Establishing and maintaining colonies of *Forcipomyia taiwana* in the laboratory

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ABSTRACT: Successful colonies of the biting midge *Forcipomyia taiwana* (Shiraki) were established and maintained in the laboratory by feeding blood with an artificial blood-feeding apparatus, rearing larvae on a soil substrate employing algae liquid, and setting suitable mating cages. The feeding rates of *F. taiwana* fed on pig blood (69.9%) and artificial blood (72.7%) were not significantly different from those fed on human blood (67.0%). The mean numbers of adults produced by females fed on the artificial blood and the human blood were 32.0 and 33.0, respectively. The algae liquid, *Chlorella vulgaris*, was suitable for rearing larvae, with larval hatching rate, pupation rate, and emergence rate of midges fed with artificial blood and human blood meal cohorts observed as 76.0%-88.8%, 98.2%-96.4%, and 98.0-94.3%, respectively. Swarming and copulation occurred 1 h before and 2 h after the lights were turned on (07:00-10:00). The average female mating rates were approximately 50-60%, and males were observed to mate with multiple females. ***Journal of Vector Ecology* 43 (2): 328-333. 2018.**

Keyword Index: Forcipomyia taiwana, artificial blood-feeding, larvae rearing, swarming and copulation, mating rates.

INTRODUCTION

Forcipomyia (Lasiohelea) taiwana (Shiraki) is a hematophagous insect that is considered to be a nuisance. Its presence was first reported in central Taiwan by Shiraki (1913). These extremely tiny midges (1 to 1.5 mm) can easily pass through window screens, although they generally feed outdoors. Female *F. taiwana* midges attack exposed parts of the body during the day (Chuang et al. 2000, Chen et al. 1981). Although no midge-borne diseases have yet to be reported in the Taiwanese population, *F. taiwana* bites can cause intense itching and redness in sensitive individuals. *F. taiwana* is one of the most irritating blood-sucking midges widely distributed in urban and suburban Taiwan, as well as in China (Chen et al. 2005).

Although the biology and ecology of the biting midge have been well studied (Liu et al. 1964, Sun 1967, Sun 1974, Chen et al. 1979, Chen et al. 1981, Lien et al. 1988, Yeh and Chuang 1996, Chuang et al. 2000), attempts to establish and maintain large laboratory colonies of *F. taiwana* have been unsuccessful (Yeh and Chuang 1996, Chen et al. 1982, Tan et al. 1989). The maintenance of laboratory colonies for mass production is important and necessary for the experimental study of their biology, behavior, and mutual relations with disease agents and for testing new methods of pest control (Lawyer et al. 2017). Thus far, the insect has not been amenable to laboratory manipulation, and its sexual behavior has not been studied in detail. In preliminary field observations, mating occurred with the formation of male swarms (Yeh and Chuang 1996). The establishment of laboratory colonies for species such as *F. taiwana* can be challenging because mating and insemination either do not occur or require a prohibitive amount of laboratory space for success (Albeny-Simões et al. 2015).

The other problem is that blood-feeding species such as *F. taiwana* are anthropophilic and the biting cycle follows a circadian rhythm. The diurnal biting cycle of *F. taiwana* is unimodal with a peak between 13:00 and 15:00 (Russell et al. 2013). Female midges of the Ceratopogonidae will feed on a chicken, a baby mouse, or citrated human blood through a membrane prepared from the inner membrane of human placenta (Tan et al. 1989, Blackwell et al. 1994). Even when bioethical rules permit the use of immobilized or anesthetized live animals as a source of blood for biting midges (Benedict 2009, Pothikasikorn et al. 2010), their care and housing is time-consuming and expensive (Kasap et al. 2003). It is important to develop an inexpensive, convenient, and effective artificial membrane blood-feeding technique that takes animal welfare into consideration.

The aim of this study was to assess the parameters for establishing and maintaining colonies of *F. taiwana* in the laboratory. An improved technique was achieved by employing a soil substrate with algae liquid that facilitated the larval rearing and an artificial blood-feeding apparatus and blood meal for maintaining the midge colonies. We varied the amount of laboratory space in this experiment to determine when mating and insemination were successful.

MATERIALS AND METHODS

Insect collection

Adult midges were collected by aspirator from the field using the human-attractant method in Tainan. The captured *F. taiwana* individuals were placed in acrylic cages, 20x20 cm, fed on 3% honey and 5% sucrose solution from moist paper towels rolled in a vial and transferred to the laboratory. After identification, captured female midges were blood-fed using an artificial blood-feeding apparatus.

