Laboratory colonization of *Anopheles sinensis* (Wiedemann, 1828)

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Abstract: After a strain of *A. sinensis* from Kanoya, Kyushu (Japan) was established by induced copulation with a human blood source for its first 26 generations, it has been further colonized for 19 generations in a small cage (30×30×30 cm) by free mating at 25±1°C, 70-80% R. H. Larval stages were mass reared on the pulverized food consisting of equal weights of wheat germ, dry yeast and oatmeal. The mating ability of males during induced copulation was investigated; 3 day-old males were completely successful at mating but the quantity of sperm in the spermathecae was very low. The insemination rates were about 16% in the 31st generation, 41% in the 41st generation. *A. lesteri* has also been maintained mainly with a mouse blood source in a small cage for 15 generations without induced copulation. The insemination rate of this species was about 8% at the 11th generation. The tolerance of the eggs of *A. sinensis* to desiccation was tested and it was found that the eggs hatched normally after 2 days of desiccation but the hatchability decreased down to 29% after 4 days.

Anopheles (*A.*) *sinensis* is a member of the *A. hyrcanus* species group, and is found in northeastern India, Malaysia, Thailand, Vietnam, Indonesia, Korea, China, Japan and eastern Siberia. It is the important vector of *Brugia malayi* (Filariæ) in China (Feng, 1931). Recently, Kanda *et al.* (1975) have found that *A. sinensis* is one of important vectors of *Brugia malayi* in the inland areas of the Korean peninsula. From these facts *A. sinensis* can be regarded as a species of considerable medical importance. However, Harrison (1973) studied the identities of the "*sinensis*" found naturally infected with filarial and malarial parasites and suggested that the vector role of *A. sinensis* is decreasing.

In Japan, *A. sinensis* is the most widely distributed and common species of anopheline mosquitoes. It may be reared only with

difficulty in the laboratory, and only a few studies have been made on the laboratory rearing of this species (Keegan *et al.*, 1964; Tanaka and Hatoyama, 1967). They collected many larvae in the field at the start of colonization and obtained matings in a large cage and then gradually adapted the colony to smaller cages in the laboratory. They also changed rabbits to guinea pigs as a source of blood. However, their method is not directly applicable for our experiments because of the difficulties in obtaining enough mosquitoes in the field for colonization and in running the risk of contamination with sympatric species from the field. Therefore we tried to establish the laboratory colonies of *A. sinensis* by the induced copulation method according to Baker *et al.* (1962), for the purposes of genetic, biological and also epidemiological studies as the vectors of malaria and filariasis. After generations of the established colonies they have been selected to copulate naturally in cages and to take mouse blood instead of human blood.

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The purpose of this paper is to report the successful colonization by induced or natural copulation in small cages of an indigenous strain of *A. sinensis* in Japan.

INDUCED COPULATION METHODS

Materials and methods

About 70 blood-fed females of *A. sinensis* were obtained for laboratory colonization by using an aspirator during the night or day in pigpens and cattle shed in breeding areas at Kanoya City, Kagoshima Prefecture in Kyushu, approximately 1,000 km southwest of Tokyo. Females were anesthetized with ether and more than half of each of the wings was cut off by a knife. Then about five females were put in each container (6 cm in diameter, 3 cm in depth) containing wet filter paper, and these containers were kept in a cool box (about 23°C) and carried to our laboratory by train. The oviposition was already observed to have started in transit and back in the laboratory the eggs were transferred to a white pan (40×25×6 cm in depth) made of polyvinyl and lined with filter paper. The eggs hatched well after 4 days. Pulverized food, consisting of equal weights of wheat germ, dry yeast, and oatmeal was sprinkled on the water surface in the rearing pan one or two times a day and the rearing water was not changed during larval development. The larvae were diluted serially during their growth to prevent overcrowding, and the larval density ranged from several thousand in the first instar stage to less than 500 per pan in fourth instar; pupae were gathered with a wide-mouthed pipette and transferred to small cups. After their emergence in a small cage (20×20×20 cm) the adult nutriment was furnished by cotton pads saturated with about 3% sugar solution. Three days

and again 5 days after emergence human blood was given to female mosquitoes; each of the blood-fed females was artificially copulated with 3 day-old males. About 50 copulated females were kept in the small cage with 3% sugar solution until the wings of parous females were cut off to promote oviposition. The laboratory humidity could not be controlled for the first 7 generations but after the 8th generation mosquitoes were reared at a controlled temperature of 25±1°C with a controlled relative humidity of 70–80%. After 27 generations the Kanoya strain maintained by this induced copulation method copulated successfully even in the small cages and took blood meals from mice.

