Laboratory rearing method of *Anopheles minimus* (Diptera: Culicidae) from Ishigaki Island, the Ryukyu Archipelago, Japan

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Abstract: Biological studies on larval development, insemination and oviposition of *Anopheles minimus* from Ishigaki Island, the Ryukyu Archipelago, were conducted under laboratory conditions. The larval developmental period was shortened and high pupation and emergence rates were obtained when larvae were reared under the conditions of the non-aeration method, standard food volume and 200–300 larvae per container. Insemination rate was increased with adult density per cage as well as cage size. The number of eggs per fed female was dependent on the method for oviposition. The study showed the following conditions to be suitable for rearing and obtaining a large number of *An. minimus* originated from Ishigaki Island, the Ryukyus: (1) the drip method for larval rearing should be used in the early generation to estimate suitable food volume and after that, by non-aeration; (2) 200–300 larvae per pan with food volume of 25 mg for the 1st, 50 mg for the 2nd, 100 mg for the 3rd and 150 mg for the 4th-instar larvae; (3) adult cage larger than at least the medium-sized cage; and (4) individual oviposition method in small vials.

Key words: *Anopheles minimus*, Ishigaki Island, Ryukyu Archipelago, laboratory rearing

INTRODUCTION

Anopheles minimus Theobald is widely distributed in rural areas from the Ryukyu Archipelago, Japan, to Southeast Asia and Assam, India. It is an effective vector of rural malaria in these countries. Many field studies have been conducted to examine the insecticide impact on behavior (Nutsathapana et al., 1986a), feeding behavior such as host preference and endo/exo-phagy (Nutsathapana et al., 1986b; Dev, 1996), and temporal and spatial movement of adults (Tsuda et al., 1999a, b). *An. minimus* in Ishigaki Island, Ryukyu Archipelago was once highly anthropophilic but recently has been suggested

as zoophilic also (Miyagi and Toma, 1978).

In addition to field studies, laboratory colonization is important for performing studies that require large numbers of individuals. Wilkinson et al. (1974) and Ye et al. (1982) attempted to colonize *An. minimus*. They showed larval mortality to be related to rearing water conditions and larval density, and it was difficult for the species to mate in limited space under laboratory conditions, which were critical factors for successful colonization of *An. minimus*. In spite of repeated attempts, Muirhead-Thomson (1941) failed to colonize this species by natural mating in 60 × 60 × 60 cm sized cage. Somboon and Suwonkerd (1997) established a stenogamous colony of *An. minimus* species A in Chiang

Mai, Thailand. These studies suggested that successful colonization was dependent on larval rearing and mating under laboratory conditions.

Detailed taxonomical studies and biochemical and molecular techniques have revealed that *An. minimus* is genetically diverse among populations and constitute a species complex. Important behaviors such as host preference also vary according to each species type in the complex (Kanda et al., 1984; Yuan, 1987; Komalamisra, 1989; Christopher et al., 1990; Sucharit et al., 1988, 1995; Sawabe et al., 1996; Van Bortel et al., 1999). This indicates that information gathered on *An. minimus* collected in various localities so far may not be wholly applicable to *An. minimus* of all regions. The Ryukyu Archipelago, Japan, is the northernmost point of *An. minimus*, and there people suffered from malaria before and after World War II (Miyagi et al., 1996). Somboon et al. (2001) had recently demonstrated that *An. minimus* in the Ryukyu Archipelago, Japan, could be treated as a new sibling species as evidenced by reproductive isolation and genetic difference from other *An. minimus* in Asia. It was important to gather more information such as biology, molecular biology and taxonomy from the aspects of the new sibling species, however, there have been few studies on *An. minimus* in the Ryukyu Archipelago because of the relative difficulty of colonization.

Toward obtaining biological information for establishment and maintenance of a laboratory colony of *An. minimus* from Ishigaki Island in which a taxonomical study of the species was formally conducted by Somboon et al. (2001), it is essential to know the effect of at least the rearing conditions on larval development, mating, feeding and oviposition behavior of the species. In the present study, these subjects were examined.

