Journal of the American Mosquito Control Association, 18(4):359–363, 2002

SCIENTIFIC NOTE

COLONIZATION OF *ANOPHELES MACULATUS* FROM CENTRAL JAVA, INDONESIA[1]

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ABSTRACT. The routine colonization of *Anopheles maculatus,* a reputed malaria vector from Central Java, is described. The strain is free mating and long lived in the laboratory. This species will readily bloodfeed on small rodents and artificial membrane systems. Either natural or controlled temperatures, humidity, and lighting provide acceptable conditions for continuous rearing. A simple larval diet incorporating a 10:4 powdered mixture of dried beef and rice hulls proved acceptable. Using a variety of simple tools and procedures, this colony strain appears readily adaptable to rearing under most laboratory conditions. This appears to be the first report of continuous colonization using a free-mating strain of *An. maculatus.* Using this simple, relatively inexpensive method of mass colonization adds to the short list of acceptable laboratory populations used in the routine production of human-infecting plasmodia.

KEY WORDS *Anopheles maculatus,* Central Java, colonization, larval diet, malaria vector, Indonesia

Anopheles (*Cellia*) *maculatus* Theobald belongs to the Theobaldi group of the Neocellia series, which also includes *Anopheles karwari* (James) and *Anopheles theobaldi* Giles (Subbarao 1998). The *An. maculatus* species complex is considered an important malaria vector assemblage over certain parts of its wide Oriental distribution, namely southern Thailand, western Indonesia, peninsular Malaysia, and the Philippines (Reid 1968). It is often reported as an important malaria vector in hilly areas of Central Java Province and southern Sumatra (Mardihusodo 1989, Takken and Knols 1990). In contrast, because of its more zoophilic behavior and normally low human-biting densities, this species is of little or no medical importance on other islands of the Indonesian archipelago—Kalimantan, Sulawesi, and eastward to Timor Island (Hoedojo 1989). Eight species have been formally recognized in the *An. maculatus* complex based on morphologic and cytogenetic studies of polytene chromosomes (Rattanarithikul and Green 1986, Rattanarithikul and Harbach 1990). Recent DNA evidence indicates the complex may likely contain more members awaiting formal description (Rongnoparut et al. 1999). The taxonomic status of *An. maculatus* in Indonesia is not known; however, there is evidence that the Javan populations are significantly divergent in phylogenetic terms from other members of the complex and may represent one or more separate species awaiting formal description (Rongnoparut, personal communication). For purposes of this article, the Central Java strain will be referred to as *An. maculatus* [sensu stricto, "species B"] (Subbarao 1998).

In nature, *An. maculatus* occupies a wide range of shaded to partially sunlit larval habitats, including small rock pools and ground depressions. In Central Java, this malaria vector is closely associated with the hill regions' numerous small steams, which serve as a primary breeding habitat. This species prefers water that has collected in drying streambeds and seepages or along the margins of slow-running creeks and small garden irrigation ditches. Generally, clear fresh water with no or limited amounts of emergent and floating vegetation are preferred oviposition sites. Little else is definitively known about the bionomics and adult behavior of this species in Java.

The reputed importance of *An. maculatus* as a disseminator of malaria prompted an investigation into the selection and colonization of this species to facilitate scientific studies on insecticide resistance, vector capacity/competence, and life history by ensuring a reliable and continuous supply of mosquitoes. Previous attempts at continuous colonization of *An. maculatus* appear limited, based on only 2 published accounts from Sri Lanka (Jayewickrema 1952) and peninsular Malaysia (Ow Yang et al. 1963). Jayewickrema's brief description did not mention mating conditions or egg output for this species, while Ow Yang et al. (1963) were able to maintain a colony only by induced artificial mating procedures. Soon afterward, *An. maculatus* was obtained by the Centers for Disease Control (CDC) and Prevention (Atlanta, GA) from the Institute for Medical Research (Kuala Lumpur, Ma-

[1] The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the U.S. Naval Service or the Indonesian Ministry of Health. Send reprint requests to the Publications Office, American Embassy Jakarta, U.S. Naval Medical Research Unit No. 2, FPO AP 95620-8132.

