ARTÍCULO CORTO

Does marking affect the mating performance of sterile males of *Anastrepha ludens* (Diptera: Tephritidae)?

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Abstract

In fruit flies three methods of marking are commonly used for the evaluation of the sexual performance of adults in field cages. These methods are marking with label, marking with acrylic paint, and marking by dyed food intake. Some of these techniques can have a negative effect on the sexual performance. Our objective here was to determine the marking effect and color on mating performance of sterile males of *Anastrepha ludens* (Loew) (Diptera: Tephritidae*)*. The results of the study did not indicate significant differences on mating performance between marked and unmarked sterile males. In the evaluation of marking colors, there were also no significant differences on mating behavior. In conclusion, any of the three methods and colors of marking evaluated do not affect the selection choices of wild females and therefore the sexual performance of sterile males of *Anastrepha ludens*. The advantages and disadvantages of their application are discussed.

Keywords:
Identification technique
Mexican fruit fly
Sexual behavior
Sterile insect technique

Palabras clave:
Técnica de marcaje
Mosca mexicana de la fruta
Comportamiento sexual
Técnica del insecto estéril

¿Afecta el marcaje el desempeño de apareamiento de machos estériles de *Anastrepha ludens* (Diptera: Tephritidae?

Resumen

En moscas de la fruta se utilizan comúnmente tres métodos de marcaje para la evaluación del desempeño sexual de adultos en jaulas de campo. Estos métodos son el marcaje con etiqueta, el marcaje con pintura acrílica y el marcaje mediante la ingesta de alimento teñido. Algunas de estas técnicas pueden tener un efecto negativo en el desempeño sexual. Nuestro objetivo aquí fue determinar el efecto del marcaje y el color en el desempeño de apareamiento de machos estériles de *Anastrepha ludens* (Loew) (Diptera: Tephritidae). Los resultados del estudio no indicaron diferencias significativas en el desempeño de apareamiento entre machos estériles marcados y no marcados. En la evaluación de los colores de marcaje, tampoco hubo diferencias significativas en el comportamiento de apareamiento. En conclusión, ninguno de los tres métodos y colores de marcaje evaluados afecta las opciones de selección de hembras silvestres y, por lo tanto, el desempeño sexual de machos estériles de *Anastrepha ludens*. Se discuten las ventajas y desventajas de su aplicación.

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1. Introduction

The sterile insect technique (SIT) is an environmentally friendly method that is being used in various parts of the world to suppress insect pests (Dyck et al., 2005). The SIT involves the rearing, irradiation, and release of a massive number of sterile insects in the field (Knipling, 1955). Estimating the level of efficacy of the SIT requires a protocol that allows discrimination between released and wild insects (Enkerlin et al., 1996). A fluorescent dye is commonly used to mark fruit flies (Day-Glo fluorescent, neon red, Cleveland, Ohio), and this powder is internalized by the insect during the process of eclosion. Dye adhering to the pupae and dye on surrounding pupae is contacted by the ptilinum at adult eclosion and the material is retracted into the head of the fly along with this structure. Dye particles that adhere to other body parts are usually disposed of by the continual "cleaning" activity of the adult (Schroeder and Mitchell, 1981). This technique is only efficient in identifying insects during the release and capture process, and it is not recommended for the observation of sexual behavior. According to Hagler and Jackson (2001), the selection of a specific marker depends on the type of study that the researcher is planning, which involves the identification of insects with and without movement.

Currently, the estimation of sexual behavior is evaluated under field conditions in cages, which are more complex than laboratory tests because they evaluate a broad combination of non-controllable parameters (Chambers et al., 1983). This approach involves the development of marking techniques that allow for individual identification and monitoring of released insects. In fruit flies three methods of marking are commonly used for the evaluation of the sexual performance in field cages: marking with label, marking with acrylic paint and marking by dyed food intake.

In the genus *Anastrepha* and *Ceratitis*, insects have been marked using a numbered label (Meza et al., 2005) or printed letter (McInnis et al., 2002) stuck to the side of thorax that allows evaluation of the sexual competition of individual adults. In the genus *Ceratitis* and *Bractocera*, the application of acrylic paint to the thorax of laboratory and wild insects in the evaluation of sexual behavior has been reported (McInnis et al., 2002; Shelly et al., 2000). In the genus *Rhagoletis*, *Ceratitis* and *Drosophila* a new technique of identification using a dye added to the diet of adults was applied (Arévalo et al., 2009; Ramirez-Santos et al., 2017; Verspoor et al. 2015).

Although the use of these techniques has been reported in numerous studies, no detailed analysis has been conducted on the effect of these marking and color methods on sexual performance of the flies.