MATERIALS AND METHODS

An. minimus used in the present study

originated from about 500 larvae collected in May 1994 from Ishigaki Island, located 360 km south of the main island, Okinawa. All experiments were conducted in an insectarium at 25°C and 80% relative humidity under a 16:8 (L:D) photoperiod. Larvae were reared in plastic pans (40×30×6 cm) containing dechlorinated water. Water volume was kept constant at 3 liters. Larvae were fed a 1:1 mixture of mouse pellets and dry yeast twice a day. Standard food volumes were 25 mg for 1st, 50 mg for 2nd, 100 mg for 3rd and 150 mg for 4th-instar larvae per pan, commencing with 200 1st-instar larvae. When 30–50% of larvae in a pan developed to the next instar, food volume given to larvae was increased according to the regimen. Pupae were removed from the rearing pans by a pipette every day, transferred to a small plastic container and placed inside a cage for adults. Usually, every generation was initiated by 800 larvae from the former generation, and about 300 males and females respectively were produced. Excess of individuals other than the 800 initial larvae were used for experiments. In the first 7 generations, 60×60×30 cm sized adult cage (large cage) was used for adaptation to laboratory conditions. Ten days after emergence, feeding and insemination rates were examined for females survived in the large cage. For the insemination rate, 15 females in the cage were taken and dissected to check the presence of sperms in spermathecae. After the 8th generation, 20×20×30 cm-cage (medium cage) was used for adult experiments. Adults were provided a cotton pad soaked with 3% sugar solution every 3 days. *Anopheles minimus* from 6th to 16th generation was used for all experiments except for feeding and insemination rates in the large cage.

Larval development

The effect of rearing methods, larval food volume and larval density on larval development was examined.

Effect of rearing method: Three met-

hods of larval rearing, "Drip", "Aeration" and "Non-aeration" were used. The drip method was a newly devised method with a siphon system for water circulation (Toma et al., 2001). The water drips from a plastic bottle tap run down from one container to another. The bottom-most container acted as a collecting reservoir (without a tubal outlet) that was emptied every day. The non-aeration method was quite simple, easy to handle and conventional in that the rearing pans were just left undisturbed with no devices. However, scum sometimes formed on the water surface, which might cause bad water quality and, therefore, larval mortality. The aeration method has been used for *Aedes* and *Culex* mosquito rearing in many laboratories (Gerberg et al., 1994). It was quite useful to prevent scum formation. Two hundred larvae were reared with each rearing method and fed twice a day according to the food regimen.

Effect of food volume: Two hundred larvae were fed 0.5, 1 (standard) and 1.5 times of standard food volume of the regimen, respectively, and reared by non-aeration method.

Effect of larval density: Using the non-aeration method, larvae were reared with 100, 200, 300, 400 and 500 individuals per pan. The amount of food per larva was kept constant among different densities. For example, 100 larvae were given half of the standard food volume and 300 larvae were given 1.5 times the standard.

Insemination rate

Insemination rate was examined under three conditions. Fifteen pairs of newly emerging adults were introduced into a small cage of 16×12×12 cm, and 15 or 30 pairs in medium cages. The effect of adult density was observed by comparison of 15 vs 30 pairs in a medium cage, while the effect of space was provided by the small vs the medium cages containing 15 pairs. Ten small cages with 15 pairs, and 8 medium cages each with 15 pairs and 30 pairs were prepared. One cage each from

the three conditions was randomly selected every two days, and all females in the cages were dissected to check for the presence of sperms in spermathecae. The experiment was conducted with individuals from 7th to 13th generations when a sufficient number was obtained, and repeated 3–4 times.

For one aspect of the mating behavior, the number of females inseminated by a single male was examined. One male and 20 females that emerged on the same day were released into a medium cage. On the 16th day after emergence, all females in the cage were dissected to check for insemination. The experiment was repeated 7 times.