Blood-feeding

The blood-feeding device for *F. taiwana* was constructed from a glass dish with an inlet feeding unit (Figure 1A). Pig

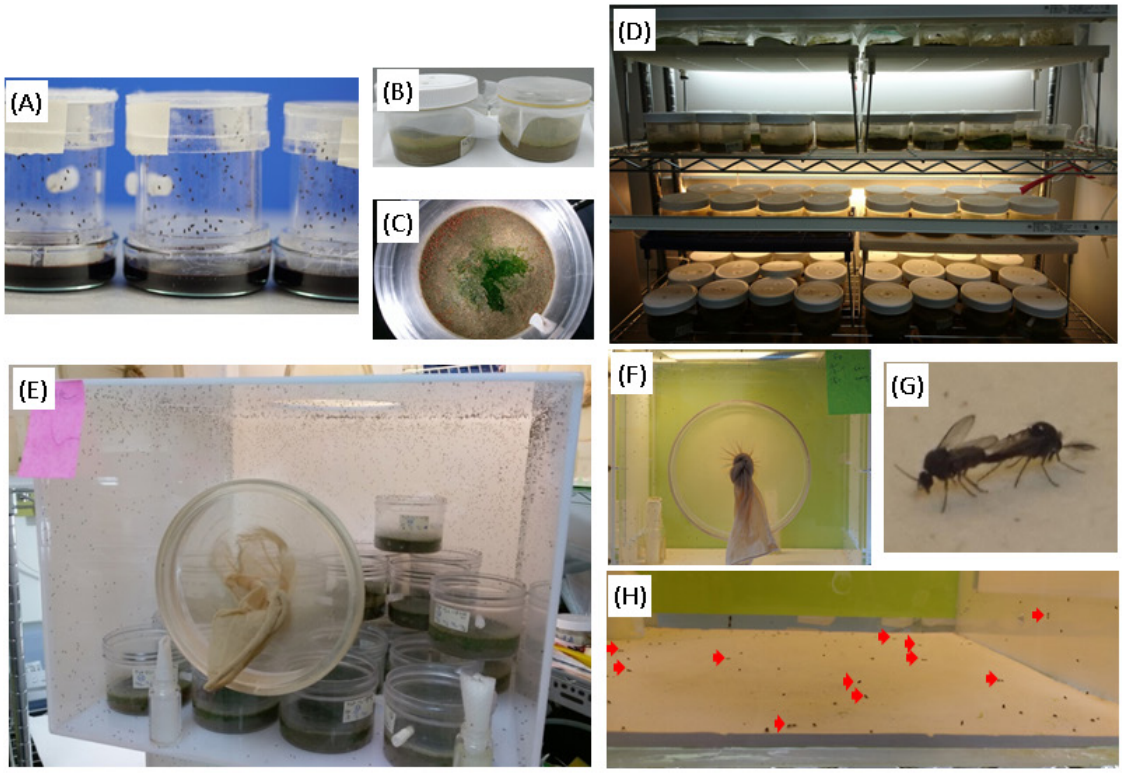

Figure 1. Methods for establishing and maintaining colonies of *Forcipomyia taiwana:* (A) Blood-feeding device was constructed from a glass dish with an inlet feeding unit. (B) Oviposition/rearing pots were made from clear plastic containers. (C) Larvae fed on algae liquid added on top of the soil substrate surface. (D) Oviposition/rearing pots were stored under standard conditions. (E) Adults emerged in the cage. (F) A custom-made acrylic cage. (G) Copulating midges. (H) Copulation occurred in the custom-made acrylic cage (arrowed).

blood was obtained from freshly slaughtered hogs with citrate phosphate dextrose (CPD) as an anticoagulant (CPD: blood = 1:7) that was freeze-dried for blood-feeding. The freeze-dried blood powder was stored at 4° C before use and 1.0 g was rehydrated in 5.0 g of deionized water that contained 10^{-3} M ATP. The glass dish containing the pig blood or artificial blood meal was placed on a hot plate preheated to 39° C. Females were captured in an acrylic tube feeding unit (45 mm inside dimension (ID) x 60 mm) that had been glued with glass fiber mosquito netting and sealed with Parafilm-M® stretched on both open sides. The feeding unit with Parafilm-M® sides was fitted into the glass dish where the liquid meal was placed to blood-feed the midges. Direct feeding was done by placing the feeding unit containing batches of 40 females on legs to allow blood-feeding through the net. The number of fully blood-fed midges was counted after 30 min. Each set of experiments was conducted at least three times. The blood-feeding rate was calculated as the number of blood-fed midges/number of midges tested x 100.

