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# LABORATORY STUDIES OF *OCHLEROTATUS SAMOANUS* IN ASSOCIATION WITH LEAF AXILS OF *FREYCINETIA* (PANDANACEAE) IN SAMOA

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ABSTRACT. Three species of *Freycinetia* (Pandanaceae) (*F. reineckei, F. storkii,* and *F. hombronii*) were tested for oviposition preference of *Ochlerotatus samoanus,* a vector of filariasis in Samoa. Laboratory tests indicated that *F. reineckei* was preferred by this mosquito for oviposition. Eggs were preferentially deposited on a peeled or a moist dried leaf. The percentage hatch was highest when eggs were kept moist for longer than 4 days before submersion. Hatching was complete in less than 6 h. These studies allowed us to successfully rear *Oc. samoanus* in the laboratory, facilitating future studies on the biology and control of this important vector.

**KEY WORDS** *Ochlerotatus samoanus, Freycinetia,* colonization, filariasis, Samoa

## INTRODUCTION

*Ochlerotatus samoanus* (Gruenberg), a sibling species of *Ochlerotatus oceanicus* (Belkin) and *Ochlerotatus tutuilae* (Ramilingam and Belkin), is an important vector of subperiodic bancroftian filariasis in the Samoan islands (Ramalingam and Belkin 1964). Despite its importance as a vector, information on the ecology and bionomics of the species is very scanty.

Larvae of *Oc. samoanus* are known to develop in leaf axils of plants belonging to the family Pandanaceae, mainly *Freycinetia,* a climber on forest trees, and *Pandanus,* the screwpine (Ramalingam 1968). *Freycinetia,* a genus of 180 species of lianas, ranges from French Polynesia to Sri Lanka, and is a prominent feature of the forests of Samoa, Tonga, and Fiji (Cox 1990). Preliminary studies in inland villages where large numbers of *Oc. samoanus* invade homes at night implicated *Freycinetia* as the primary mosquito breeding site. Biting densities in these villages commonly exceed 200 bites/man-hour (Uchida et al., unpublished data).

Three species of *Freycinetia* are found in Samoa (Martelli 1934, Stone 1965): *Freycinetia reineckei* Warburg, *Freycinetia storkii* Seem (syn. *F. samoensis* Warburg), and *Freycinetia hombronii* Martelli. All 3 species are climbing lianas on forest trees and are characterized by an unwhorled tristichous leaf arrangement, dioecy (Huynh and Cox 1992), spicate inflorescences, and females that produce numerous berries in an aggregate fruit. *Freycinetia reineckei* differs distinctively from the other 2 species in some morphological characteristics. Both *F. storkii* and *F. hombronii* produce inflorescences on lateral, drooping branches, which shed rather than collect rainfall. In contrast, *F. reineckei* has sympodial growth of hapaxanthic axes, with each axis terminated by an inflorescence. Shoots develop from buds located in the axil of a leaf beneath the inflorescence. Thus, *F. reineckei* has an erect orientation that leads to rainfall accumulation in leaf axils, similar to the capture of rainwater by many species of *Pandanus.*

*Freycinetia reineckei,* also in contrast to *F. storkii* and *F. hombronii,* has large well-developed auricles (ear-shaped flanges at the base of the leaves). These auricles connect the basal part of the leaf to the main axis, forming a small reservoir or basin at the axil of each leaf. Because the leaves have an upward inclination in the 1st half of their length, with the concave erect portion of the leaves serving as miniature funnels, they form conduits for the accumulation of rainfall at the leaf bases. The auricles of *F. storkii* and *F. hombronii* are much smaller and do not contribute to the formation of a reservoir at the leaf axil. Because of these morphological differences, only *F. reineckei* apparently is capable of storing sufficient water in leaf axils for mosquito larval development. Field surveys on the islands of Savai'i and Upolu, Samoa, commonly found mosquito larvae and pupae in leaf axils of *F. reineckei,* but never in the axils of *F. storkii* or *F. hombronii* (Uchida et al., unpublished data).

Laboratory colonies of *Oc. samoanus* were needed to better understand its biology and the epidemiology of filariasis in Samoa. Initial attempts to colonize *Oc. samoanus* in the laboratory by using ordinary protocols for rearing aedine mosquitoes were not successful because gravid females would not readily lay eggs on materials such as wet filter paper or paper towels. Therefore, obtaining eggs was difficult and, accordingly, the number of emerged adults was insufficient to produce successive generations in the laboratory. We decided to use leaves of plants of the family Pandanaceae as oviposition-inducing materials in the laboratory.