Oviposition

The number of eggs laid was examined, when different oviposition methods were used. Three days after blood feeding, females were introduced singly into small plastic vials (3.0(D)×5.0(H) cm) with a small amount of water; this was the individual oviposition method. Fifteen females were housed in a small/medium cage for the group oviposition method. A plastic container (8.0(D)×5.0(H) cm) containing water was provided for females to oviposit freely in small and medium cages. The total number of eggs oviposited was counted, and the number of eggs per fed female was calculated.

Statistical analyses

The effects of rearing methods, larval food volume and larval density on the number of days to pupation were analyzed as mean developmental day to pupa by ANOVA. For pair-wise comparisons among different conditions of each experiment, Turkey's multiple comparison was conducted. The chi-squared test was used to compare the following parameters under different conditions: (1) pupation rate (the number of pupae/the number of larvae×100); (2) emergence rate for larvae (the number of emerged adults/the number of larvae×100) and pupae (the

Table 1. Larval developmental attributes of a sibling species of *Anopheles minimus* from Ishigaki Island, the Ryukyu Archipelago.

Experiment		No. exp	Total larvae used	Days to pupae	Pupation %	EmergenceL % ¹⁾	EmergenceP % ²⁾	No. ♀	No. ♂	Sex ratio (♀/♂)
Method	Drip	4	800	13.9±1.2 ^c	87.4 ^a	82.3 ^a	94.1 ^a	363	295	1.23 ^a
	Non-aeration	4	800	14.4±1.3 ^b	83.6 ^a	79.4 ^a	94.9 ^a	322	313	1.03 ^a
	Aeration	2	400	22.1±3.4 ^a	9.5 ^b	3.3 ^b	34.2 ^b	9	4	2.25 ^a
	Statistics			$F=678.7$	$\chi^2=887.8$	$\chi^2=851.1$	$\chi^2=203.9$			$\chi^2=3.9$
	Prob.			$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$			$P>0.05$
Food	0.5	4	800	14.4±2.0 ^a	82.1 ^b	67.6 ^b	82.3 ^a	311	230	1.35 ^a
	1.0	4	800	12.9±1.3 ^b	92.5 ^a	78.0 ^a	84.3 ^a	357	267	1.34 ^a
	1.5	2	400	12.8±1.3 ^b	86.0 ^b	67.3 ^b	78.2 ^b	183	86	2.13 ^b
	Statistics			$F=188.6$	$\chi^2=38.7$	$\chi^2=26.1$	$\chi^2=6.1$			$\chi^2=10.4$
	Prob.			$P<0.001$	$P<0.001$	$P<0.001$	$P<0.05$			$P<0.01$
Density	100	4	400	12.3±1.0 ^c	97.8 ^a	89.0 ^a	91.0 ^{ab}	205	151	1.36 ^a
	200	2	400	13.0±1.6 ^b	94.8 ^a	87.0 ^a	91.8 ^{ab}	197	151	1.30 ^a
	300	6	1,800	12.8±1.5 ^b	85.1 ^b	72.8 ^b	85.6 ^c	710	600	1.18 ^a
	400	3	1,200	12.5±1.6 ^c	60.4 ^c	56.9 ^c	94.2 ^a	308	375	0.82 ^b
	500	4	2,000	18.5±2.5 ^a	34.0 ^d	30.5 ^d	89.8 ^b	284	326	0.87 ^b
	Statistics			$F=1,518.1$	$\chi^2=1,496.6$	$\chi^2=1,070.0$	$\chi^2=44.4$			$\chi^2=29.9$
	Prob.			$P<0.001$	$P<0.001$	$P<0.001$	$P<0.05$			$P<0.01$

The values in the same column followed by the same letter were not significantly different ($P>0.05$) by ANOVA for days to pupae and χ^2 test for pupation, emergenceL and emergenceP %.

¹⁾ emergence rate to the number of larvae

²⁾ emergence rate to the number of pupae

number of emerged adults/the number of pupae $\times$ 100); and (3) sex ratio. If significant difference was detected among the group, multiple comparisons with Bonferroni correction were applied.

