

## Short Communication

## Efficiency of the induced mating technique for *Toxorhynchites theobaldi* (Diptera, Culicidae)

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## ABSTRACT

*Toxorhynchites* mosquitoes play important ecological roles in aquatic microenvironments, and are frequently investigated as potential biological control agents of mosquito disease vectors. Establishment of *Toxorhynchites* laboratory colonies can be challenging because for some species, mating and insemination either do not occur or require a prohibitive amount of laboratory space for success. Consequently, artificial insemination techniques have been developed to assist with mass rearing of these species. Herein we describe an adapted protocol for colony establishment of *T. theobaldi*, a species with broad distribution in the Neotropics. The success of the technique and its implications are discussed.

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Over the last 50 years, both interest in and knowledge of mosquitoes in the genus *Toxorhynchites* (Diptera, Culicidae) have increased. *Toxorhynchites* species have been used as laboratory models for isolation and propagation of viruses and protozoans (Rosen, 1981), for developmental and morphological studies (Pascini et al., 2011), and in an ecological context, on the role of *Toxorhynchites* species in structuring aquatic communities (Kneitel and Miller, 2002). However, the primary interest in mosquitoes from this genus involves their potential role as natural ecological barriers against the spread of mosquito vectors of disease (Kristan III, 2003), and as potential biological control agents (Miyagi et al., 1992). This is because *Toxorhynchites* larvae reside in both natural (e.g., tree holes, bromeliads) and artificial (e.g., discarded tires, planters, cemetery vases) “containers”, where they voraciously predate upon other insects and aquatic organisms, including other mosquitoes (Schreiber, 2007; Steffan and Evenhuis, 1981). Adults are non-hematophagous, presenting no known health risks to humans or other animals.

Manipulative experiments involving *Toxorhynchites* require maintenance of laboratory colonies for mass production. This can be problematic, as many species either will not mate under lab conditions or require a prohibitive amount of space to do so (Choochote et al., 2002), and egg viability can be low in some species (Steffan and Evenhuis, 1981). These difficulties with *Toxorhynchites* and other

genera or species poorly suited for lab colonization have been overcome by the development of a manual (induced) copulation technique (Baker et al., 1962; McCuiston and White, 1976). Herein we describe a step-by-step induced copulation procedure (modified from Baker et al. [1962]) that has been successful in developing laboratory populations of *Toxorhynchites theobaldi* (Dyar & Knab, 1906), a prominent species distributed broadly in the Neotropics (Gaffigan et al., 2001). This protocol has resulted in the first successful establishment of a *Toxorhynchites* species colony in Brazil.

Five automobile tires were randomly placed on a tract of land in an Atlantic Forest fragment located at the Universidade Federal de Viçosa, Viçosa, MG, Brazil. The tires were filled with 3L of tap water. Fifteen days later, *Toxorhynchites theobaldi* eggs and larvae were removed by sieving all tire water content. The tire water was saved, for later use, in oviposition containers. Eggs and larvae were taken to the laboratory for positive identification. *T. theobaldi* larvae were kept in 100 mL polypropylene cups and fed with *Aedes aegypti* (Linnaeus, 1762) (laboratory colony) larvae ad libitum. Upon eclosion, *T. theobaldi* adults were held in a 60 × 60 × 60 cm cage and continuously provided with water-honey solution (1:1). Fourteen virgin *T. theobaldi* females between 4–7 days old were selected for induced copulation using our modified technique. The full procedure is as follows:

### Male preparation

Step 1: Five males (3–4 days old) should be prepared per virgin female. Males are removed from the colony by aspiration and housed

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individually in 20 mL glass vials covered with cotton. Males are anesthetized by the exposure of the glass vials to CO<sub>2</sub> by placing the end of a one-meter hose connected to a metal cylinder CO<sub>2</sub> tank over the cotton (inside of the glass vial) for 3-5 seconds. Care must be taken to avoid excessive anesthetizing of males, prevent death, and to allow time for full recovery (five minutes or more) prior to mating.

Step 2: After immobilization, the dorsal thoracic region of the male is fixed to a Petri dish with a small drop of water-soluble glue, ventral side facing up (Fig. 1A).

Step 3: After the glue is dry, the male abdomen is touched using a forceps. Vigorous movement of the legs upon touching indicates commencement of recovery from anesthesia. Males are then decapitated using forceps to remove the subesophageal ganglion, an action that stops nervous system inhibition of copulation. The legs are also removed to allow better contact between male and female genital organs (Fig. 1B). After decapitation, *T. theobaldi* males will actively copulate for approximately 30 minutes.

### Female preparation

Step 4: Prior to male preparation, virgin females are removed from the colony and housed individually in 20 mL glass vials covered with cotton. After males are prepared, females are lightly anesthetized with chloroform (two or three drops placed on the cotton cover). The chloroformed cotton should remain in place for roughly 30-60 seconds, or until the female ceases movement and the legs are relaxed. Fully immobilized females are then removed from the vial and held by a suction pipette applied gently to the mesonotum. This positions the female and allows her to be handled for the mating procedure (Fig. 1C). Chloroformed females remain motionless for roughly 10-15 minutes; the induced mating procedure must be completed within this period of time. Care must be taken to avoid female injury. This is accomplished by ensuring that anesthesia is sufficient to allow relaxed body posture, and by adjusting the pipette suction to a low level in order to handle the female by the mesonotum without damaging the body.

Step 5: The Petri dish holding the male is then placed under a stereomicroscope (10-20×). The female genital opening should be held in contact with the male phallosome (Fig. 1C). A slight vibratory movement of the female indicates that copulation is occurring. If the male is not responsive within one minute, discard the individual and attempt copulation with the next male. Responsive males should successfully inseminate a female within 1-3 minutes.

