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COLONIZATION OF THAILAND STRAINS OF *ANOPHELES NIVIPES* AND *ANOPHELES PHILIPPINENSIS*

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ABSTRACT. Two Thailand strains of *Anopheles nivipes* and one of *An. philippinensis* have been colonized by the artificial mating technique. The essential methods and colonization techniques are described. *Anopheles philippinensis* was more easily colonized than *An. nivipes*. Differences were detected in the laboratory feeding propensity and ovipositional behavior of these species. Other small biological differences also were noted.

INTRODUCTION

For years the name *Anopheles philippinensis* Ludlow has been used for a common mosquito with a wide distribution in the Orient. Recently, Knight and Stone (1977) listed this distribution as the Philippines, Borneo, Java, Sumatra, Malaya, French Indochina, Thailand, Burma, India, Andaman Islands, China and Hainan Island. However, Reid (1967) elevated the name *An. nivipes* (Theobald), previously considered a synonym of *An. philippinensis,* to species status based on specimens from Malaya, Burma and the extreme southern peninsular region of Thailand. Separation of these 2 species was based on slightly overlapping morphological characters in several life stages (Reid 1967, 1968). Reid suggested the need for progeny studies to confirm the species status of these 2 taxa. Since Reid's studies, there has been no further elucidation of the status or distribution of *An. nivipes*.

Anopheles philippinensis has been incriminated as a vector of human malaria parasites in India (Covell 1944), and recently (Harinasuta et al. 1976), has been considered a suspected vector in Thailand. The role of *An. nivipes* in malaria transmission has not been examined. Furthermore, the presence of *An. nivipes* in Thailand has not been recognized by the Thailand Malaria Division (unpublished reports), even though Reid (1967) discussed Thai specimens.

In late 1978, studies were initiated by the Department of Medical Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, to clarify (1) the specific status of *An. philippinensis* and *An. nivipes* by cross-mating and cytogenetic studies; (2) the distributions and bionomics of these 2 species in Thailand; and (3) the susceptibility of the 2 species to malaria parasites.

To date, preliminary cross-mating and cytogenetic studies (unpublished data) at our laboratory have firmly established

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that *An. philippinensis* and *An. nivipes* are distinct species. Furthermore, the pupal characters described by Reid (1967, 1968), i.e., the ratio of the refractile margin to paddle length, and the number of fringe teeth on the paddle, were found highly reliable for differentiating these species in Thailand. The adult differences described by Reid (1967, 1968) must be used with caution since these characters overlap within progeny broods of both species. Using the above pupal characters, an examination of adults with associated immature skins that were reared from larval collections or reared as progeny from adults taken in biting collections from various areas of Thailand, indicates that *An. nivipes* is much more common and widespread than previously thought. This species extends throughout Thailand, and is usually the species that previously has been called "*philippinensis*" in central, northeastern and northern Thailand. On the other hand, *An. philippinensis*, which previously was considered widespread and common throughout Thailand, has a more restricted range. Accordingly, previous distributional records of *An. philippinensis* in Thailand (Scanlon et al. 1968; Thailand Malaria Division, unpublished reports) apply to both *An. philippinensis* and *An. nivipes*, or *An. nivipes* alone. A revised interpretation of these 2 species in Thailand will be published elsewhere.

Colonization attempts began in 1979 because large numbers of both species were essential for the above studies. The resulting colonies and colonization techniques for *An. nivipes* and *An. philippinensis* are described below.

MATERIALS AND METHODS

The *An. philippinensis* colony originated from more than 500 females collected at Ban Chat Makrut, Amphoe Klaeng, Rayong Province, Thailand, during the periods 24–26 October and 6 November 1979. The *An. nivipes* (Korat strain) originated from more than 500 females collected at Thepnimit (formerly, Ban Nong Sakae), Mu 10, Tambon Chaliang, Amphoe Khonburi, Nakhon Ratchasima Province, Thailand, during the periods 1–2 and 6 October 1980. Three previous attempts to colonize this *nivipes* strain failed earlier in the year. The *An. nivipes* (Phrae strain) colony originated from an unknown number of females collected during October 1980 at the Forestry Station, Suan Pak Mae Kammee, 25 km from Nan, Phrae Province, Thailand. Adults of both species were obtained from bovine biting collections and resting collections from vegetation and cowsheds near the host. Specimens biting the collectors were also taken. Both *An. philippinensis* and *An. nivipes* were collected from Ban Chat Makrut, however, only *An. nivipes* was collected from the other 2 localities.

