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Possibility of Free-Mating Malaria Vector in the Laboratory

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Abstract

Anopheles dirus mosquitoes are significant malaria vectors that have recently been successfully maintained in the laboratory. However, this mosquito is complicated to breed, requiring additional artificial mating processes, taking time and needing a large amount of manpower. Then, we focused on free-mating experiments to establish a free-mating *An. dirus* colony without a mating stimulant, as well as a highly productive laboratory colony to minimize work time. Males and females of *An. dirus* (F219) were maintained in $45 \times 45 \times 45$ cm mosquito cages at insectary condition with $26 \pm 1°C$ and $70 \pm 10\%$ relative humidity under 12 : 12 h light-dark cycle. The male-female ratio is approximately 1 : 1, and no stimuli are used to induce free-mating. The result of the free-mating of *An. dirus* is demonstrated by the spermatheca of female generations insemination from three generations, F0 to F2, is supported by the free mating process. The insemination of free-mating F0 to F2 was dissected and exhibited in each generation, F0 (16.10%), F1 (31.60%), and F2 (35.29%), respectively. Most females produce the healthy eggs that can develop into adult stage. The adult stages have a high survival rate of approximately 98.19%, 98.17%, and 99.05%, respectively. While the artificial mating colony has an unstable survival rate. The ability to conduct independent mosquito breeding experiments in each generation of *Anopheles*, mosquitoes, along with sperm insemination from F0 to F2 generations, increased the number of naturally fertilized mosquitoes. Therefore, there is an opportunity for enhanced breeding until free mosquito colonies are established.
KEYWORDS: Artificial mating, Free-mating, Oviposition, Hatching, Survival, Feeding, Sustainable colony

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1 INTRODUCTION

Malaria is a major concerned on the international borders of Southeast Asia (Nguitragool et al., 2019). Although the most of Thailand is malaria-free, the border areas with Myanmar still contain endemic pathogens (Parker et al., 2015), and malaria remains one of the significant problems in the Thai border area (Jongdeepaisal et al., 2022; Lertpiriyasuwat et al., 2021). There is still an ongoing disease surveillance plan (Giglio et al., 2015; Ocampo et al., 2013). The National Malaria Eradication Strategy 2017-2026 recommends strategies 1-3-7 for effective surveillance and prevention by prioritizing evidence-based action and completing it promptly (Lertpiriyasuwat et al., 2021). Malaria vectors were found throughout an infectious disease vector surveillance in Thailand (Jongdeepaisal et al., 2022), and seven *Anopheles* species have been indicated as important malaria vectors in Thailand (Taai et al., 2017; Tananchai et al., 2019). *Anopheles dirus* remains the primary vector transmission carrier, which provides to support research studies in this area. *Anopheles* mosquitoes, including *An. dirus*, *An. minimus*, and *An. maculatus*, are the most important local malaria vectors in two malaria-endemic areas along the Thai-Myanmar border (Tainchum et al., 2014; Tananchai et al., 2012).

Mating is a key physiological process for mosquito population maintenance and is necessary for next generation reproduction (Hamady et al., 2013). Therefore, many studies and experiments on mosquito population expansion encourage various research from nature to be developed in laboratories. To support research and other knowledge, sound and flight frequencies have been studied to play an essential role in the mating behavior of mosquitoes, particularly *Aedes* mosquitoes (Cator et al., 2011). Natural *Anopheles* mosquito mating begins with the males swarming at night, followed by the female flying into the swarm, and physical factors such as light and temperature affect mosquito mating (Wang et al., 2021). The Mosquito Ecology Research Center was established as a semi-field to study the swarming behavior of male malaria mosquitoes, and *An. gambiae* complex are known to mate in swarms at specific locations at dawn and dusk (Niang et al., 2019). The hotspot pattern of small formations corresponds to the relationship between herd size and mating success. Mating success of individual males does not increase with herd size (Diabate et al., 2011).