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laysia) for use in a series of investigations on infectivity and transmission of different *Plasmodium* species and strains (Collins et al. 1976, 1980, 1986). The CDC colony still had the distinct disadvantage of requiring artificial mating for its propagation (Chin et al. 1966). In 1999, a colony of *An. maculatus* form B was established in Thailand, but again, colony maintenance has required artificial mating (Chareonviriyaphap, personal communication). The materials and methods described herein for maintaining a continuous breeding stock were developed using mostly local, inexpensive products and standard handling procedures. The only significant items purchased from outside of Indonesia were the collapsible aluminum adult cages (Bio-Quip, Gardena, CA, and American Biological Supply Co., Gainesville, FL). General collection, rearing, and handling methods for anopheline mosquitoes were based on extractions from standard insectary methodology and procedures (Coluzzi 1964, Gahan 1966, Foster 1980, Gerberg et al. 1994).

In 1994, natural bloodfed *An. maculatus* were obtained from outdoor and animal shelter resting collections in Desa (village) Hargotirto, Subdistrict Kokap (07°49′64″S; 110°06′07″E, elevation ~775 ft above sea level) within the Special Administrative Province of Yogyakarta in central Java. This site is located in the Menoreh Hills of Kulonprogo District, one of the most malaria-endemic areas in Java. This strain was first established in the Disease Vector and Reservoir Research Unit, Salatiga, Central Java, within a spacious rearing room provided with ample natural light from windows and ceiling skylights. Temperature and humidity in the facility were not controlled and the mosquitoes were subject to prevailing tropical ambient temperatures, humidity (27 ± 6°C; 75 ± 15% relative humidity), and lighting conditions. This strain later was adapted under more controlled conditions of temperature and humidity with a natural 12 h light:12 h dark regimen.

In Salatiga, individual bloodfed females derived from field samples were placed in paper cups for oviposition. Each cup (200-ml volume) was lined with filter paper and filled one third with nonchlorinated water. The tops of the cups were covered with a fine mesh synthetic screen and females were provided with a 10% sucrose solution soaked into cotton wool. Eggs were freely laid on the water surface and allowed to hatch, occurring within 2–3 days of oviposition. Approximately 400 first instars were transferred into white enameled metal pans (25-cm diameter × 5-cm depth) containing 500 ml clear (nonfiltered) spring water.

After laboratory adaptation, the following procedures were found useful for routine colonization. Fired clay bowls containing eggs were removed from cages daily and eggs were carefully transferred to hatching bowls by sweeping eggs out using a wash bottle. Enameled metal bowls were lined with 4-cm-wide filter paper strips placed along the inner walls to prevent stranding and drying of eggs. After hatching, approximately 400 first-stage larvae were transferred into larger enamelware or plastic rearing pans (35 × 24 × 5 cm) containing approximately 2 liters of clean, nonchlorinated water from either well or bottled sources. Finely powdered larval food was spread evenly onto the water surface twice daily, beginning with approximately 50 mg divided on day 1 and increasing by 50 mg incrementally to 350 mg by day 7 and beyond as needed. Larval food was decreased as late fourth instars and pupae developed. In general, daily food requirements can vary by rearing pan and careful observation was necessary to adjust amounts to avoid over- or underfeeding. The amount of food was adjusted to the developmental stage and numbers present. Several different food combinations for larvae were tried (Barodji et al. 1985). A mixture (10:4) of low-fat (lean), dried, powdered beef and finely ground rice hulls (bekatul) as previously described with the colonization of *Anopheles aconitus* Doenitz and *Anopheles barbirostris* Van der Wulp (Barodji et al. 1985, Soelarto et al. 1995) was found the most acceptable food for all instars. The ingredients are readily available at relatively low cost compared with many other food mixtures that use commercially refined products like liver powder, powdered brewer's yeast, finely ground food formulated for laboratory animals, aquarium fish, dog food, or dog biscuits, to name a few. This beef/bekatul mixture also helps reduce rearing maintenance because many larval foods (e.g., commercial dog food) are more likely to create unfavorable conditions (e.g., scum, bacterial growth) in the aquatic medium because of their excessive fat content. To further reduce scum formation on the water surface, positioned electric fans provided a light flow of air over the pans. Direct low-volume aeration through the water using small electric aquarium pumps was also found acceptable. In some instances, water temperature was regulated at a constant 28–30°C by placing rearing pans on flat silicone rubber-coated electrical heating strips (Cole-Parmer, Vernon Hills, IL). Fresh water was added to the rearing pans daily to replace loss from evaporation.