The wild caught females were brought to the insectary in Bangkok for colonization. *Anopheles philippinensis* and both strains of *An. nivipes* were maintained in 3 separate rearing rooms to prevent mixing. The insectary rooms were maintained at 22–32°C (daily mean, 25–27°C) at 50–80% RH, with only minor room variation. The insectary rooms were illuminated with a combination of natural daylight and fluorescent lighting for approximately 12 hours a day. These females were handled with care since they were collected from malaria endemic regions and potentially infected with human malarial parasites. Fully engorged females were transferred to individual screened 6 dr (24 × 84 mm) vials and provided a 5% solution of a commercially produced multi-vitamin syrup, which included 2% sucrose. On the second day, 5 ml of water were added to each vial to permit oviposition. Bottled drinking water was used for this purpose and for rearing the larvae of both species throughout the study. Females were held in the oviposition vials until they either oviposited or died. Adults which oviposited were placed with the unengorged females and treated as described below.

Unengorged and partially engorged females of *An. philippinensis* were put in a screened cage 21 cm square and allowed

to feed on a hamster restrained in a wire container. Unengorged females of *An. nivipes* were put in screened paper drinking cups (20 cm^3) and a hamster was placed on the screened top. Blood-engorged females of both species were removed from the cages or cups and were treated as above for fully engorged females.

Eggs from each feral female were transferred as a batch from the vial to a paper parasitological specimen cup containing water. The eggs were surrounded by a plastic drinking straw formed into a circle to prevent them from becoming stranded on the sides of the specimen cups. Upon hatching, the larvae and remaining eggs of each *An. philippinensis* female were put in a plastic larval rearing pan, three-fourths full of water. *Anopheles nivipes* larvae, upon hatching, were removed daily for a period of 14 days and put in larval rearing pans. At the end of the 14-day period, all unhatched eggs were discarded. A small amount of mud slurry (heat treated clay soil in water) was added to each of the larval rearing pans for both species. Larvae were fed a mixture of finely ground mouse and guinea pig laboratory chow twice daily. The first pupae of progeny from each feral mother were placed in individual rearing plastic vials. Upon emergence, the pupal skins were mounted and identified as either *An. philippinensis* or *An. nivipes* based on Reid's (1967) criteria. The remaining pupae from each brood were put into paper specimen cups, placed into the appropriate screened cage and provided a 5% multivitamin solution after emergence.

Reared females that were at least 3–4 days old were allowed to feed on a hamster daily as previously described. The 5% multi-vitamin solution was removed from the cage several hours before and during exposure to the hamster to enhance the feeding response. Engorged females were removed, mated with 3–4 day old males using the forced mating method described by Ow Yang et al. (1963) and put in a screened cage for 2 days. Blood-fed females were removed on day 2 after feeding and placed in individual 6 dr vials with water, and held for 6 days or until they oviposited. Engorged females which oviposited or remained in the vial after 6 days were returned to the screened cage or a screened paper cup and allowed to feed on a hamster again. Eggs collected from F_1 and later generations of *An. philippinensis* were counted, separated into lots of 200 or 300, and put into specimen cups with water for hatching. When a majority of the eggs had hatched, usually by day 2 or 3, the larvae and unhatched eggs were placed in larval rearing pans and treated as described above.

RESULTS AND DISCUSSION

The colony of *An. philippinensis* was established from 113 (22.6% of all females) females collected in Rayong Province during the latter part of 1979. The colonies of *An. nivipes* were established from 107 (20.5% of all females) females collected in Nakhon Ratchasima (Korat) Province, and 22 (total number of females unknown) females collected in Phrae Province, during October 1980.