Experimental results

Table 1 shows the number of oviposited eggs, their hatchability, the viability of the larvae in 8th and 19th generations of the Kanoya strain, and also in the 6th generation of the Yakumo strain. The latter strain was collected to establish another colony at Yakumo, Hokkaido. It took 2 days from oviposition to hatching, and at least another 8 days to appearance of the first pupae, but additional 2 weeks or more were required for the pupation of all larvae. The males of the 19th generation of the Kanoya strain were tested for their mating ability by induced copulation, using 20 individuals of each age from 1 through 6 days. The results are shown in Table 2. The 3 day-old males had completely successful mating at the highest mating rate, but in order to confirm an ejaculation into the females artificially mated with the 3 day-old males the spermathecae in the females were dissected to observe the quantity of sperm in them. In this experiment 80% of the

Table 1 Fecundity of *A. sinensis* after induced copulation

Strains and generation	No. of females tested	Total no. of eggs oviposited	Mean no. of eggs/♀ ₁ oviposited	Hatchability (%)	Viability (%) from 1st to 4th instar
Kanoya F ₈	4	940	235.0	31.4	—
Kanoya F ₁₉	6	1,676	279.3	22.4	78.0
Yakumo F ₆	5	1,645	329.0	18.0	68.0

Table 2 The mating ability of males used in induced copulation

Days after emergence	No. of males tested	No. of males which copulated	% of males which copulated
1	20	2	10
2	20	17	85
3	20	20	100
5	20	19	95
6	20	14	70

spermathecae dissected contained very few sperm, no sperm being in 20% of the spermathecae. It is, therefore, considered that the quantity of the sperm ejaculated by only one male at the induced copulation was not sufficient for one female to lay eggs.

NATURAL COPULATION WITH UTILIZATION OF MICE AS A BLOOD SOURCE

Materials and methods

In order to establish a colony capable of freely mating in a cage without induced copulation (after the 27th generation of the Kanoya strain), the following experiment was done: a total of about 1,000 newly emerged females and males were gathered in a cage (30×30×30 cm) for a week and a mouse restrained with screen mesh was exposed in the cage every night during a 2 week period. An egg deposition cup with rearing water was provided in the cage.

The insemination rates were tested in the 31st and the 41st generations of the Kanoya strain of *A. sinensis* using two kinds of the small cages (20×20×20 cm and 30×30×30 cm). About 500 newly emerged adults had

been gathered in smaller cage and 1,000 adults into the other cage, during one week, then after five days 20% of the females were sampled from each cage to be dissected. *A. lesteri* was also tested at its 8th generation using a 30×30×30 cm cage.

In order to examine tolerance of the eggs to desiccation, eggs deposited within 12 hours were completely desiccated on filter paper and then their hatchability was checked. For the comparison, hatchability of the eggs which were kept at 90% relative humidity was observed. Two hundred eggs were tested a day.

Experimental results

The insemination rates are shown in Table 3. Only slight differences between the rates obtained in the two kinds of small cages of the same generation of *A. sinensis* were found, but the insemination rates of *A. sinensis* were raised by about 20% in each small cage between the 31st and the 41st generations.

Blood-fed females began to deposit eggs two days after each blood meal, and repeated

Table 3 Insemination rates using by natural copulation in *Anopheles sinensis* and *A. lesteri*

Species and generation	Cage* used	No. of females	No. of males	No. of females dissected	Insemination rate (%)
<i>A. sinensis</i>	A	500	500	100	16
Kanoya F ₃₁	B	250	250	50	18
	A	500	500	100	41
Kanoya F ₄₁	B	250	250	50	42
<i>A. lesteri</i> Yakumo F ₈	A	500	500	100	8

* A: 30×30×30cm cage

B: 20×20×20cm cage

their oviposition several times. The numbers of eggs laid the second and third times were greater than the first time, and decreased afterwards; the number of the eggs deposited at the second time in case of a $30 \times 30 \times 30$ cm cage was about 10,000 to 15,000 and hatchability was 92% in the 41st generation.

The results of tolerance of the eggs to desiccation are shown in Fig. 1. It was

Fig. 1 Results of tolerance of eggs to desiccation using *Anopheles sinensis* Kanoya F₄₂

A: Eggs desiccated completely. B: Eggs kept at 90% relative humidity.

found that the eggs hatched normally after 2 days of desiccation but the hatchability decreased down to 29% after 4 days with no hatch occurring beyond 5 days. On the other hand, 70% of the eggs kept at 90% relative humidity for 6 days hatched and a hatchability of 23.5% was observed even after 10 days.

Discussion

Difficulties in colonization of *A. sinensis* in the laboratory might be due to at least two factors; one was that adults were not capable of copulation in the small cage and the other was that they died easily in low humidity. It was very difficult to maintain the strain by induced copulation in our laboratory in the early generations, especially in winter because the humidity was not controlled, but the adult mortality decreased after the humidity could be controlled.

A cooler box was very useful for transportation of the female mosquitoes to the laboratory, especially from a long distance since the lower temperature prevented the hatch or development of eggs deposited by some blood-fed females. It was possible to carry alive adults without many deaths in a paper box in which sufficient humidity was maintained; but during transportation of the mosquitoes in this way for more than 3 days they require another blood meal in transit.

Tap water was used at first for larval rearing, but thereafter we switched to de-ionized water for the sake of safety. Rearing water was not changed in most of the pans during larval development. Finely ground mouse pellets gave good results for larval growth as did oatmeal; the males which emerged from good larval rearing conditions were capable of being used for induced copulation for more days; almost all the males of this kind, even up to 10 days old, were able to be utilized for induced copulation. On the contrary, the males produced by insufficient rearing condition such as undernourishment or overcrowding could mate only for a limited time or even could not.

