evaluation of its toxicity to the mosquito fish, *Gambusia affinis*. The LC_{50} determination indicates a very low toxicity for the compound.

INTRODUCTION. The Naval Medical Field Research Laboratory, Camp Lejeune, North Carolina, has become interested in determining if DEET (N,N-diethyl-meta-toluamide) is effective as an area repellent when applied on talc or other inert carrier. Since ground application is indicated, the question of run-off and the resulting toxicity to aquatic organisms has been raised. In response to this question, a study was developed to determine the effects of DEET on fish. The mosquito fish, *Gambusia affinis*, was chosen as the test animal due to its availability and use in mosquito control on military bases (Sholdt, *et al.*, 1972).

METHODS AND MATERIALS. Experimental fish were drawn from two 30-gallon aquaria maintained in the laboratory. Two females, 3.5 to 5.0 cm in length, were added to 3-liter glass jars containing 2 liters of water drawn from the aquaria. Aeration was provided (Lagler, 1969) and all tests were conducted at room temperature (70° F). Covers were placed over the glass jars to keep the fish from jumping out and to reduce evaporation of the test solutions. Feeding was curtailed 24 hours before testing and was not resumed during the experimental period. Test concentrations of DEET were produced by pipetting the desired amount of technical material (95% N,N-diethyl-meta-toluamide and 5% other isomers) into 500 ml of water from each test container. The material

[1] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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