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Table 1. Oviposition preference of *Ochlerotatus samoanus.*

| | Number of eggs laid | | | | | |
|---|---|---|---|---|---|---|
| | Trial 1 ($n$ = 2,048) | | | Trial 2 ($n$ = 1,387) | | |
| Plant species | Leaf blade | Auricle | Total | Leaf blade | Auricle | Total |
| *Freycinetia reineckei* | 48 | 1,332 | 1,380 (67.1%) | 80 | 944 | 1,024 (73.8%) |
| *Freycinetia storkii* | 11 | 277 | 288 (14.1%) | 13 | 73 | 86 (6.2%) |
| *Freycinetia hombronii* | 14 | 233 | 257 (12.5%) | 187 | 90 | 277 (20.0%) |
| *Pandanus* sp.[1] | 123 | — | 123 (6.0%) | — | — | — |

[1] *Pandanus* sp., which has no auricles, was examined with others only in trial 1.

Here we report on that study and describe effective techniques for rearing *Oc. samoanus.*

## MATERIALS AND METHODS

Preliminary field and laboratory studies on Upolu, Samoa, indicated that female *Oc. samoanus* laid most of their eggs on the proximal, rather than the distal, portion of *Freycinetia* leaves. Therefore, only that portion of the leaf was used in these studies.

To determine if mosquitoes had a host plant species oviposition preference, a leaf from each of the 3 species of *Freycinetia* (*F. reineckei, F. storkii,* and *F. hombronii*) with intact auricles was each placed in a 50-ml beaker with water filled up to the midpoint of the auricles. For comparison, the basal end of a single leaf of the common pandan, *Pandanus tectorius,* also was placed in another beaker during the 1st replication of this study. The beakers were placed equidistant from each other in a cage (30 × 30 × 30 cm) containing 100 gravid *Oc. samoanus.* Leaves were removed from the cage and eggs were counted after 48 h. This study was repeated twice with fresh leaf material and female mosquitoes.

Leaf surface preference for oviposition was examined with 6 types of *F. reineckei* leaves. These were middle of a fresh leaf, middle of a dry leaf, fresh leaf with epidermis peeled off, proximal end of a young leaf with green auricles, proximal end of a mature leaf with withered brownish auricles, and proximal end of a mature leaf with withered auricles removed. Leaf sections were approximately 11 cm long. The leaves were arranged in a 50-ml beaker with water inside a cage with 100 gravid *Oc. samoanus.* Eggs were counted after 48 h. This experiment also was repeated twice with fresh leaf material.

The effect of wetting and drying of eggs on hatching was studied in the following manner. *Freycinetia reineckei* leaves bearing freshly laid *Oc. samoanus* eggs were placed on wet filter paper in a petri dish (10-cm diameter), with the addition of small quantities of water from time to time to keep the eggs moist without submerging them. Other leaf sections bearing eggs were dried at room temperature and kept in open air. The wet and the dried eggs then were submerged in water for hatching at intervals of 1, 2, 3, 4, 5, 6, and 11 days (the wet eggs), and 2, 6, 11, and 13 days (the dried eggs). Three pieces of egg-bearing leaves were submerged immediately after egg deposition. To study hatching under more natural conditions, leaves bearing eggs were held in a beaker with water added regularly to maintain the level prevailing at the time of oviposition. In each trial, the number of hatched larvae was counted daily. Because of time limitations, we could not conduct sufficient trials in some experiments to provide statistical validity, and therefore only those data comprising 3 or more trials were subjected to statistical analysis (see Table 3).

## RESULTS

### Oviposition preference of *Oc. samoanus*

Almost 73% of the eggs were laid on *F. reineckei,* suggesting that it was the preferred plant for oviposition (Table 1). On *Freycinetia* leaves (all 3 species), almost all (89%) the eggs were located in the grooves, slits, and depressions of the wet withered areas of the auricles. Very few eggs were laid on *Pandanus* leaves, which lack auricles. On *Pandanus* leaves, eggs were found along the grooves between leaf veins close to the water surface.

When the *F. reineckei* leaves were artificially treated, the greatest numbers of eggs were laid on freshly peeled leaves (Table 2). Most eggs were laid on depressions or grooves between leaf veins exposed by this treatment. Large numbers of eggs also were laid on dried leaves and intact leaves with mature auricles, both of which also offered pronounced grooves for oviposition. In the former, the eggs were deposited on the wet area of the leaves 0–3 cm above the water, and in the latter, the eggs were mostly limited to the edges of the auricles,

Table 2. Preferred surface of *Freycinetia reineckei* leaves for oviposition of *Ochlerotatus samoanus*.