Hatchability of the eggs obtained from the hand-copulated females was very different from the naturally inseminated females; the former was 20% and the latter 92%. Judging from observation of the sperm in the spermathecae ejaculated by the induced copulation, the males appeared to have accomplished incomplete insemination; therefore, after the 21st generation of the Kanoya strain, two or more males were used for copulation for each of the females of this strain; the hatchability increased to about 50%. On the other hand most of the spermathecae of the freely mated females in the cage in the 41st generation of the Kanoya strain were filled with sperm and not a case was found in which an insufficient amount of sperm was in the spermathecae, although the insemination rate was about 40%. From this fact it seems that by natural copulation in the cages spermatheca could be filled with many sperm not by multiple mating, but by only one mating.

However, in the 31st generation of the same strain only a few of the freely mated females had the spermathecae filled with sperm and there were lesser amounts of the sperm in the spermathecae in the other females as compared with those of the 41st generation. It seems, therefore, that the amounts of the sperm ejaculated by one free copulation increased along with the rise in the insemination rate in the cage.

Tanaka and Hatoyama (1967) colonized this species in a large cage (90×72×90 cm); and after selection of eggs from the cage they finally could maintain this strain in a small cage (45×45×45 cm); a hatchability of 66% to 72% could be obtained in this small cage. This hatchability appeared to be due to the high mating rate of the strain. They initially used a rabbit as a source of blood and although the biting rate was low at first it gradually increased in subsequent generations. In the 8th generation this colony was able to take blood from guinea pigs although it refused this blood source in its first generation. Our strain showed an insemination rate of 16% at the first time after released into a small cage (30×30×30 cm) for free copulation and then the rate increased by more than 20% after ten generations. It is not reasonable to compare our data with those of Tanaka and Hatoyama (1967), since the strain used in our experiment had been maintained by induced copulation for the first 28 generations. Our strain refused guinea pig, pigeon or mouse as a blood source in its early generations; the strain also needed a long period of time even for taking human blood until the 20th generation; in the 27th generation it showed a blood sucking rate of about 60% for mice, and therefore mice have been utilized for blood sources. Finally, nearly 90% of the mosquitoes took blood from mice at the 35th generation. At present about 200 pupae per day are collected for one week and a mouse is supplied overnight for the adults every second day after the 10th day in the cages. Oviposition is observed 2 days after the blood meal.

The experiments of Tanaka and Hatoyama (1967) and those of the present authors suggest that after the biting rate in the early

generations reaches to a certain level with the animal which the mosquitoes prefer as a source of blood, we may change the animal to another which is perhaps more handy but which the mosquitoes do not prefer originally as a blood source. As a matter of fact human blood probably could have been replaced by mouse blood in the earlier generations of our strain sooner that it actually was.

A. sineroides and *A. koreicus* were maintained by induced copulation for several generations but eventually were discontinued since to maintain many species of anopheline mosquitoes requires much labor. When colonization of the mosquitoes has to be started from a very few individuals, which perhaps must be collected at a distant locality, the induced copulation method is a good and effective technique for increasing the numbers of individuals, since an increase in numbers is essential for a freely mating strain of anopheline mosquitoes in a small cage. There are, of course, several species of anopheline mosquitoes including *A. nigerrimus* and *A. pseudopictus* which can copulate in small cages with few individuals even in the initial generations without need of induced copulation.

The Yakumo strain of *A. lesteri* being maintained in our laboratory bites man or mice more readily than does *A. sinensis*. After the Yakumo strain was maintained by induced copulation for the first 6 generations, it has copulated freely in a 30×30×30 cm cage, showing an insemination rate of 8% in 11th generation. This strain is now in its 15th generation, naturally copulates, and feeds freely on man or mouse.

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摘 要

Anopheles sinensis の実験室内飼育

1970年7月鹿児島県鹿屋市の豚舎、牛舎内にて吸血蚊を採集した。この吸血蚊の翅を半分以上ナイフで切断し、湿った濾紙をしいた密閉容器（径6cm、深さ3cm）に5～6頭ずつ移した。3日後に産卵が見られ、卵を白色ポリビニール樹脂バット（40×25×6cm）へ移した。この際バットの内側に幅約4cmの濾紙をはりつけておいた。水は脱イオン水を使用した。幼虫には微粉にしたドライ・イースト、小麦胚芽、オートミールを等量混合した餌を若令期には1日1回、3～4令期には2回水面に浮くように与えた。羽化後3日目の雌を人の手から吸血させ、雄と強制交尾を行ない次代の卵を得た。実験室は25±1°C、70～80%湿度である。この方法で27代まで飼育され、その後ケージ内での自然交尾、マウス吸血への移行を試みたところ成功した。雄の交尾能力は羽化後3日目が最高であった。卵の耐乾燥性について調査したところ、孵化率は2日目までは正常であったが、4日後には29%に減少した。