Laboratory-raised mosquitoes, such as *An. atroparvus* European Species Colony Laboratory are important for investigating arthropod-borne diseases (Birnberg et al., 2020; Masters et al., 2020). Furthermore, females of *An. belenrae* and *An. pullus* from the Republic of Korea, are forced to mate in order to maintain their colonies. Additionally, breeding techniques were used to create *An. kleini* and *An. sinensis* (Phasomkusolsil et al., 2014; Phasomkusolsil et al., 2018). After optical induction in subsequent F1 to F6 generations, independent mating was achieved using an automatic coupling induction system (Araujo et al., 2019). There was also an experimental process using force mate by *An. dirus*, *An. campestris* and *An. sawadwongporni* (Phasomkusolsil et al., 2017). It has been reported that *An. minimus* and *An. harrison* may mate naturally in smaller cages (Taai et al., 2017). Furthermore, light stimulation experiments with *Nyssorhynchus deaneorum* from Brazil colonization can grow naturally and be free-mating in the 10th generation (Lima et al., 2004). Light-stimulated experiments in breeding *An. darlingi* from the Peruvian Amazon began in free resolution in the F14 generation and were successful in the F26 generation (Villarreal-Trevino et al., 2015). In the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Thailand, we raise

mosquitoes in the laboratory for research services, with *Aedes* spp., *Culex* spp., and *Anopheles* spp., which are free-breeding mosquitos. However, *An. minimus* is the only species that can breed freely among reared *Anopheles* spp. We are raising *An. dirus* at generation F229, but artificial mating is still mandatory. This approach has lengthened the working duration and needs the usage of experience. So, the purpose of this study experiment was to focus on establishing a free-mating colony of *An. dirus* mosquitoes in the laboratory without a mating stimulant, in order to manage the appropriate time of work.

2 MATERIAL AND METHOD

2.1 Mosquito rearing

2.1.1 Larvae stages

The eggs were deposited on an oviposition plate with moistened cotton that had been covered with filter paper. Eggs on the filter paper were submerged in water and hatched into larvae, with approximately 200 larvae per plastic tray (23x33x5 cm) containing 1L of distilled water. The larvae were reared in distilled water and fed fish powder food on a daily basis at a frequency of 0.011 g per tray for the first instar, 0.22 per tray for the second instar, and 0.066 g per tray for the third-fourth instar. The distill water in the tray was changed once a week until pupation. These pupae were collected daily and transferred to a 3L plastic container (500 pupae /container). These pupae were maintained until they reached the adult stage.

2.1.2 Adults stages

The newly emerging adults were placed in cages of size $30 \times 30 \times 30$ cm and maintained in an insectary at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University with the present condition at 26°C and 70°RH with a 12 : 12 h light-dark cycle. They were given 5% sugar and a vitamin syrup solution as food $(1 : 1)$.

2.1.3 Artificial mating

Female *An. dirus* mosquitoes (F219), aged 5-7 days, 250 adults per cage, were artificially fed blood for 30 minutes. The engorged females were mated with one male at a time, by gently stroking the male's abdomen over the female's abdomen to induce the claspers to open before the man clasped the female. Until a total of 250 mosquitoes are produced. The engorged females were kept apart in cages (250 each cage) for the next four days and fed vitamins and sugar. After that, the cage received an oviposition plate composed of moistened cotton that had been protected with filter paper for a day. The eggs that had been placed on the filter paper were soaked in water.

2.1.4 Free-mating

To build up the free mating, males and females of *An. dirus* (F219) were separated and reared in standard insectary protocol at an insectary 26 ± 1°C and 70 ± 10% relative humidity under a 12 : 12 h lightdark cycle. Free mating was induced as follows. After emergence, male and female *An. dirus* (F0) in 1:1 ratio was released in the net cage, size $45 \times 45 \times 45$ cm until they were 5-7 days old. Only female *An. dirus* was selected to give blood by the artificial-

feeding method. After three days, female adults completely digested blood, and laid eggs. The oviposition plate was kept in the cage and changed daily until three days. The number of eggs and larvae was counted after hatching. The larvae were reared in standard protocol until they became adults and repeated the free-mating procedure.