| Leaf surface | No. eggs laid | |
|---|---|---|
| | Trial 1 ($n$ = 1,986) | Trial 2 ($n$ = 959) |
| Fresh leaf blade | 1 (0.05%) | 12 (1.3%) |
| Dried leaf blade | 430 (21.7%) | 219 (22.7%) |
| Peeled leaf blade | 933 (47.0%) | 474 (49.4%) |
| Proximal portion of young leaf with green auricles | 98 (4.9%) | 63 (6.6%) |
| Proximal portion of mature leaf with brown auricles | 326 (16.4%) | 132 (13.8%) |
| Proximal portion of mature leaf with brown auricles excised | 198 (10.0%) | 59 (6.2%) |

where the tissue had already withered and was kept wet by absorbing water. This is probably similar to field conditions. In contrast, very few eggs were laid on fresh green leaves or the proximal end of leaves with young green auricles. Both of these are fairly waxy and have few grooves or other types of surface relief to provide locations for eggs to be laid. Removal of mature auricles also reduced oviposition rate compared with the intact mature auricles.

### Effect of wetting on egg hatch

Significant ($P < 0.05$) differences were found between the treatments in percentage of egg hatch (Table 3). Under different conditions of drying and wetting of eggs, the percentage hatch was highest when eggs were kept moist for longer than 4 days after oviposition before submersion. Hatching was very synchronous, with 80% of hatching occurring within 6 h of submergence in water. Submerging eggs immediately after laying or keeping the eggs moist for only 1–2 days resulted in low rates of hatching, delayed the onset of hatching, and prolonged the hatching period. Allowing the eggs to dry immediately after oviposition seemed to be fatal to the embryos, although 34% of eggs kept dry for 2 days still hatched. When the eggs were maintained under simulated field conditions, wide variation occurred in percent of egg hatch (10.7–84.4%; Table 4). Hatching was delayed and less synchronous relative to eggs held in laboratory conditions, averaging 7 days (range 4–13 days) for the commencement of hatching and 11 days (range 10–14 days) for 80% of hatch.

### *Ochlerotatus samoanus* colonization

Based on our studies, the following protocol has been established for rearing and maintaining *Oc. samoanus* in the laboratory. Gravid mosquitoes are provided with fresh leaves of *F. reineckei*. Eggs laid on *F. reineckei* leaves are transferred with a fine brush to wet paper towels in a covered plastic tray (10 cm wide × 15 cm long × 5 cm deep). The paper towels are kept moist for 4 days. On the 5th day, enough water is added to the tray to submerge the eggs. Freshly hatched larvae are then transferred to a rearing medium prepared by diluting stock medium with water (1:4, stock:water). The stock medium consists of a 3-day-old suspension of 1.5 g of dried powdered fish food, available locally at pet stores, per liter of water. A typical culture consists of a 25-cm-diameter glass dish holding 250 larvae in 1 liter of medium. Approximately 100 mg of dried yeast powder is added to each culture every 2 days. Pupae are collected daily and placed in 100-ml beakers containing approximately 80 ml of water and placed in a cage for adult emergence.

Adults are maintained in 20-cm cube cages. The base and 2 sides of our cages are made of hardboard. The other sides are made of plastic screening. Room temperature fluctuated between 27 and 30°C. Humidity is maintained by draping a wet towel over the cage. A fresh twig of a plant in a flask of water is placed inside the cage to provide resting sites for the adults. Adults are fed a 10.0% sugar solution on cotton wool pads that are replaced every 2 days. Mating occurs in the cages. Females are fed blood on the 4th day between 1900 and 2100 h. Females are gravid in 3 days, at which time *Freycinetia* leaves are added to the cage to provide oviposition sites. By using these techniques, *Oc. samoanus* was maintained through 3 generations in the laboratory.

## DISCUSSION

Of the 3 species of *Freycinetia* found in Samoa, only *F. reineckei* seemed to possess the morphological characteristics to provide breeding sites for *Oc. samoanus*. This was supported by the significant preference we observed of *Oc. samoanus* for *F. reineckei* as an oviposition site. This finding led to the colonization of *Oc. samoanus*, and thus studies of the distribution, population dynamics, and control of the species. The principal requirement of *Oc. samoanus* for oviposition seems to be a rough surface of grooves and slits, as exemplified by the number of eggs obtained on dried leaf surfaces. In nature, *Oc. samoanus* can find such conditions in the auricles of *F. reineckei*.