Our objective here was to determine the marking effect and color on mating performance of sterile males of *Anastrepha ludens (*Loew). Our hypothesis was that marking and color on insects does not affect mating performance of *Anastrepha ludens* sterile males. We conducted this study in field cages and compare three commonly used methods of marking on fruit flies.

Our results should provide reliable evidence for the potential application of these techniques in quality tests of flies used for SIT programs. The advantages and disadvantages of the methods' application are also discussed.

2. Materials and Methods

2.1. Biological material

Wild and sterile adults of *A. ludens* were used in this study. Sterile insects were obtained from irradiated pupae as a product of mass rearing of *A. ludens* in the Metapa de Domínguez Moscafrut Facility, Chiapas, Mexico (14°49'49.2"N, 92°11'44.8"W, and altitude 102 m above sea level). For the sterilization process, the pupae were irradiated under hypoxia conditions 48 h before peak adult emergence to a radiation dose of 80 Gy in a Gamma Beam 127 irradiator with a Cobalt-60 gamma source (series no. 226, dry storage source; Nordion International, Kanata, ON, Canada).

Wild insects were obtained by the recovery of larvae from bitter orange (*Citrus aurantium*, L.) collected in the region of Soconusco, Chiapas, Mexico. Once the larvae reached maturity, they were placed in containers with wet vermiculite to promote pupation and remained in those containers until they emerged as adults (Orozco et al., 2013).

2.2. Insect management

The pupae of both strains were placed in wooden cages (30x30x30 cm) covered at the ends with tulle fabric (2 mm) until the emergence of the adults. Upon emergence, females and males were isolated in separate cages (to avoid any contact of pheromones before testing) and maintained under a photoperiod of 12 h of light (from 7:00 to 19:00 h at a light intensity of 550 ± 50 lux) and 12 h of darkness, a temperature of 25 ± 1 °C, and relative humidity of 65 ± 5%. The adults were fed *ad libitum* with diet formulated with water and a mixture of sugar and hydrolyzed protein (3:1 ratio), yeast hydrolysate enzymatic, BP Biochemicals, LLC. In all of the experiments, sexually mature, virgin wild and sterile adults of 18 and 10 days of age after emergence, respectively, were used.

2.3. Study site

Tests were performed in a mango orchard (*Mangifera indica* cv. Ataulfo) close to the Moscafrut facility, which is located at 14°49′33.9″N, 92°11′46.2″W at 97 m above sea level in Metapa de Dominguez, Chiapas, Mexico. Field cages of 3 m in diameter by 2 m in height were used and were supported by a metal structure (Calkins and Webb, 1983). The field cage covered a citrus (*Citrus x aurantium* L.) of approximately 1.8 m in height.

The tests were performed under the following environmental conditions: 25-29 ºC, 70-80% relative humidity, and 3,000-0 Lux. Observations were made from 16:00 to 17:00 h, which is considered to be the period of maximum sexual activity for this species (Aluja et al., 2000).

2.4. Insect marking

Two days before the test, sterile insects were marked according to the following methods: 1) Mark with label. The flies were marked with a small paper tag (2 mm diameter) and glued on the fly thorax for individual identification (Meza et al., 2005). The tag was glued using a toothpick and white school glue of the mark Resistol^{MR} 850 of manufacturing in Mexico. 2) Mark with acrylic paint. The flies were marked with a small amount of acrylic paint (white, green, yellow, blue or red) applied on the surface of the thorax. The paint was added with a toothpick and placed gently on the insect. The methodology was like described in the manual of the FAO/IAEA/USDA (2014). 3) Mark by dyed food intake. The flies were marked by the intake of food coloring (DEIMAN®), which was added to the water (1 mL of coloring in 50 mL of water) two days prior to the test. This dye enters the intestines of the fly, and the abdomens were observably colored one day after exposure. 4). Unmarked. This treatment corresponds to the control, and unmarked sterile flies were used.

2.5. Sexual performance of males with different marking colors

Tests were conducted between marked sterile males (with different colors) and wild females (unmarked). In this study the three marking methods mentioned above were used. In each field cage, 10 males marked of each color (blue, green, red, white, yellow) and 50 wild females (unmarked) (1:1:1:1:1:5 ratio) were released. The males were released 30 min before the females to allow them to establish territories. The number of mating (which in this case served to evaluate the selection of wild females), was recorded in each cage.

Each field cage was considered as an experimental unit. Three different production batches were evaluated, with four replicates for each batch. In total, 12 replicates were performed for each one of the marking methods.