The reproductive capacity of different blood meals

Oviposition/rearing pots (ovipots) were made from clear plastic containers (82 mm inside dimension (ID) x 63 mm) (Figure 1B). Three holes were drilled in the bottom of the container for moisture regulation and one hole in the wall to inoculate the midges. The container was then filled with 75 cm^3 sterilized soil and 12 ml of water. The soil layer was tamped down using a wooden stick, and a sprinkle of algae liquid (*Chlorella vulgaris*, CHL, Freshwater Bioresource Center, National Chiyi University, Chiyi, Taiwan) was put on the surface of the soil layer. The soil substrate helped to maintain the humidity in the pot and provided a resting surface without water condensation. The pot was closed with fine gauze and a screw-on lid, which had two drilled pores. Twelve gravid females were put in the pot through the hole in the wall with an aspirator and the hole was topped with a plug. Cotton balls soaked in 3% honey and 5% sucrose solution were smeared over the screen tops of the loaded pots daily as an energy source and the pots were stored under standard conditions (26±2° C, 70±5% RH and 12:12 photoperiod) (Figure 1D). After two to three days post-engorgement, gravid females laid their eggs on the soil substrates supplied with algal liquid. The number of eggs laid on the soil increased with the height of the light intensity. If light came from a lateral direction above the soil layer, *F. taiwana* always preferred to lay eggs on the lower surface of the soil. Eggs hatched in two to three days and 1st instar larvae fed on the algal liquid added to the soil surface. With the increased growth of the larvae, a frequent supply of food material became necessary (Figure 1C). There are four larval instars and a fully grown larva is only approximately 2.7 mm long. In warm climates, larval development is completed within seven to ten days. *F. taiwana* pupated on the edge of the soil layer where the moisture levels were lower. The pupal stage typically is formed on the same site as the last larval stage,

and adults emerge in three days (Figure 1E). The fecundity of different blood meals was evaluated as the number of eggs laid, larval survival, pupation, and adult emergency. Each set of experiments was conducted with 25 replicates.

Swarming and copulation

A custom-made acrylic cage was used to contain adult *F. taiwana*. This custom-made acrylic cage (30x30x30 cm) was lined with a thin layer of plaster on the bottom, fitted with white corrugated board on the bilateral exterior side and light green painting on the back panel (Figure 1F). The screen on the top of the cage allowed for good ventilation. Upon emergence, adult midges were released into mating cages and fed 3% honey and 5% sucrose solution from moist paper towels rolled in a vial. The mating behavior (Figure 1G, 1H) of the biting midge *F. taiwana* was characterized by a number of distinct stages using laboratory-bred insects. In the first experiment, we investigated whether insect age influenced mating behavior. Three age cohorts (1-day old♀/1-day old♂, 2-day old♀/1-day old♂, and 1-day old♀/2-day old♂) were tested. The trials were conducted by releasing approximately 100 pairs of adults of distinct ages in the custom-made acrylic cage. In the second experiment, three sex ratios of *F. taiwana* were obtained by releasing 20♀/100♂, 100♀/100♂, and 100♀/20♂ adults in the custom-made acrylic cage. Mating behavior was observed for five days. For both tests, the number of mating pairs was counted from 07:00-17:00. Each set of experiments was conducted at least three times.

Statistical methods

The results were expressed as the mean ± standard error (SD). The data were analyzed with one-way analysis of variance (ANOVA) followed by the Tukey HSD test to determine the significance of differences among various groups. A p value < 0.05 was considered to be statistically significant.

RESULTS

The feeding rates of *F. taiwana* fed on pig blood (69.9%) and artificial blood (72.7%) were not significantly different from on human blood (67.0%) (Figure 2). The feeding rates were positively associated with the female mating rates. The feeding rate of field-caught females on artificial blood was up to 81.0%. While *F. taiwana* fed on pig blood, the anti-coagulated pig blood was allowed to stand vertically in a glass dish, allowing the red cells to progressively settle to the bottom leaving the clear plasma above. *F. taiwana* fed with the layered blood meal may experience an imbalance of nutrients that could affect the development of eggs. The artificial blood meal was rehydrated from the freeze-dried blood powder and homogeneously mixed during the feeding process. The composition was identical to the pig blood meal, but the physical properties of the artificial blood meal were better than those of the pig blood meal.

