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Laboratory colonization of *Anopheles pseudopunctipennis* (Diptera: Culicidae) without forced mating

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Abstract

Anopheles pseudopunctipennis is one of the main malaria vectors in the Andean regions of South America. Few experimental data exist on this species because it is not very available in laboratories due to its eurygamic status that makes colony maintenance difficult. Indeed, individuals do not mate in the confined space of insectary cages. To avoid this problem, forced artificial mating can be used. However, this technique is time consuming, requires a well-trained technician, and is inadequate for easy mass production, which is sometimes necessary for certain experimental works. This study presents a technique based on exposure of adult mosquitoes to a blue stroboscopic light for 20 min during several nights, which encourages them to copulate naturally under laboratory conditions. After some generations, a self-free-mating strain was obtained. The technique is simple, inexpensive and is probably effective whatever the *An. pseudopunctipennis* strain considered. ***To cite this article: F. Lardeux et al., C. R. Biologies 330 (2007).***

Résumé

Colonisation en laboratoire de *Anopheles pseudopunctipennis* (Diptera : Culicidae) sans copulation forcée. *Anopheles pseudopunctipennis* est l'un des principaux vecteurs du paludisme dans les régions andines d'Amérique du Sud. Peu de données expérimentales existent pour cette espèce, car elle est peu disponible en laboratoire en raison de son statut eurygame, qui rend son élevage difficile. En effet, les sexes ne s'apparient pas naturellement dans les cages d'insectarium. Une manière contourner ce problème est de forcer artificiellement l'accouplement. Toutefois, cette technique est lente, nécessite l'intervention d'un technicien particulièrement entraîné et n'est pas adaptée à la production de masse d'insectes, qui est parfois nécessaire pour certaines recherches expérimentales. Cette étude présente une technique basée sur l'exposition des moustiques adultes à une lumière stroboscopique bleue durant une vingtaine de minute pendant plusieurs nuits, qui les incite à copuler dans les conditions du laboratoire. Après quelques générations de ce traitement, une souche qui se reproduit seule et naturellement a été obtenue. La technique est simple, peu chère et est vraisemblablement performante quelle que soit la souche *d'An. pseudopunctipennis* considérée. ***Pour citer cet article : F. Lardeux et al., C. R. Biologies 330 (2007).***

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1. Introduction

The mosquito *Anopheles pseudopunctipennis* Theobald is the most widely distributed anopheline mosquito in the New World and occurs from southern USA (south of 40°N) to northern Argentina and Chile (30°S) along the Andes, with an eastern extension to Venezuela and the Lesser Antilles [1]. It is the most important malaria vector in the foothills and mountainous areas (up to 2800 m) of its distribution range [2].

The ecology of the species and its relations to malaria transmission have been studied to some extent in the field, but few experimental data exist because of the difficulties in establishing laboratory colonies and obtaining mosquitoes in sufficient number. *An. pseudopunctipennis* is eurygamic and does not mate in the confined space of laboratory cages because it needs swarming to induce its mating behaviour [3]. As such, experimental studies have been limited to artificial cross mating for genetic experiments [4,5] or susceptibility to malaria parasites [6]. When mating was needed, mosquito copulation was artificially induced, forcing each female to mate with males by manipulating them under the stereomicroscope [7–10]. Forced mating is a tedious and time-consuming technique that needs a well-trained technician. It may be adequate to maintain small laboratory colonies during a limited period, but it is inappropriate for long-term mass production.

Few references exist on the adaptation of *An. pseudopunctipennis* to the laboratory environment [11–13]. First attempts to maintain this species in insectary met little success and did not last more than a few months, although forced mating was used [14,15]. To obtain free-mating colonies and avoid the use of forced mating, modifications of insectary conditions such as exposure of the mosquitoes to cycles of low-intensity light (red or blue) and/or rearing of adults in large-capacity cages were proposed. In such conditions and without using forced copulation, at least 40 generations of a Panama strain of *An. pseudopunctipennis* were produced [16], but other strains failed [17], or were reared with many difficulties [18]. Other insectary refinements were proposed, such as the use of mud for oviposition [19], which seemed to induce natural insemination in *An. pseudopunctipennis* [17]. However, this stimulus only provoked natural pairing in the first generation [17]. More recently, exposing the mosquitoes to a light beam and a drop in temperature simulating natural sunset conditions proved to be successful for selecting a stenogamic colony from a Mexican strain of *An. pseudopunctipennis* [20], but this complicated technique has not been tried elsewhere since then.