2.6. Sexual performance of marked and unmarked males

In each field cage, 20 marked males, 20 unmarked males and 20 wild females (unmarked) (1:1:1 ratio) were released. The males were release as described above. Tests were conducted for each one of the marking methods. The number of mating's was recorded in each cage. Each field cage was considered as an experimental unit. Three different production batches were evaluated. Ten replicates were performed for each one of the marking methods.

2.7. Data analysis

Data normality and the homogeneity of variance were determined using the Shapiro test and the Bartlett test, respectively. The data of sexual performance of males with different marking colors were analyzed using an analysis of variance (ANOVA) followed by a comparison of means with Tukey's test ($P<0.05$) (Zar, 1999). The data of sexual performance of marked and unmarked males were analyzed by a paired Student *t* test. The data were analyzed using the statistical software Minitab 16 (2010) from Minitab Inc. (www.minitab.com).

3. Results

3.1. Sexual performance of males with different marking colors

The evaluation of sterile males marked with different colors indicated no significant differences in the selection of the wild females in the different marking methods evaluated ($F_{4,55}=1.07$, $P=0.38$ Mark by dyed food intake; $F_{4,55}=0.74$, $P=0.57$ Mark with label; and $F_{4,55}=1.95$, $P=0.12$ Mark with acrylic paint) (Figure 1).

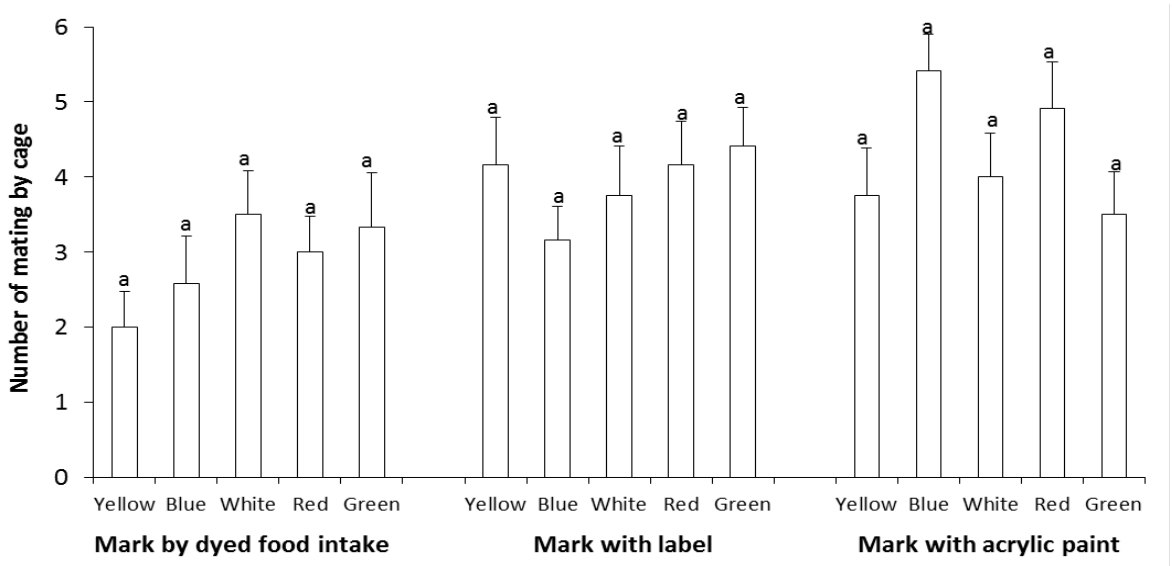

Figure 1. Sexual performance of sterile males marked with different colors under different marking methods. Bars marked with the same letter in each marking method are not significantly different ($P>0.05$).

3.2. Sexual performance of marked and unmarked male

The results of the study did not indicate significant differences between marked and unmarked males among the different marking method evaluated (t_9=0.61, P=0.56 Mark by dyed food intake; t_9=2.08, P=0.067 Mark with label; and t_9=0.53, P=0.606 Mark with acrylic paint).