Pupation normally occurs between days 8 and 10, sometimes sooner, depending on the mean water temperature (~28–30°C). Pupae were transferred daily using a hand pipette from rearing pans into plastic cups (100 ml) containing clean water. Enough cups are placed inside mosquito cages (45 × 45 × 45 cm) to allow approximately 5,000 adults (male and female) to emerge per cage. Care was taken to avoid overcrowding of adults that can influence host-seeking behavior and greatly reduce bloodfeeding success. After eclosion, adults were provided ad libitum 10% sucrose solution and a solution of multivitamin B_{12} complex syrup (Calcidol, Kimia Farma, Indonesia) at a dilution of 20 ml syrup/480 ml water. The solutions were placed sepa-

Fig. 1. View inside adult mosquito holding cage for maintaining *Anopheles maculatus*. Center: Two hundred fifty-milliliter terra-cotta water-holding container used as an adult resting and oviposition site for gravid mosquitoes. The moist, cool terra-cotta surface serves an as ideal resting site for adults while also providing additional humidity. Left: One hundred-milliliter plastic container to hold pupae before eclosion. Right: Erlenmeyer flask containing multivitamin solution with thick cotton wicks (separate flask with 10% sucrose solution not shown).

rately in a 125-ml Erlenmeyer borosilicate flask with thick cotton wicks to absorb nutrients. Water-filled clay bowls (nonglazed, terra-cotta, 12-cm diameter, 250-ml capacity) provided an essential source of free water and additional humidity inside the holding cage, preventing adults from dehydrating (Fig. 1). In the Salatiga insectary, mean rearing temperature was dependent on diel ambient fluctuations, normally ranging from 21 to 33°C. Relative humidity varied between 50% (midday) and >90% (late evening), with exposure to a natural light:dark cycle. To increase surrounding humidity and promote adult survival, cages were routinely covered with wet toweling, especially during the hot, drier periods of the year. This species adapted quickly to open laboratory-rearing conditions and even more rapidly under controlled insectary conditions (26–30°C, 65–80% relative humidity).

Anopheles maculatus was found to be naturally free mating regardless of the size of the holding cage (minimum 32 × 32 × 32 cm). Mating activity was most often observed during dusk and declined into the early evening hours. This species is anautogeneous, requiring blood for egg development. After mating, 2- to 3-day-old females were provided access to live guinea pigs (*Cavia cobaya*), with one adult animal per cage. To provide a denuded surface area for hungry mosquitoes, each guinea pig was first shaved on the hind dorsal aspect using an electric hair clipper, followed by placing the animal inside an elastic plastic sleeve with expanded openings to allow for easy feeding access (Bangs et al. 1994). Most mosquitoes feed readily on restrained animals placed inside the cage for periods of up to 30 min. Other blood sources, such as mice and rabbits, were found to be less acceptable for our insectary work because of small and excessive body size, respectively. This strain also feeds readily on human and other animal blood, either directly or when placed in an artificial membrane system connected to a circulating, heated (~37°C) water bath (Rutledge et al. 1964). In general, Baudruche membranes made from a bovine intestinal preparation (Joseph Long, Inc., Belleville, NJ) were found more acceptable than stretched Parafilm® (American Can Co., Greenwich, CT) when placed over the glass feeding apparatus. Deprived of a carbohydrate source (provided water only) 12–24 h before blood feeding, starved female mosquitoes consistently gave >90% feeding success. Females commonly exhibit diuresis shortly after beginning blood ingestion, facilitating blood protein intake. When undisturbed, bloodfeeding often results in a discharge of copious quantities of bright red fluid containing erythrocytes from the mosquito's anus. Primigravid gonotrophic development and oviposition generally occur 2–3 days after the second successive bloodmeal (using *Cavia*), with subsequent ovarian development and oviposition requiring only a single bloodmeal thereafter. One wide-mouthed (12-cm diameter) terra-cotta bowl, lined with filter paper and filled with clean water, was placed in the cage for oviposition by gravid females. Wet filter paper prevented eggs from becom-