RESULTS

Larval development

Effect of rearing methods: The mean number of days to pupation was 13.9 days with the drip method, 14.4 days with the non-aeration method, and 22.1 days with the aeration method (Table 1). When the drip and non-aeration methods were used, high pupation rate (drip: 87.4%, non-aeration: 83.6%) and emergence rate for larvae (82.3% and 79.4%) and pupae (94.1% and 94.9%) were observed. On the other hand, the corresponding rates in the aeration method were low (9.5, 3.3 and 34.2% respectively), showing high larval and pupal mortality. There was no significant difference in the sex ratio among the different rearing methods; however, more females emerged than males in each rearing method.

Effect of larval food volume: The mean number of days to pupation was 12.9 days for the standard food volume, 12.8 days for 1.5 times the standard, and 14.4 days for 0.5 times the standard (Table 1). Compared to the standard food volume, the pupation rate and emergence rate for larvae were low when both small and

large food volume were provided although larvae developed rapidly with large food volume.

Effect of larval density: The effects of larval density were marked on larval development (Table 1). High larval density (400 and 500 per pan) prolonged the larval stage and lowered pupation and emergence rates. On the other hand, when larval density was 100, 200 and 300 per pan, the number of days to pupation was 12.3, 13.0 and 12.8 days, respectively, and pupation and emergence rates were higher (more than 80%), indicating low larval and pupal mortality. The sex ratio was 1.18–1.36 with density of 100–300. The ratio at high larval density (400 and 500) was 0.82–0.87, indicating more males than females.

Insemination and feeding rates

Insemination and feeding rates from 1st to 7th generations in the large cage are shown in Table 2. The insemination rate of 1st and 2nd generations was as high as 100% and 80%, respectively. Although the rate fell from the 3rd generation, after that the rate was relatively constant, 46.7–60.0%. On the same day the rest of the females in the cage were fed on a mouse the whole night. Next day, the number of fed females was counted. *An. minimus* fed easily on the mouse in laboratory conditions. The feeding rate was 70.7–100% and high through the generations.

Table 2. Feeding and insemination rates (%) of *Anopheles minimus* from Ishigaki Island, the Ryukyu Archipelago in the large cage.

Generation	No. of females examined	Insemination rate ¹⁾	Feeding rate (no. fed) ¹⁾
F1	22	100.0	100.0 (7)
F2	40	80.0	88.0 (22)
F3	37	46.7	95.5 (21)
F4	97	53.3	82.9 (68)
F5	106	60.0	97.8 (89)
F6	189	53.3	70.7 (123)
F7	258	60.0	76.1 (185)
Total	749	64.8	87.3

¹⁾ Ten days after emergence, 15 females were dissected, and the rest in the cage was fed on mouse in the same day.

Fig. 1. Insemination rate of *Anopheles minimus* from Ishigaki Island, the Ryukyu Archipelago under conditions of different cage size and density. M30, 30 pairs in a medium cage; M15, 15 pairs in a medium cage; S15, 15 pairs in a small cage.

Table 3. Oviposition rate (%) and the number of eggs of *Anopheles minimus* by individual rearing in small vials and group rearing in small and medium cages.

Oviposition method	Individual	Group	
	Vial	Small	Medium
Size	3.0(D)×5.0(H) cm	12×12×16 cm	20×20×30 cm
No. experiments conducted	3	2	2
Total no. fed females (A)	104	30 ⁴⁾	30 ⁴⁾
Total no. females oviposited (B)	33	—	—
Oviposition rate (B/A)	31.7	—	—
Total no. eggs oviposited (C)	2,900 ¹⁾	584	81
No. eggs/females oviposited (C/B)	100.0 ²⁾	—	—
No. eggs/fed females (C/A)	64.4 ³⁾	19.5	2.7

¹⁾ Oviposited by 29 females

²⁾ 2,900/29 (4 females out of 33 were dead)

³⁾ 2,900/45 (59 females out of 104 were dead)

⁴⁾ 15 females/cage

When adults were housed in a small cage, none of the females was inseminated (Fig. 1). With a medium cage, the first insemination occurred on the 5th day for 15 pairs, and on the 3rd day for 30 pairs. The rate increased faster in 30 pairs than in 15 pairs, with 50% of females inseminated by the 7th day for 30 pairs but by the 11th day for 15 pairs. By the 11th day, more than 80% insemination was observed for 30 pairs.