The reproductive capacity was determined using cohorts of adult midges (12 gravid females per trial) instead of individual female adult trials. However, it is important to note that individual differences in the engorgement and

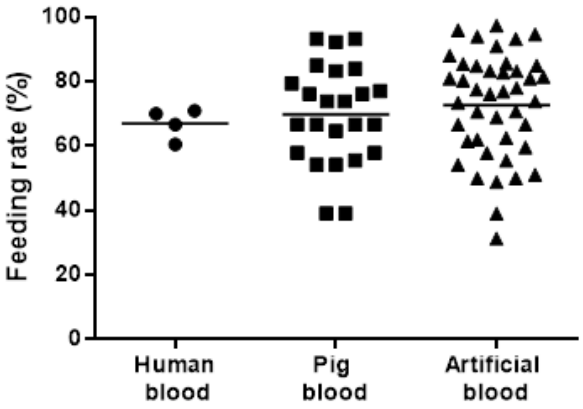

Figure 2. Average feeding rates of *Forcipomyia taiwana* on human blood, pig blood, and artificial blood. The means of feeding rates are not significantly different (P>0.05, Tukey adjustment).

feeding rates exist within the population, which may have an effect on the evaluation of the efficacy of the blood meal for the mass rearing of midges. A comparative evaluation of reproductive capacity of the female midges that fed on human blood and artificial blood was calculated as the number of eggs, larva, pupa, and adults produced by the females. The mean numbers of eggs, larvae, pupae, and adults produced by females fed human blood were 40.9, 36.3, 35.0, and 33.0, respectively. The mean numbers of eggs, larvae, pupae, and adults produced by females fed the artificial blood were 42.9, 32.6, 32.0, and 31.5, respectively. The reproductive capacity of *F. taiwana* fed on human blood and artificial blood was not significantly different (Figure 3).

Establishment of *F. taiwana* laboratory colonies can be challenging for mating and insemination which require appropriate environmental rearing conditions for success. The amount of cage space was not the main factor affecting mating success. Three sizes of plastic cages, 20, 30, and 40 cm on all sides, were used, and swarming and copulation occurred in all sizes. Other factors such as cage wall

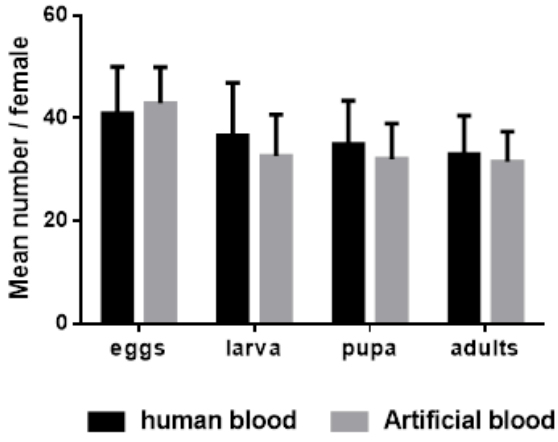

Figure 3. The reproductive capacity of *Forcipomyia taiwana* fed with human blood and artificial blood meals. The mean number of eggs, larvae, pupae, and adults are not significantly different (P>0.05, Tukey adjustment).

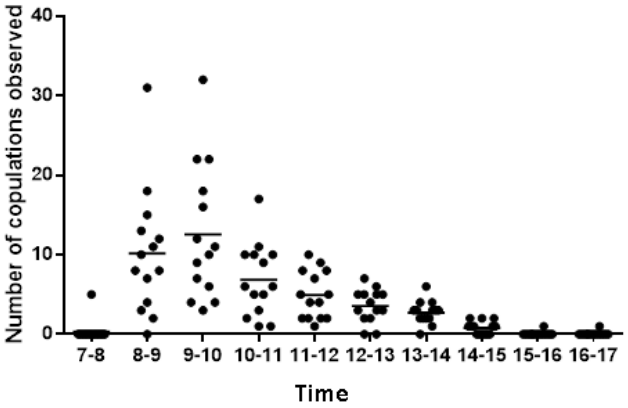

Figure 4. Number of copulations observed during 07:00-17:00.

transmittance, light illumination and shadow, sex ratio, and age affected the mating rates. A custom-made acrylic cage was lined with a thin layer of plaster on the bottom that cushioned the coupling midges' falls. The bilateral side and back panel were impenetrable to light, but the screen on the top of the cage allowed a light source to shine from above. The interior design created three-dimensional visual space that facilitated *F. taiwana* mating in the laboratory. When 100 pairs were kept in a custom-made acrylic cage, swarming and copulation occurred at 1 h before and 2 h after lights on (07:00-10:00) (Figure 4). The experiments were conducted fourteen times and copulation occurred in up to 81.2% of the pairs during this interval.