ing stranded and drying out. Eggs were deposited on the water surface during the evening. The approximate egg production ranged from 60–100 per brood batch, similar to fecundity reported by Ow Yang et al. (1963). Oviposition bowls were inspected daily, eggs carefully removed, and bowls replaced in cages with clean water.

Generally, *Anopheles* species are far more difficult to adapt to captivity than species of most other genera of mosquitoes (Gahan 1966). Commonly, colonization of anophelines from field material is frustrating, as many species do not adapt readily to laboratory culture, often involving difficulties in mating, oviposition, feeding, and survival. Nevertheless, this strain quickly adapted to laboratory conditions from the wild. Mortality seen in F_1 progeny derived from wild-caught female adults was surprising low. With each succeeding generation, the colony rapidly adapted, showing synchronized preimaginal development with minimal mortality (<10–20%) from immature stages through to eclosion. Up to the present, this colony has completed over 100 generations (~17 per year). Under ideal rearing conditions (adequate food and space), adults are generally robust and relatively long lived (3–4 weeks).

Successful colonization of mosquitoes from the field becomes paramount for many investigations on the biology and behavior of species. Mosquito colonies are still a prerequisite to any facility working with malaria and requiring sporozoites, studying the extrinsic cycle of plasmodia, and the mechanisms of transmission. Colonization is also the first step to developing and maintaining standardized and specific reference strains of variable genetic background and character and for genetic and nongenetic research purposes (e.g., mutant markers, insecticide resistance, sibling species, parasite–vector competence, etc.) (WHO 1966). Routine procedures for colonization of *An. maculatus* will permit increased laboratory experimentation on the vector biology and competence of this species as a disease vector. An investigation describing the relative susceptibility of this Central Java population to malaria parasites continues. Without deliberate artificial selection, *An. maculatus* has already proven valuable in the routine production of coindigenous strains of *Plasmodium falciparum* and *Plasmodium vivax* sporozoites for use in immunologic assays and malaria vaccine research. This colonized species adds to a relatively short list of valuable laboratory strains routinely used in the study of human plasmodia (Ward and Kitzmiller 1963, Vanderberg and Gwadz 1980).

This appears to be the first report of continuous colonization of *An. maculatus* from a free-mating population. The rapid success at colonizing this species may be due, in part, to the routine use of clear, natural spring water, an acceptable high-protein larval food combined with ground rice hulls, and exposure to ambient temperature conditions and natural photoperiodicity. Notably, the advantage that this strain of *An. maculatus* can mate and oviposit within the confines of a small cage contributed greatly to practicable colonization. This achievement is also owed to the knowledge, skill, and attention of personnel that contributed to the success of the initial rearing.

We thank William Ryan for his review and editorial assistance. This study was supported by funds provided by the Indonesian Ministry of Health and Social Welfare and the U.S. Naval Medical Research Center, Work Unit 62787A/870/U/D0013/W2902. In all aspects of colony maintenance requiring either direct feeding or blood draws from animals, the investigators strictly adhered to the Guide for the Care and Use of Laboratory Animals as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, U.S. National Research Council, No. 86-23 (1996).

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