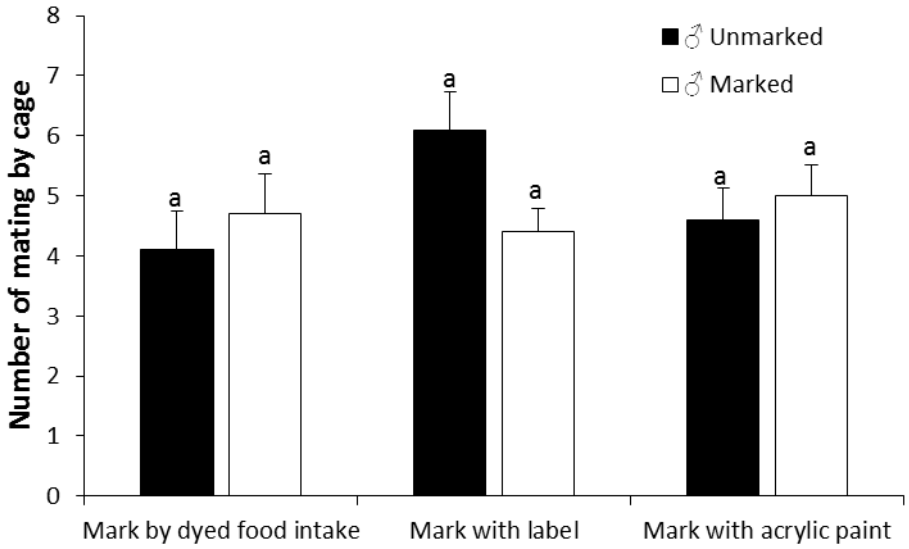

Figure 2. Sexual performance of marked and unmarked sterile males using different marking methods. Bars marked with the same letter in each marking method are not significantly different (P>0.05).

4. Discussion

Our results confirmed our hypothesis that color and marking on insects does not affect mating performance of *Anastrepha ludens* sterile males. These results were similar to those documented in *Ceratitis capitata* (Wiedemann), where the marking color does not affect the performance of the flies in tests of sexual behavior (Holbrook et al. 1970; Liedo et al., 2002). Three reasons can explain this observed behavior. The first is that the color in the thorax and / or abdomen of the flies is not perceptible to wild females. Second, the colors used are not a cause of rejection for wild females and the third, color is not a factor of discrimination of wild females. Although the latter is unlikely, for *A. ludens* a preference for the colors green, yellow and orange by females is reported (Robacker et al., 1990).

In *Anastrepha ludens* it is inferred that the type of marking does not interfere with the sexual activity of the flies (Meza et al., 2005; Meza-Hernández and Diaz-Fleischer, 2006). This study confirms these results and is similar to those documented in other species. In *C. capitata*, there are no differences between marked and unmarked males in mating tests (Guerfali et al., 2011). In the ectoparasite *Dinarmus basalis*, there is also documented no effect of marking on sexual performance tests (Lacoume et al., 2007). According to the results, the three marking methods evaluated are viable to evaluate the sexual behavior of the flies.

According to Hagler and Jackson (2001), to evaluate the movement of insects in their natural habitat, it is essential to develop a wide variety of markers that permit the evaluation of different insect behaviors, such as population dynamics, dispersion, territoriality, feeding behavior, trophic-level interactions, and other ecological interactions of insects, and the ideal marker must persist without inhibiting the normal biology of the insect. In this study, the three marking methods evaluated meet these premises.

Although there are no significant effects of marking on the sexual performance of insects, not all of the methods allow for the individual assessment of certain behavioral aspects, such as male calling, leks, and remating, as is the case for acrylic paint and dyed food intake. The greatest advantage of using the Marking method with label is that has low cost, is long lasting, and can be used to individually identify insects. This method has been successfully applied in species such as *A. ludens* (Liedo et al., 2010; Meza-Hernández and Díaz-Fleischer, 2006), *A. obliqua* (Quintero-Fong et al., 2011; Telles-Romero et al., 2011), and *C. capitata* (McInnis et al., 2002). However, one of the disadvantages is that individual application is tedious and time-consuming. Additionally, there is a risk that the label will detach from the thorax during handling or exposure to water during the rainy season.

Despite the limitation of differentiating individual insects, the marking method with acrylic paint is practical and reliable, and the marking is retained in the insect for a sufficient period. This method is generally the most used for the evaluation of the sexual behavior of fruit flies (FAO/IAEA/USDA, 2014). However, the risk of using toxic paint restricts the use of many solvents (Southwood, 1978).

The Marking method by dyed food intake offers many advantages compared to other methods, primarily in that adding the dye to the diet requires a minimum amount of

additional work. Secondly, the procedure avoids the handling of insects. Finally, the detection of the marking inside the insects is easy. However, it is possible that some dyes are not visible, have a retention period that is too short, or are harmful to the insects. In addition, there is the limitation of not being able to differentiate between individual insects. In *C. capitata* this method was documented to evaluate the sexual competence of a genetically modified strain (Ramirez-Santos et al., 2017).

In conclusion, in *A. ludens* the marking does not affect the sexual performance of sterile males or the selection of wild females. Any of the three methods and colors evaluated can be used to mark flies in sexual behavior tests.

Conflict of interests

The authors declare that they have no conflict of interest.

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