Number of females inseminated by a single male: On the 16th day, a single male inseminated two females on the average. The maximum inseminated by a single

male was 5.

Oviposition

The number of eggs oviposited (64.4) per fed female was higher in the individual oviposition method (Table 3). On the other hand, the number of eggs/fed female in the group oviposition method was 19.5 with small cages and 2.7 with medium cages. The oviposition rate was 31.7% in the individual method.

DISCUSSION

In the present study, larval develop-

ment of *An. minimus* from Ishigaki Island, the Ryukyus, was affected by the rearing methods, modified rearing water conditions and larval food volume, which Wilkinson et al. (1974) suggested although not examined in their study. The breeding sites of *An. minimus* in the Ryukyu Archipelago were stagnant pools of clear running streams at the forest edge but not those with high organic matters such as paddy-fields and swamps (Miyagi and Toma, 1978, 1980). The drip method newly devised in our laboratory, for rearing of *Anopheles saperoi* larvae which breed in edges of small streams (Toma et al., 2001), produced water circulation, and kept the water condition clean, hence simulating a clear stream. In the present study, this method resulted in low larval mortality although it was time-consuming to prepare. The non-aeration method which was easy to use, resulted in short larval developmental period and higher pupation and emergence rate (Table 1). However pupation and emergence rate were decreased due to poor water quality under the conditions with high food volume and the non-aeration method (Table 1). It is, therefore, recommended that larval rearing should be started by the drip method with estimation of proper food volume in the early generations. After that, the non-aeration method could be utilized.

The aeration method might be appropriate to avoid scum on the water surface and to keep the water conditions clean; however, the aeration method caused very high larval mortality, especially in the earlier stage in this study. The food on the water surface was trapped in the bubbles and sank quickly to the bottom, and larvae could not obtain enough food for development. Therefore the aeration method is not suitable for larval rearing of *An. minimus*.

The effect of larval density on larval development also was confirmed in the present study. Larval mortality of *An. minimus* under high-density condition

was high (400 larvae/pan=3 cm²/larva: 39.6%; 500 larvae/pan=2.4 cm²/larva: 64.0%) as shown by Ye et al. (1982) (1.5–3 cm²/larvae, 14.2–31.8%), compared to those under low-density condition (100–300 larvae/pan=4–12 cm²/larvae: 2.2–14.9%) in our experiments. Larval developmental period also was prolonged at 18.5 days in extremely high density (500 larvae/pan), while 12.3–13.0 days in the density of 100–300 larvae/pan. Since food volume per larva was constant in the present study, high larval mortality at high density might not be caused by food deficiency but intra-specific competition for limited space as in *An. stephensi* Liston (Reisen, 1975) or deterioration of water quality due to excretion by the larvae (Ike-shoji, 1993).

Sex ratio was biased to females in the larval experiments of this study. In the study of Wilkinson et al. (1974), excess of *An. minimus* females from Thailand was also encountered. Ye et al. (1982) also reported biased sex ratio in *An. minimus* from China, showing that males (52.3%) were predominated over females (47.7%) in their study. This may be due to selective mortality to either sex by the rearing techniques rather than to a population characteristic, since generally it would be assumed that the sex ratio in a population was 1 : 1.

Rearing at high larval density (400 and 500 larvae/pan) produced more males than females. The results indicated that high larval density caused especially high mortality of females at the larval stage and, therefore, a serious effect on the next generation. Abnormal recovery of one sex over the other under high larval density rearing was often observed in *Anopheles* mosquitoes such as *An. sacharovi* Favre, (Zakharova et al., 1990) and *An. stephensi* (Reisen, 1975).