To carry on with experimental work on *An. pseudopunctipennis*, a simple and efficient rearing protocol is urgently needed. The present study claims to present the successful colonization of that species without forced mating, at the Medical Entomology Laboratory of the 'Instituto Nacional de Laboratorios de Salud' (INLASA) in La Paz, Bolivia, using a simple technique that, unlike others, may be easily implemented and is likely to work with all the strains considered.

2. Rearing protocols

2.1. Larvae

Eggs deposited on filter paper are immersed in 2 l of water at the insectary temperature (27 °C), in a plastic tray of 33 × 22 × 5 cm, so that about 500 L1 larvae may hatch. About two days later, larvae are separated in various trays at densities of ∼200 larvae/tray. They will stay there until pupation. Food is provided daily and consists of one meal for L1 larvae, made of finely grounded tropical fish-food flakes mixed with ∼10% yeast, and two meals for the others instars. At 26–27 °C, eggs hatch in 1–2 days, the first larval stage lasts 1–2 days, and the next three ones ∼2 days each. First pupae appear at day 9–10 and obviously are not fed.

Pupae are collected with pipette and placed in small bowls in 30 × 30 × 30-cm mosquito cages where adults emerge ∼2 days later.

2.2. Adults

Adults are supplied with cotton wool soaked in a 10% sucrose solution and maintained in controlled conditions at 27 °C, 70% relative humidity and a 12:12 h day:night photoperiod. Day light is a weak blue light.

2.2.1. Forced-mating technique

After emergence, ∼250 adults are transferred in 4-l plastic pots, whose walls are covered with paper, en-

abling the mosquitoes to pose easily. Females are blood fed on rabbit (30-min to 1-h exposure) for two successive nights if the first night is not fully successful. The mosquitoes remain one day in the pot, enabling the females to begin digestion and excrete faeces following alimentation. Then, the forced mating is carried out following [7,8], and modified as follow. Four- to seven-day-old males are slightly anaesthetized to a 'knock-down' state in a 60-ml hermetic glass tube supplied with cotton soaked with 3 ml diethyl ether (∼20 s exposure or less), and are immediately pinned laterally in the thorax with a minutin needle inserted in a 15-cm-long wooden stick. They are left 2–3 min until recovery (their legs begin to move again). Then they are decapitated and their legs are pulled off. When decapitation is delayed, mosquitoes survive longer and may be prepared up to 30 min prior to processing. A series of 10–15 decapitated males may be prepared at a time so that if one male is not responsive for copulation, others are immediately available. Virgin females are then slightly anaesthetized to the point of relaxation using the same technique as for the males (a series of 5–10 females, depending on the ability of the technician to practice the forced copulation technique) and put ventral side up below a stereomicroscope. Using the wooden stick, the male is brought close to the female, at an angle of ∼90°. Touching the female's genitalia with the male's claspers several times stimulates it, as evidenced by the movement of its claspers and abdomen. Correctly positioning the male will result in clasping and copulation. The confirmation of mating is that when the stick with the male is raised, the female is firmly attached. The mosquitoes remain tightly joined for 5–20 s, enabling the operator to transfer the female in a separate cage before the male withdraws. If a male is not responsive, the female may be offered to another male of the series. A male may copulate with two or three females and may remain functional 5 to 10 min before dying. It is best to wait a little between successive attempts with the same male. Inseminated females are left two days in their cage, until complete blood digestion. Then, they are put in oviposition vials individually or in groups of two to three individuals. Oviposition vials are plastic vials of ∼100 ml, in which cotton soaked with water and covered with filter paper is used to collect the mosquito eggs. Females lay eggs for two days and filter papers with eggs are immediately put in water for egg hatching and larvae rearing.