FEEDING BEHAVIOR. The first few generations of *An. philippinensis* and both *An. nivipes* strains fed poorly on hamsters (<10%) in the laboratory. Therefore, the host exposure time was increased to obtain a satisfactory number of blood-fed adults. This was accomplished for *An. philippinensis* by leaving a hamster in a screened cage, 21 cm square, overnight. The most recent generations of *An. philippinensis* are now better adapted to feeding on hamsters, and the host exposure time has been reduced to approximately 5 hours, 0900 to 1400 daily.

When the host exposure time was increased by leaving a hamster in the screened cage overnight for both strains of *An. nivipes*, there was a high mosquito mortality rate, often more than 50%. This problem was overcome by placing 3–4 day old female mosquitoes in a screened cardboard drinking cup and laying a hamster on the screened top for 2–3 hrs.

In this manner, a feeding response of more than 50% was usually obtained and mosquito mortality rates were drastically reduced. However, with each generation fewer and fewer females were willing to feed on hamsters, and eventually the *An. nivipes* colonies were fed on a human blood source. This species feeds very eagerly on humans.

OVIPOSITION BEHAVIOR. Females initially were placed in oviposition vials and held until they oviposited or died. Of 1,168 females placed in vials, 44.3% (517) oviposited. It soon became evident, however, that if females had not oviposited by day 6 after the blood meal, it was unlikely that they would oviposit without an additional blood meal. Of 246 *An. philippinensis* females that oviposited, 241 (98%) oviposited from days 1–6, while only 5 (2%) oviposited from days 7–10. Thereafter, blood-fed females of both *An. philippinensis* and *An. nivipes* that had not oviposited by day 6 were returned to either a screened cage or cup and offered another blood meal.

Maintaining females in individual oviposition containers and subsequently removing eggs from these containers was very time consuming. Therefore, on several occasions blood-fed females were retained in a screened cage 21 cm square and provided 2 different oviposition substrates: (1) a white ceramic bowl containing water and (2) a petri dish packed with moist cotton and covered with filter paper. By the F_7 generation, in excess of 1,500 eggs were collected daily from *An. philippinensis* females using the cage substrates. Accordingly, isolating female *An. philippinensis* in vials was discontinued. Attempts to maintain both *An. nivipes* strains by using oviposition substrates in cages, however, were not successful, and both colonies are still being maintained by isolating females in vials for oviposition.

It appears that there is a direct relationship between close confinement and an increase in oviposition by both species. In all initial trials where blood-fed females were maintained in 21 cm square cages, *An. philippinensis* produced only a few eggs, while *An. nivipes* failed to oviposit. When *An. philippinensis* females were placed in a screened cage 38 cm square, even fewer eggs were deposited than in the smaller cages.

Davis (1928) noted that a second blood meal prior to oviposition increased egg production in *An. pseudopunctipennis* Theobald. Accordingly, blood-fed females of *An. philippinensis* were retained in a screened cage and offered a second blood meal prior to oviposition. Following the second blood meal, 150 females were put in oviposition vials containing water. Seventy-one (47.3%) of the females oviposited with an average of 58.4 eggs per female. The percentage of females ovipositing after 2 blood meals increased only slightly, while the average number of eggs produced per female was nearly the same as that from females having only one blood meal. Therefore, females were subsequently provided with only one blood meal prior to exposure to an oviposition substrate.

Overall, the number of eggs oviposited per female of *An. nivipes* (Korat and Phrae strains) and *An. philippinensis* ranged from 7–170 ($\bar{x}$ = 69.0), 9–174 ($\bar{x}$ = 71.3) and 10–220 ($\bar{x}$ = 57.1), respectively (Table 1).

EGG HATCHING. Eggs from the first 4 generations were removed from the individual oviposition vials (later generations of *An. philippinensis* came from screened cages), and were placed in specimen cups containing water and held for 14 days to determine the percentage of daily hatch. The larvae were removed, counted and placed in rearing pans daily. Eclosion did not occur during the first 24 hours post-oviposition, however, a major portion of the eggs of both species hatched during the 2nd and 3rd days. The percentage of *An. philippinensis* hatching by 72 hours was 81.0% for eggs from females maintained in vials and 74.1% for eggs from caged females, while the percentage of eggs hatching by 72 hours for *An. nivipes* (Korat and Phrae strains) females maintained in vials, was 96.0% and 98.2%, respectively (Table 1). Eclosion occurred

Table 1. Fecundity and daily egg hatch of *Anopheles philippinensis* and *Anopheles nivipes* (Korat and Phrae strains).