2.2 Insemination Status

The insemination status was determined by dissecting the spermatheca of adult females. The females were sampled from each generation. Generations F1 and F2 are almost completely dissected following the standard methods (WHO, 2015). Briefly, the anesthetized females were placed ventral side up with the abdomen resting on the slide with the PBS solution and removed the Terminalia using a finetip needle. The slide was covered with a cover slip and seen under 100X magnification on a microscope. If a female mosquito was mating, it was seen the long thread-like spermatozoa in the spermathecal. Uninseminated females had fairly transparent spermathecal.

2.3 Statistical Analysis

2.3.1 The number of samples for dissection

Free-mating estimates a finite population proportion sampling F0 generation of *An. dirus* mosquitoes to verify sperm in females. It is calculated from a preliminary study of equally male and female adults with free mating, which found 20% positive female spermatheca. Then sample size was calculated to form Formula (Ngamjarus, 2016), as shown below:

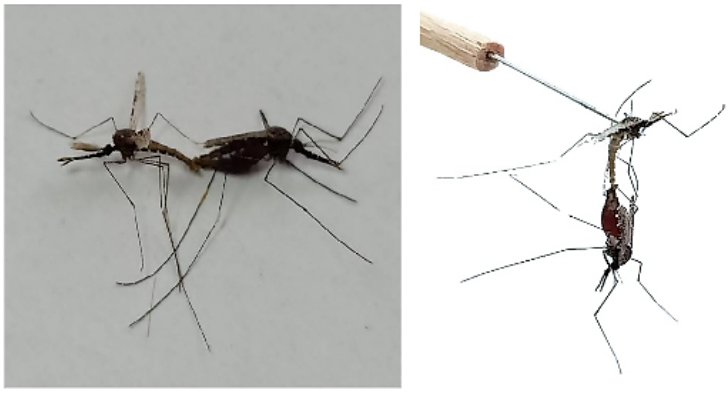
$$n = \frac{z^2_{1-\frac{\alpha}{2}} p(1-p)}{d^2}$$

Population (N) = Infinite
Proportion (p) = 0.2 (preliminary study)
Error (d) = 0.05
Alpha (α) = 0.05
Z = 1.96
N = 246

The parameters used to calculate the hatching rates, survival rates, oviposition. Reduction of larva and adult mortality was evaluated by non-parametric tests changes in eggs larva and pupa productivity was tested using.

Feeding rate = total female mosquito blood fed/total female mosquito
Hatching rate = (total larvae $\times$ 100)/total egg
Oviposition = total egg /number blood fed of female
Survival rate = (total adult $\times$ 100)/ total number larva

Using non-parametric tests assessments to analyze and a 95% confidence interval of the difference, statistical analyzes were performed using GraphPad Prism (GraphPad Software, Inc.), and a $p < 0.05$ was considered significant for all tests



(A) (B)

Figure 1: *An. dirus during artificial mating. (A) The artificial mating processes. (B) A successful artificial mating of An. dirus*

Table 1: *Total number of engorged females, eggs, larvae, pupae, and adults; and percentage of larval and pupal mortality of An. dirus free and artificial mating.*

Method	Generation	Engorge female	Male	Total eggs	Unhatched (%)	Total larva	Larva-pupa (mortality%)	Total pupae	Pupa-adult mortality (%)	Total adults
Free mating	F0	36500	39500	7044	9.24	6393	1.78	6279	0.03	6277
	F1	2959	3318	2020	21.58	1584	1.83	1555	0	1555
	F2	730	825	1340	21.64	1050	0.95	1040	0	1040
	Total	40189	43643	10404		9027		8874		8872
Artificial mating	212	226	226	15000	4	14400	2.41	14053	0.3	14010
	220	297	297	19000	6	17860	5.09	16950	0.14	16925
	230	326	326	17600	3.66	16955	0.88	16805	0.06	16795
	Total	849	849	51600		49215		47808		47730