2.2.2. *Protocol to induce natural mating*

To get rid of the preceding forced-mating technique, and obtain a free-mating colony that could be reared using standard insectary protocols, natural mating is induced as follow. After emergence, males and females are exposed to a stroboscopic blue light (Skytec model 2505, 20 W) for 30 min, and for seven consecutive days at the beginning of the night cycle when the insectary lamps are turned off. The strobe gave 140 flashes per minute, with each flash lasting ∼0.2 s. The blue filter was ∼470 nm. In each cage (standard mosquito cage of 30 × 30 × 30 cm), about 1000 adults (500 males and 500 females) are used. At days 6 and 7, a mouse is provided for female blood feeding inside the cage. At day 8, a complementary blood meal may be provided if necessary. Unfed females are discarded, the others being left there until complete digestion and egg laying. After several generations of such a treatment, a self-free-mating strain that copulates without the stroboscopic light stimulus is obtained (see results) and can be reared following simple standard protocols for *Anopheles* rearing, as detailed in [13].

3. Results

Under the insectary conditions, the development time of one generation (from eggs to eggs) is about three weeks.

With the forced-mating technique, all the Bolivian strains of *An. pseudopunctipennis* captured in the field were successfully maintained in our laboratory. These strains came from various localities from all over the distribution range of *An. pseudopunctipennis*, with different environmental conditions: Mataral (Cochabamba) (S18.60, W65.14, alt. 1500 m), Novillero (Cochabamba) (S18.28, W65.22, alt. 2240 m), El Chaco (Chuquisaca) (S18.89, W65.11, alt. 1900 m), Corpus Cristi (Caranavi, La Paz) (S15.84, W67.54, alt. 650 m), Teoponte (La Paz) (S15.48, W67.81, alt. 420 m), El Barrial (Tarija) (S21.56, W63.56, alt. 600 m), Caiza (Tarija) (S21.79, W63.55, alt. 574 m), El Saladito (Tarija) (S21.31, W64.16, alt. 950 m), among others. None of these strains failed in laboratory colonization; and were voluntarily stopped when needed. The oldest one, the Mataral strain, has been reared in our laboratory since November 2002 and to date has reached the F60 generation. In general terms, a single male may copulate with several females, but success in insemination varies. An experiment made with one male mating with three successive females showed that the first mating led to 70% of fertilized females, the second to 90% and the third to 40%. Best results were obtained with the second mating, when males were free of the anaesthetic effect of ether (or chloroform), not weak and still harbouring high spermatozoid quantities. Colonization of

other species of Bolivian *Anopheles*, especially within the *Nyssorhynchus* subgenus, was attempted using the same forced mating technique. These species were *An. albitarsis*, *An. argyritarsis*, *An. darlingi*, *An. rangeli*, *An. triannulatus*, and *An. trinkae*. None of these species was successful. Although the males were able to clasp the females and apparently copulate, there was no insemination, as confirmed by the observation of empty spermathecae and the absence of egg laying.

In the 'natural-mating' experiment, the El Chaco strain was used. This strain has been raised in the laboratory with the forced-mating technique for more than 50 generations. The parental generation (F0) for the 'natural mating' experiment came from this laboratory force-mated colony. To date, and using the 'natural-mating' protocol, the El Chaco strain has reached the F9 generation. At various generation times, insemination rates were determined by examining the spermathecae of females. In the parental generation, as well as in other strains from the insectary, no spermatozoid was observed, indicating that without the use of the blue flashing light, mating did not occur naturally. With the blue flashing light stimulus, the F1 generation of the El Chaco strain exhibited an insemination rate of 21% (17 females with positive spermathecae/82 dissections). The F2, F3, and F4 generations exhibited insemination rates of 39% (19:48), 28% (27:95), 36% (13:36), respectively. To test whether the strain was self free mating after some generations of stroboscopic treatment, the F7 generation was divided into two groups, one of which was exposed to the blue stroboscopic light to induce mating as in the preceding generations, the other not. The two groups exhibited an insemination rate of 15% (6:39) and 14% (8:57), respectively, indicating that the El Chaco strain was now able to mate without the help of the blue flashlight stimulus. The same experiment was carried out with the F8 generation, and gave similar results, with insemination rates of ∼40%.