Species	Oviposition site	Eggs/female Range	Eggs/female Mean	% egg hatch	% hatch/day post-oviposition 2	3	4	5	6	7	8	9	10	11
An. philippinensis	Vial	10–220	57.1 (9,081/159)	73.9	42.2	38.6	7.1	4.8	2.8	1.9	1.2	1.0	0.1	0.1
An. philippinensis	Cage	ND	ND	81.3	30.5	43.6	13.3	5.0	4.2	2.0	0.8	0.4	0.1	0.1
An. nivipes (Korat strain)	Vial	7–170	69.0 (30,339/440)	75.1	56.7	39.3	3.4	0.3	0.1	0.1	0.1	—	—	—
An. nivipes (Phrae strain)	Vial	9–174	71.3 (18,200/255)	81.1	72.8	25.4	1.2	0.5	0.1	—	—	—	—	—

ND = not done.

Table 2. Pupation of *Anopheles philippinensis* and *Anopheles nivipes* (Korat and Phrae strains).

Species	Replicates	Larvae per replicate	% larval mortality	Mean date of pupation	% pupation/day post-eclosion 8	9	10	11	12	13	14	15	16	17	18
An. philippinensis	20*	100	18.2	11.9	0.5	9.2	17.7	17.4	17.1	16.4	14.5	4.8	1.8	0.4	0.2
An. philippinensis	20**	100	14.6	11.1	0.6	8.3	23.9	34.7	14.9	12.4	4.0	0.7	0.4	0.1	—
An. philippinensis	40***	100	16.4	11.5	0.5	8.7	20.9	26.3	16.0	14.4	9.1	2.7	1.1	0.2	0.1
An. nivipes (Korat strain)	67*	Progeny broods Range 6–119 Mean 56.9	32.4	12.1	—	1.9	13.8	25.4	25.9	15.6	8.6	3.3	3.8	1.4	0.3
An. nivipes (Phrae strain)	36*	Progeny broods Range 3–100 Mean 49.3	65.4	13.1	—	0.7	6.3	11.7	20.3	18.2	23.4	11.0	5.8	2.2	0.4

* Mud slurry added to water.
** Water without mud slurry.
*** Total, with and without mud slurry.

during the period, 2–11 days post-oviposition for eggs of *An. philippinensis*, while eclosion for eggs of *An. nivipes*, Korat and Phrae strains, was reduced to the period, 2–8 and 2–6 days post oviposition, respectively. Furthermore, less than 1% of the *An. nivipes* eggs hatched after day 4, while more than 10% of the *An. philippinensis* eggs hatched after day 4. A number of egg batches from both strains of *An. nivipes* completed hatching within a 24 hr period once eclosion started. This was not observed in egg batches from *An. philippinensis*.

During the initial attempts to colonize *An. philippinensis* the percentage of eggs hatching from females held in screened cages appeared to be lower than that for females isolated in vials. Accordingly, 20 replicates with 100 eggs each oviposited in screened cages after the F_7 generation, were allowed to hatch and the number of larvae counted and the day of hatch recorded. The percentage of eggs hatching (81.3%) from these females was actually slightly higher than the percentage hatching (73.9%) from females isolated in vials (Table 1).

DEVELOPMENTAL PERIODS. Forty replicates of 100 newly hatched first instar *An. philippinensis* were put in rearing pans to determine the length of time for pupation under laboratory conditions. A mud slurry was added to 20 of the replicates to determine if this affected pupation and if there was a significant difference in larval mortality between the 2 lots, i.e., (1) with and (2) without mud slurry. The length of time for pupation in lot 1 ranged from 8–18 days ($\bar{\chi}$ = 11.9 days, while in lot 2 the length of time for pupation ranged from 8–17 days ($\bar{\chi}$ = 11.1 days) (Table 2). It appears that the replicates without a mud slurry were slightly more synchronized than those containing a mud slurry. However, there was no statistically significant difference between the length of pupation times of the 2 lots. The mortality rate of the lot with a mud slurry was 18.2%, only slightly more than the lot without a mud slurry, 14.2%.