The insemination rate of females was influenced by space size and adult density. In this study insemination did not occur in a small sized cage. This might be because they were unable to form a swarm in the

limited space as described for *An. minimus* species A (Somboon and Suwonkerd, 1997). To overcome the difficulty of mating in the laboratory, Wilkinson et al. (1974) and Ye et al. (1982) used an artificial mating technique. However, the technique is time-consuming. In the present study, we attempted colonization by natural mating, and a larger cage ($60 \times 60 \times 30$ cm) was used for adaptation to laboratory conditions during the first several generations (1st to 7th), because the insemination rate in the medium cage was very low. With the larger cage, more than 50% insemination rate was observed by 10 days after emergence (average 64.8%, Table. 2). The higher insemination rate for the first 2 generations than the latter may be due to the fact, that a small number of females which survived were relatively healthy to mate and feed in laboratory conditions. After the 3rd generation, though the insemination rate was decreased, the rate was relatively constant at about 50% and the strain was successfully maintained (Table 2). Ye et al. (1982) also described that success of colonization by natural mating was achieved by the insemination rate in a cage of $22 \times 22 \times 30$ cm at the level of 54.7%.

In our study, *An. minimus* from Ishigaki Island was able to mate naturally in the space of $20 \times 20 \times 30$ cm-sized cage in a relatively earlier generation (7th generation) compared with Ye et al. (1982) with more than 24 generations in a cage of $22 \times 22 \times 30$ cm and Muirhead-Thomson (1941) whose attempt failed in a $60 \times 60 \times 60$ cm-sized cage. These results might suggest difference characteristic somewhat among populations of *An. minimus*.

The insemination rate of *An. minimus* from Ishigaki Island, the Ryukyus, increased with the adult density as reported for *An. minimus* sp. A (Somboon and Suwonkerd, 1997). A single male inseminated several females in this study. Higher density of males may have contributed to increasing the insemination rate. It is necessary to evaluate suitable density using the

medium cage because high density may cause competition for space and high mortality.

The number of eggs oviposited was higher in the females individually housed in small vials. Small vials should be used as the oviposition method to obtain a larger number of eggs. The number of eggs decreased by the group oviposition method in the small/medium sized cages in spite of the ability to lay more than 60 eggs per female. This may be due to not incomplete blood feeding but oviposition behavior. In nature, *Anopheles* mosquitoes were considered to oviposit in flight, and it was observed that outdoor-caged females dropped only a few eggs at once, and then repeated the oviposition behavior frequently (Russell and Rao, 1942; Clements, 1999). In a laboratory also, *Anopheles* mosquitoes may perform oviposition in flight if the size of space in which they were placed was large enough. However, when a number of females were placed in a limited space in which the females could barely lay eggs in flight, the behavior was probably interfered by each other and the opportunities of oviposition by one female reduced as a result. Therefore, it might be difficult to lay all eggs and fewer eggs were obtained than expected with group rearing in the cages used in our study. On the other hand, in individual rearing with a small vial in this study, a female was almost in trapped condition in which the females had no interference with each other and oviposited in settled conditions rather than in flight. In such a situation, the female may lay more eggs than those with group rearing in the cages. These results were supported by McCrea (1984) in which females of *An. gambiae* s. l. that settled tended to lay all eggs. The importance of cage size may be different according to mosquito species.

The present study showed that the following conditions were suitable for rearing and obtaining a large number of *An. minimus* originated from Ishigaki Island, the Ryukyus: (1) larvae should be reared

by the drip method in the early generation to estimate suitable food volume and after that, by the non-aeration method; (2) 200–300 larvae per pan with food volume of 25 mg for the 1st, 50 mg for the 2nd, 100 mg for the 3rd and 150 mg for the 4th-instar larvae; (3) adult cage larger than at least medium-sized cage (20×20×30 cm); and (4) individual oviposition method in small vials.

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