4. Discussion

The forced-mating technique is widely used to rear mosquitoes, in particular *Anopheles* [13]. Unlike with *An. pseudopunctipennis*, it may help in selecting self-copulating strains after several forced mating cycles [21]. Nevertheless, with the forced mating technique, several Bolivian strains of *An. pseudopunctipennis* are maintained in our laboratory in La Paz (Bolivia) at an unusual altitude of 3600 m far from optimal rearing and natural conditions (the field limit for that species is ∼2800 m).

If *An. pseudopunctipennis* can successfully be reared with forced mating, that was not the case with the tested species of the *Nysshorynchus* subgenus. In the *Nysshorynchus* species, the male claspers are larger than those of *An. pseudopunctipennis* (*Anopheles* subgenus), and although the male clasps the female and appears to copulate, the introduction of the male aedeagus in the female bursa inseminalis and insemination are likely to be problematic when forced mating is used.

Rearing mosquitoes is one of the tedious tasks in an entomology laboratory. Ways to reduce the time and effort spent on the insectary are always sought. In that sense, using a time-consuming and only moderately effective forced-mating technique to maintain colonies is a barrier to some experimental research. Unfortunately, obtaining self-free-mating strains of eurygamic species is always a challenge because of their particular mating behaviours, which are difficult to realize in insectaries, like swarming. Swarming is common amongst mosquito species and in *Anopheles* in particular [22]. This behaviour is likely an important component of mating process and is probably what makes establishment of free-mating colonies of most of the important South American malaria vectors so difficult. Mosquitoes might not swarm in the confined space of the laboratory cages. To permit swarming to some extent (and thus 'natural' mating), large cages have been proposed, with some success for some *Anopheles* species. However, even placed in large cages, *An. pseudopunctipennis* needed modifications of environmental conditions to mate [16,20], in particular simulation of sunset, which is not easily feasible in all insectaries. Moreover, the use of large-capacity cages may be inconvenient for routine use or for rearing several strains in small insectaries. Our attempts to use large cages (100 × 80 × 60 cm) were unsuccessful because of the death of the mosquitoes after a few days. Simulating sunset to increase sex encounters gives one cue to mating: light may be one stimulus. The use of a blue flashlight was very successful and since the experiments with the El Chaco strain, success has also been also obtained with the Mataral strain and with a field strain from El Chaco. The use of a stroboscopic light to induce copulation has been suggested in our laboratory by the involuntary observation of copulations in the cages during experiments carried out on mosquito wing movements and using the stroboscopic light to slow down the apparent movements of the wings. This appeared strange because usually mating does not occur easily under standard insectary conditions [3]. Since, free copulations were also observed in the 4-l pots not used in the 'natural mating' experiment, but involuntarily exposed to the flashlight.

Insemination rates obtained with the stroboscopic light during the experiment were smaller than those with the environmental condition modification technique were [20]. However, at each generation time, the rate was large enough to permit mass rearing of the mosquito. Fluctuations in the insemination rates with the stroboscopic light (range 14–40%) are due to the physical condition of the insects: well-fed larvae give strong adults that mate more easily, especially if they are under calm conditions during the flashlight stimulation.

Unlike other techniques such as [20], the use of the stroboscopic light may work well whatever the strain exposed. As a preliminary proof, all the Bolivian strains from our insectary are at present reared successfully with the 'stroboscopic technique', and it is likely that strains from other countries may succeed as well.

5. Conclusion

The use of a stroboscopic blue light permitted the easy and rapid development of a self-mating colony of *An. pseudopunctipennis*, without modifying the standard rearing conditions in the insectary (27 °C, 12h:12 h day:night photoperiod, 70% relative humidity). The role of such stimulus in inducing mating is still unclear, but seems to be a simple and effective substitute for the complex processes leading to mating in eurygamous species, in particular swarming. More research is needed to understand better the role of light in the induction of mating in such species.

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