The length of time for pupation for *An. nivipes*, (Korat and Phrae strains) was calculated for 67 and 36 replicate progeny broods, respectively. The eggs for these broods all hatched within 24 hours, once eclosion began. The number of larvae per brood ranged from 6 to 119 ($\bar{\chi}$ = 56.9) (Korat strain), and 3 to 100 ($\bar{\chi}$ = 49.3) (Phrae strain). All replicates were reared in pans containing a small amount of mud slurry. The mean length of time for pupation was similar for both strains, 12.1 days for Korat strain and 13.1 days for the Phrae strain, and ranged from 9–18 days for both strains (Table 2).

SURVIVAL RATE. The survival rate of *An. philippinensis* under laboratory conditions was determined by placing 10 replicates of 20 unfed females and 20 males each in separate screened cardboard specimen cups provided with a 5% multi-vitamin solution. The daily survival rate ranged from 20.1 to 27.0 days, ($\bar{\chi}$ = 25.4 days) for the females and 18.1 to 30.4 days ($\bar{\chi}$ = 24.7 days) for the males. The effect of blood meals on the survival rate was not determined. No explanation is available at present for the short life span of the females in this study. Daily survival rates were not calculated for the 2 strains of *An. nivipes*.

In conclusion, *An. philippinensis* was much easier to colonize and maintain than *An. nivipes*. This was due to *An. philippinensis* rapidly adapting to feeding on hamsters and to ovipositing on either moist filter paper or in bowls of water placed in the cages. The 2 strains of *An. nivipes*, however, normally would not feed on hamsters and had to be offered a human blood source on which they fed avidly. Furthermore, both strains of *An. nivipes* rarely oviposited on the moist filter paper or in bowls of water placed in the cages. The *An. nivipes* colonies are still being maintained on a human blood source and oviposition is induced by isolating individual females over water in vials. Other biological differences between the 2 species included: (1) *An. nivipes* oviposited more eggs per female than *An. philippinensis;* (2) eclosion of *An. nivipes* eggs was much more synchronous

and frequently was completed within a 24 hr period, while this was not observed in *An. philippinensis;* and (3) larval development (to pupation) was faster for *An. philippinensis* than for *An. nivipes*. These apparently are physiologic differences in response to the laboratory conditions described above, which suggest basic biological differences between *An. nivipes* and *An. philippinensis*.

ACKNOWLEDGMENTS

The assistance of Mr. Kol Mongkolpanya, Mr. Ruan Thaopha and Mr. Sanit Nakngen in collecting field specimens, and the work of Mrs. Nantana Eikarat, Mrs. Boonsong Sripongsai, Mr. Somporn Chanaimongkol and Mr. Larp Panthusiri in the insectaries, are gratefully appreciated. We also gratefully acknowledge Dr. Visut Baimai, Mahidol University, Bangkok, for assisting with collections and cytogenetic interpretations, and Mr. Chris A. Green, South African Institute for Medical Research, Johannesburg, for collecting the parental stock for the *nivipes* (Phrae strain).

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INTERNATIONAL SYMPOSIUM ON CALIFORNIA SEROGROUP VIRUSES

An International Symposium on California Serogroup Viruses will be held at the Bond Court Hotel, Cleveland, Ohio, November 12–13, 1982. This symposium will immediately follow The Annual Meeting of the American Society of Tropical Medicine and Hygiene which will be at the same site. The symposium will bring together experts to discuss the current knowledge of the ecology, epidemiology, virus variation, genetics, pathogenesis, clinical aspects, prevalence, economic impact, diagnosis and control of LaCrosse encephalitis disease and other California serogroup bunyaviruses. Emphasis will be placed on the disease, the viruses and the arthropod vectors of these diseases.

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