The mating behavior experiment of *F. taiwana* was divided into three age cohorts. When 100 pairs were kept in a custom-made acrylic cage, the copulation of the 1-day old♀/1-day old♂ trial occurred on the third day, the copulation of the 1-day old♀/2-day old♂ and the 2-day old♀/1-day old♂ trials occurred one day earlier. The results indicated that the 24-h-old males and 48-h-old females were receptive to mating (Figure 5).

The other mating behavior experiment of *F. taiwana* was divided into three sex ratio cohorts. The mean mating pairs of 100♀/20♂, 100♀/100♂, and 20♀/100♂ were 56.0, 56.4, and 10.4 pairs, respectively. The female mating rates were 56.0%, 56.4%, and 52.0%. The male mating rates were 270.0%, 56.4%, and 10.4% (Figure 6). The results indicated that resistance behavior is a method through which the female assesses the fitness of the male, allowing some degree of mate choice. In a previous study, mated females were very resistant to further attempts to mate by males. The male mating rate of the 100♀/20♂ cohort was 270.0%. This finding suggests that multiple mating was achieved through the persistent efforts of males.

DISCUSSION

These experiments demonstrated that feeding on artificial membranes using artificial blood can be successfully used to establish and maintain *F. taiwana* in the laboratory. Tan et al. (1989) indicated that the feeding rates on chicken, baby mouse, or citrated human blood through a membrane prepared from the inner membrane of human placenta were 7%, 65%, and 35%, respectively. A previous attempt using rat, chicken, duck, and guinea pig blood to blood-feed *F. taiwana* female adults in the laboratory failed (Yeh and Chuang 1996). Therefore, using legs as bait in the field collection and as a blood source in the laboratory was a conventional operating method (Yeh and Chuang 1996).

In field observations, as female midges were landing and feeding on the Hexiang pig's nose and skin, the idea surfaced to rear *F. taiwana* on artificial blood. The feeding rates and reproductive capacity of *F. taiwana* fed on pig blood, artificial blood, and human blood were not significantly different. The artificial blood meal was rehydrated from the freeze-dried blood powder and was homogeneously mixed during the feeding process. When feeding on the artificial feeder, biting midges pierced the parafilm-M® membrane and immediately located a blood pool (Fahrner and Barthelmess 1988). The reproductive capacity was determined using cohorts of adult midges instead of individual female midge trials. However, the evaluation of the efficacy of the artificial blood meal for the mass rearing of midges was likely interfered with because of the individual differences in the engorgement and feeding rates within the population. The experiments were conducted with 25 replicates, and approximately 300 female midges were used after the blood meal. One female of the F_0 after a single instance of artificial blood or human blood was

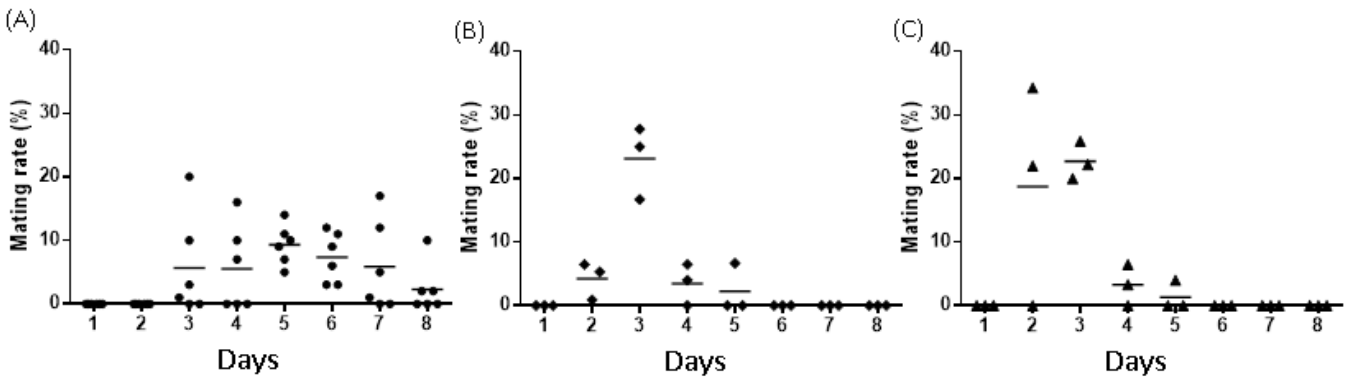

Figure 5. Age of *Forcipomyia taiwana* adults influenced mating behavior. (A) 1-day-old♀/1-day-old♂ trial (B) 1-day-old♀/2-day-old♂ trial. (C) 2-day-old♀/1-day-old♂ trial.