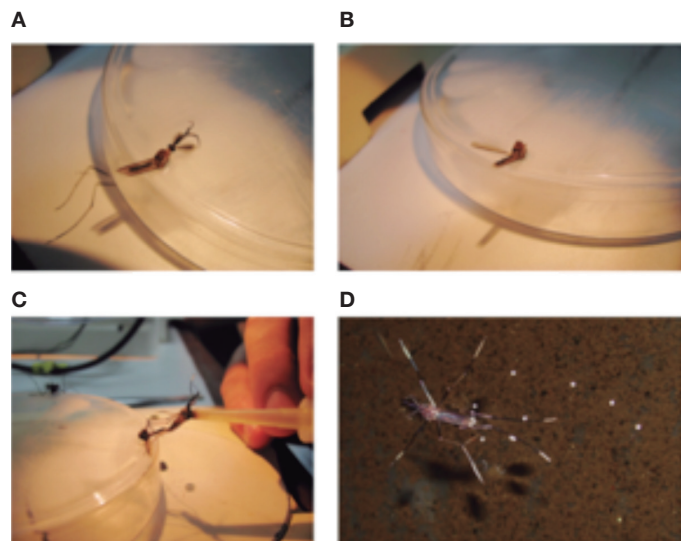
### Postmating

Step 6: As soon as male/female contact is broken, mated females should be released from the aspirator and placed in recovery. During the recovery period (15-30 minutes) females should be placed on a surface covered with a wet paper towel, each individual inside of an inverted 20 mL glass vial. Maintaining high humidity is an important aspect of the recovery process; thus, paper towels should remain sufficiently moist throughout the recovery period.

Step 7: Recovered females (upright, with some movement) are then transferred to a new 60 × 60 × 60 cm cage and given immediate access to water-honey solution, in order to facilitate full recovery.

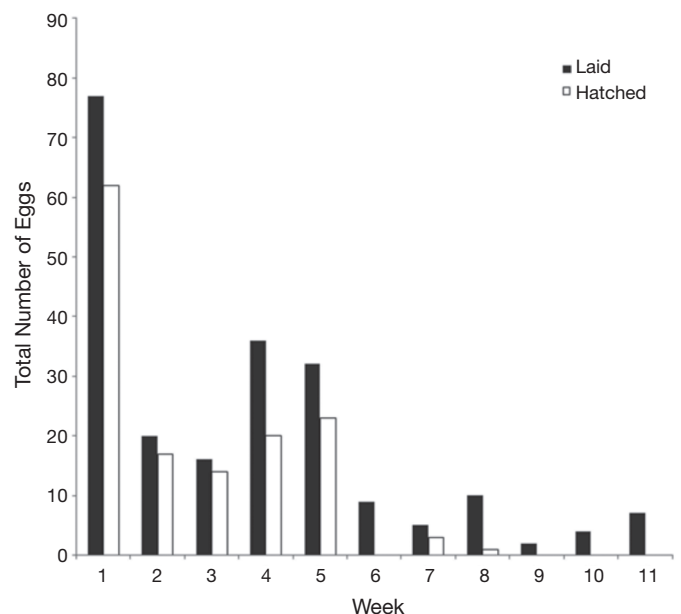
After mated females were placed in the new colony cage, an oviposition container was placed in the cage, consisting of a 2 L black plastic bottle holding 1 L of water from the field-placed automobile tires. Oviposition water was replaced weekly. We began recording oviposition on the first day of egg production, which occurred seven days after the mating procedure. Oviposition containers were checked daily for *T. theobaldi* eggs for 11 weeks (Fig. 1D). Eggs were placed individually in 30 mL glass vials filled with 20 mL of field tire water, and hatching success was evaluated.

The manually mated females laid 218 eggs in total over the eleven-week period, 140 of which hatched, for a total hatching success



**Figure 1.** *Toxorhynchites theobaldi* male preparation: legs are excised (A) and head is removed (B). *T. theobaldi* induced mating: encounter between the female genital opening and the male phallosome (C). *T. theobaldi* female resting on the water surface after laying eggs (D).

of 64.2%. We analyzed differences using generalized linear models (Crawley, 1993), performed in R Development Core Team (2011). Residual analyses were conducted to verify error distribution, including checking for overdispersion. Both the total number of eggs and number of eggs hatching were analyzed using Poisson family error. From week one to week eleven, there was a significant decrease in the total number of eggs laid per week ( $F_{1,10} = 19.796$ ,  $p < 0.001$ ) and in the proportion of eggs hatched ( $F_{1,10} = 24.039$ ,  $p < 0.001$ ) (Fig. 2). This was expected, as it is known that female oviposition in *Toxorhynchites* declines with age (Trpis, 1981) and that older eggs decrease in viability (Steffan and Evenhuis, 1981). The decrease in egg production and viability over time did not hinder successful establishment of a colony. However, for long-term sustainability, new adults must be fed into the colony on a regular basis, consisting of



**Figure 2.** Total number of eggs laid by *Toxorhynchites theobaldi* (top) and number of hatched eggs (bottom) during an 11-week period.

both colony offspring and, occasionally, field-collected individuals (e.g., to reduce unintended negative consequences of inbreeding).

This procedure has been critical for the production of a successful multi-year *T. theobaldi* colony in Brazil, and has led to new and exciting research involving chemical and behavioral ecology (Albeny-Simões et al., 2014) and morphology (Pascini et al., 2011). Our goal in publishing this induced-mating methodology is to provide a template for colony establishment and successful mass rearing (i.e., for biological control) of *Toxorhynchites* species in Brazil and beyond, ultimately facilitating increased research involving this broadly important mosquito genus.

### Conflicts of interest

The authors declare no conflicts of interest.

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