3 RESULTS

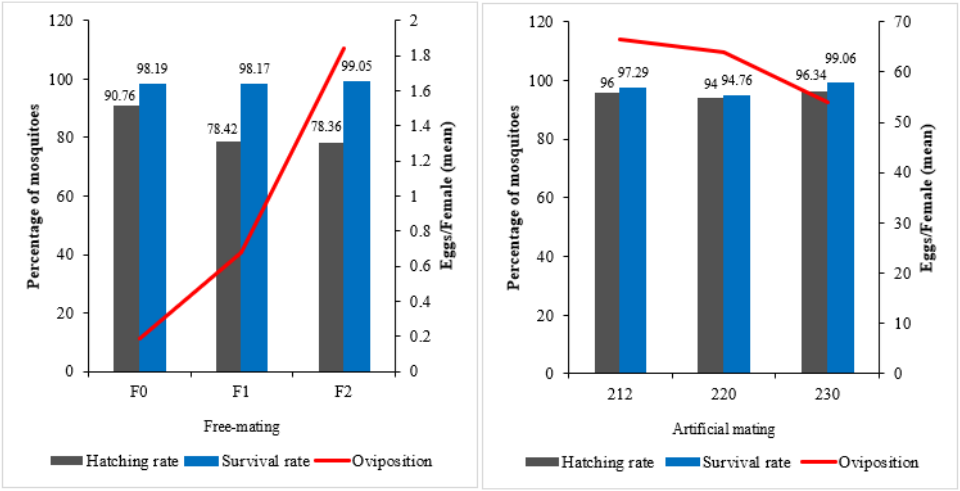
The experiments required 16 replications of the F0 generation to amass enough eggs to begin a colony, and 8 replications of the F1 generation to produce the F2 generation. It is commonly known that inefficient mating has reduced the number of mosquitoes in a generation. A large number of F0-engorged females and males were needed for subsequent generations. Finally, *An. dirus* colony free-mating has been maintained for three generations (F0-F2) in laboratory conditions at the insectary of Medical Entomology Department, Faculty of Tropical Medicine, Mahidol University. Table 1 presented all information from the two techniques of mating. Data on artificial mating is derived from the F212-230 generation, which spans the experimentation period representing baseline information of this colony. To create free mating colony, F219 of artificial mating was used as parent. Free and artificial mating are not directly compared with generation. Although the percentage of unhatched was higher in F1 and F2 than of F0 in free mating, the mean number of unhatched eggs in free and artificial mating is significantly different (Kruskal Wallis Test; $p = 0.037$).

The total larvae of the F0-F2 generation of free mating *An. dirus* ranged from 1,050 to 6,393. The percentage of larval-pupa mortality from free-mating ranged from 0.95 to 1.78, which was not statistically significant when compared to force-mating (Kruskal Wallis Test; $p = 0.317$).

The total pupae of free-mating colony produce range from 1,040 to 6,279. The pupa-adult mortality of free mating compared with the force mating was not significant (Kruskal Wallis Test; $p = 0.094$). Moreover, the results of the experiment from free-mating and force-mating showed very low mortality rates and high survival date (Table 1).

The development of free-mating *An. dirus* from egg to adult was observed in all three generations (F0-F2 generation). Each generation took approximately 14 days, much the same as artificial mating. When compared to engorge females in each cohort, there was an increase in oviposition from F0 to F2 generations (Figure 2A). This data suggests artificial mating in insectary colonies to reveal the potential for developing free-mating and successful colony establishment.

In spite of the increasing percentage of unhatched eggs, which ranges from 9.24% to 21.64%, the increase in oviposition (Figure 2A) shows that the hatching rate is still high. In this study, the observation of open operculum eggs or flattened mosquito eggs was not examined to confirm unhatching.



A B

Figure 2: *The percentage of hatching, survival and the number of oviposition of free-mating An. dirus (F0-F2 generation), (A) and artificial mating An. dirus (B)*

The laboratory *An. dirus* has been maintaining by artificial mating. The females of F232 and F233 *An. dirus* had their spermatheca completely dissected. The percentages of positive sperm were 54.25 and 74.33, respectively (Table 2). For free mating *An. dirus*, 3,560 female mosquitoes from the F0 generation, or 10% of all mosquitoes, were chosen at random for spermatheca dissection. In Table 2, only 16.10% of the ones were positive. The F1 and F2 females were completely dissected, and the insemination rates were 31.60% and 35.29%, respectively.