As observed for *Aedes aegypti* (L.) (Christophers 1960), *Aedes albopictus* (Skuse) (Do Si Hien 1975), and *Aedes polynesiensis* Marks (Ingram 1954), *Oc. samoanus* eggs need 4 days of conditioning on a moist surface for maturation of embryos. The auricles of *F. reineckei* provide ideal locations for this. As the leaves of *F. reineckei* mature, the distal part of the auricle withers, becomes papery, and is able to absorb water from the leaf axils. The auricles remain wet and provide moisture for the eggs

Table 3. The effect of moisture on hatching of *Ochlerotatus samoanus* eggs.

| Treatment | Total no. eggs examined ($n$ = trials) | Mean percentage of hatch (± SE)[1] | Time after submerging until start of hatch (±SE)[1] | Time after submerging until 80% of hatch (±SE)[1,2] |
|---|---|---|---|---|
| Dried (days)[3] | | | | |
| 2 | 35 ($n$ = 1) | 34.3 | 6 days | 12 days |
| 6 | 126 ($n$ = 1) | 0 | — | — |
| 11 | 200 ($n$ = 1) | 1.0 | 3 days | 3 days |
| 13 | 146 ($n$ = 1) | 0 | — | — |
| Moist[4] | | | | |
| 0 | 368 ($n$ = 3) | 21.26 ± 5.40 a | 5.33 days ± 2.30 a | 12.00 days ± 7.81 a |
| 1 | 330 ($n$ = 2) | 21.22 ± 2.25 | 6.00 days ± 2.82 | 14.00 days ± 5.65 |
| 2 | 747 ($n$ = 6) | 41.79 ± 14.30 b | 3.33 days ± 1.96 a | 7.83 days ± 1.72 a |
| 3 | 930 ($n$ = 5) | 36.08 ± 10.35 b | <1 day b | <1 day b |
| 4 | 740 ($n$ = 7) | 69.09 ± 6.88 d | <6 h b | <6 h b |
| 5 | 295 ($n$ = 2) | 90.40 ± 1.42 | <6 h | <6 h |
| 6 | 252 ($n$ = 2) | 77.32 ± 7.67 | <6 h | <6 h |
| 11 | 148 ($n$ = 2) | 78.73 ± 2.97 | <6 h | <6 h |

[1] Data obtained from 3 or more trials were statistically compared. Means followed by the same letter within a column are not significantly different (Fisher's least-significant difference $P < 0.05$).

[2] Days until 80% of the total larvae had hatched after egg submersion.

[3] Eggs were dried for 2–13 days and then submerged in water; unreplicated trials.

[4] Eggs were kept moist for 1–11 days and then submerged in water.

Table 4. Hatching of *Ochlerotatus samoanus* eggs laid on leaves of *Freycinetia reineckei* and maintained to simulate natural conditions.

| Trial no. | No. eggs | | Days from oviposition until | |
|---|---|---|---|---|
| | In batch | Hatched (%) | Start of hatch | 80% of hatch |
| 1 | 306 | 109 (35.6) | 4 | 12 |
| 2 | 112 | 35 (31.3) | 4 | 12 |
| 3 | 197 | 21 (10.7) | 4 | 10 |
| 4 | 159 | 43 (27.0) | 5 | 12 |
| 5 | 45 | 38 (84.4) | 13 | 14 |
| 6 | 78 | 50 (64.1) | 7 | 10 |
| 7 | 70 | 50 (71.4) | 7 | 12 |
| 8 | 83 | 46 (55.4) | 11 | 11 |
| 9 | 220 | 160 (72.2) | 7 | 10 |
| 10 | 320 | 253 (79.1) | 5 | 10 |
| Mean ± SE | | 53.1 ± 8.0 | 7 ± 1.0 | 11 ± 0.1 |

as long as water is present in the axils. Auricles of young leaves are covered with wax and do not retain water. The willingness of *Oc. samoanus* to oviposit on dried leaf blades means that they can be colonized in laboratories where fresh leaves of *F. reineckei* are not readily available.

In Samoa, *F. reineckei* grows only in primary rain forests or on tree remnants after logging. Before the logging era, Samoan villages, with few exceptions, were typically distant from primary rain forest, with large plantations of breadfruit, taro, and cocoyams providing a buffer between villages and *F. reineckei* populations. However, in recent years, as the Samoan rain forest has been progressively logged, small settlements have been established in the destroyed areas of the forests, with the result that human proximity to the breeding areas of *Oc. samoanus* has significantly increased. Epidemiological studies of disease incidence in villages that have cut their forests and settled near forest remnants should now be compared to those villages (Cox 1997) that have preserved their forests to see if rain forest conservation protects villagers from an increased incidence of filariasis and other diseases borne by *Oc. samoanus*.

The variation in percentage hatch and the time taken for hatching we observed in simulated field oviposition trials probably was due to uneven distribution of water in the auricles to moisten and condition the eggs. This situation probably occurs to a greater extent in nature because of the influence of sunlight, rain, and drought and may be a mechanism for regulating egg development and reducing overcrowding of larvae in leaf axils.

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