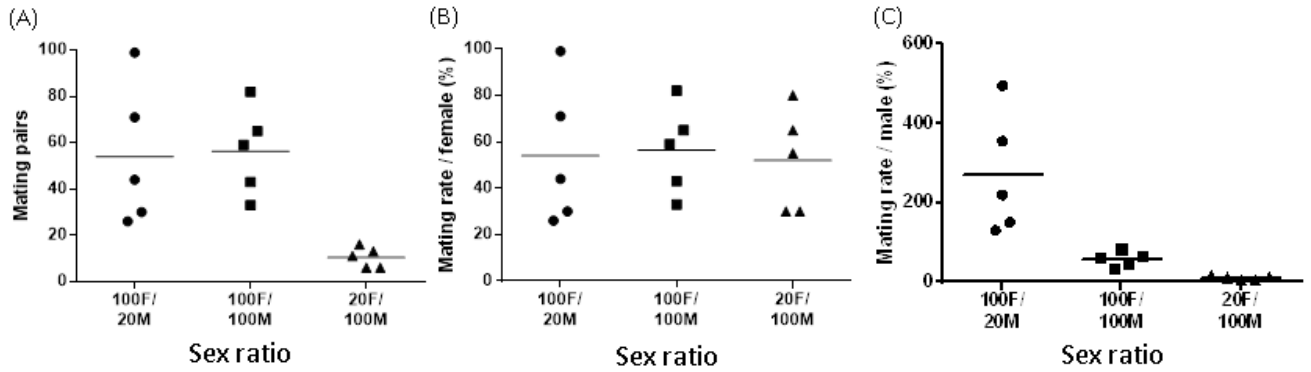

Figure 6. Sex ratio of *Forcipomyia taiwana* adults influenced mating behavior. (A) 100♀/20♂ trial (B) 100♀/100♂ trial (C) 20♀/100♂ trial.

expected to reproduce 15.8 or 16.5 females of F_1 generation. Approximately 50% of the F_1 generation was assumed to be male midges (Fahrner and Barthelmess 1988).

The larval hatching rate, pupation rate, and emergence rate of those fed with artificial blood meals were 76.0%, 98.2%, and 98.0%, and fed with human blood meals were 88.8%, 96.4%, and 94.3%, respectively. When Yeh and Chuang (1996) fed larvae with blue green algae, the pupation rate was 71.4% and the emergence rate was 80.2%. This technique was more efficient than that reported in previous studies. When using agar-based substrate or cotton substrate covered with filter paper to breed biting midge larvae, it was difficult to maintain the optimal humidity. The environmental factors seriously affected the survival rate (Liu et al. 2008). Using soil substrate for oviposition/rearing pots was a new technique that facilitated the rearing of the midges. Soil moisture content was kept constant by drilling three holes in the bottom for moisture regulation. Algae grows in soil that is always moist, and it was the best diet and most convenient rearing technique. We added the engorged females to the pots to lay eggs and the resulting larvae fed on the algae and developed into pupae. The emerged adults were released into adult rearing cages. This technical innovation will save time and labor for transferring larvae and pupae between different containers (Yeh and Chuang 1996).

The swarming and copulation of *F. taiwana* were extreme challenges for establishing *F. taiwana* in the laboratory. Sun (1974) and Yeh and Chuang (1996) had reported the successful colonization of *F. taiwana* in the laboratory. *F. taiwana* adults are eurygamous and are unable to mate in the laboratory in small cages without a crepuscular period (Yeh and Chuang 1996). We reset the lighting time at 08:00 for easy observation and designed a customized acrylic cage to make the light shine from a dome. The sides of the cage were impenetrable to light, decreasing the disturbances for the *F. taiwana* adults. Swarming and copulation occurred 1 h before and 2 h after the lights were turned on (07:00-10:00). The current study describes the successful colonization of *F. taiwana* in the laboratory. The average female mating rates were approximately 50-60%, and males were observed to mate with multiple females.

Acknowledgments

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