4 DISCUSSION

Results showed that female *An. dirus* can mate freely in a cage size 45x45x45 cm. Even though the F0 showed a meager mating percentage, a successful mating can still occur if the

Table 2: *The insemination rate of artificial mating and natural free-mating of An. dirus in each generation*

Method	Generation	Total engorge female	No. of dissection	No. of Positive spermatheca	Insemination rate (%)
Free-mating	F0	36500	3560	573	16.1
	F1	2959	1285	406	31.6
	F2	730	714	252	35.29
Artificial mating	F232	188	188	102	54.25
	F233	339	339	252	74.33

F0 is more than adequate. It can maintain the colony for the following generation and raise the females' insemination rate. That is potential proof that its free-mating colony in a lab can be established. This experiment did not use the technique to encourage free mating behavior. In numerous publications, they describe how light activates mosquito species to self-mating (Villarreal-Trevino et al., 2015). The natural swarming behavior of *Anopheles* mosquitoes is the reason for their mating. *An. gambiae* complex is known to mate in swarms at specific locations at dawn and dusk (Niang et al., 2019). Still, when reared in the laboratory, these behaviors were not demonstrated because mosquitoes were raised in a narrow cage. And the limitations in keeping with each other prevent the opportunity to fly high, swarming, and not mating.

Our preliminary experiment was performed on 100 males: 100 females (1:1) in a 3L container, and 500 males: 500 females (1:1) in a cage 30x30x30 cm in size without stimulation, but no spawning was found. In addition, 1,000 female and 1,000 male mosquitoes were tested in a cage of 30x30x30 cm, and yielded approximately 100 eggs. Hence cage size, male-to-female ratio, and mosquito density all had an effect on free mating success. We found that the most effective mosquitoes number and cage size were 2,500 and 45x45x45 cm. This agrees with Villarreal-Trevino et al. 2015. The size of the cage and the number of mosquitoes used in comparison to a medium size 46x46x46 cm cage used 1,500 males: females (1:1) and 2,400 in a large cage using thermo-period and LED light stimuli, F30 generation was successful (Villarreal-Trevino et al., 2015).

In a free-mating study of mosquitoes *N. deaneorum* from brazil (Araujo et al., 2020), a stimulus mating study using the automatic copulation induction system (ACIS) technique was used up to F4 generation, starting from F5 generation, no stimulation was used to induce mosquitoes to interbreed until F10, the standard free-mating mosquito that could breed among itself in the laboratory (Araujo et al., 2020); our study did not use a stimulus. Free-mating mosquitoes in *An. albitarsis* were studied in the F1 cohort by crossing lab strain mosquitoes with the original population. The percentage of inseminated females from the lab strain mixed with actual males was 7.6%. As a result of the crossing of the original female mosquito with the male lab strain, there were 18.8% inseminated females 20% of the crosses between females and males of the lab strain were inseminated females (Lima et al., 2004). As for our results, males and females of a lab, the strain used in cohort F1 means of inseminated rate was 31.60%. Both of these experiments did not have stimuli to mate with each other. While the results of force-mate unsampled sperm dissections a mean dissection of 64.29 relatively higher percentage than free-mate.

In the Villarreal-Treviño et al. experiment, thermo-periods were set at 30ś1 daytime and 25ś1 nighttime with a 30-min twilight LED excitation lamp. It is stimulated until F9 and F10 generations after that, allowing mating without stimuli until the development of F19 generations is free-mating mosquitoes (Villarreal-Trevino et al., 2015). Even only the F0-F2 generation was completed result in this study, the data showed increasing the percentage insemination. Then, the next experiment will be continued colonization until the stable free mating *An. dirus* colony is established in the laboratory. This data suggests artificial mating in insectary colonies to determine whether or not there is a potential of developing standardized free-mating and thriving colony settings in a larger mosquito cage.

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