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Radiation Sterilization of Mexican Fruit Fly *Anastrepha ludens* (Leow) Based on Pupal Eye Color

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This paper reports on the documented pupal eye color changes of Mexican fruit fly *Anastrepha ludens* (Leow) at different holding temperatures. In holding mature larval samples at 15, 19, and 26 ºC (standard holding temperature); 28 ºC; and at environmental temperature (24–34 ºC), the development of pupae lasted 49, 33, 16, 15, and 16 d, respectively. Holding pupae at lower temperature delays pupal development and slows down progression of eye color changes. This is very important in manipulating pupal development, especially when uncontrolled problems occur during sterile insect technique (SIT) operations. The recommended timing of pupal irradiation for *A. ludens* at 26 ºC (standard holding temperature) is 2 d before adult emergence, where the pupae are 12–14 d old and the eye colors are dark brown, very dark brown, and dark grayish green. Using this eye colors as the reference guide for irradiation of pupae, the right age when held at 15, 19, and 28 ºC and at environmental temperature (24–34 ºC) was 41–45, 28–31, 11–13, and 12–14 d old, respectively. A table using documented and close-up photograph of pupal eye color can be used as a reference guide to determine the best time for the irradiation of pupae in an SIT program.

Keywords: *Anastrepha ludens*, Mexican fruit fly, pupal eye color

INTRODUCTION

Mexican fruit fly *Anastrepha ludens* (Leow) is the major pest of citrus fruits in commercial orchards situated in higher altitudes of Mexico, Belize, Guatemala, and the Lower Rio Grande Valley of Texas (Aluja *et al.* 1996, Thomas and Loera-Gallardo 1998). It is also one of the most significant pests of commercially grown fruit from the southern United States to northern Argentina (Aluja 1994, Aluja *et al.* 1996). This pest causes major damage in the field and often cause quarantines preventing the free movement and trade of fresh fruits, which are hosts of this serious pest. Sterile insect technique (SIT) is an environmentally friendly approach of insect control that involves mass rearing, sterilizing by ionizing radiation, and releasing sterile flies in the target area in numbers large enough to outcompete their wild counterparts (Knippling 1955, Dyck *et al.* 2005). In many cases, this type of insect pest control will lead to eventual eradication of the target pest population (Hendrichs and Robinson 2009).

Success of SIT program depends on the induction of sterility at the correct age of fruit fly pupae development to preserve the mating competitiveness of the released

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sterile adults against their wild counterparts (Seo *et al.* 1987). In many fruit fly mass production facilities, irradiation of pupae takes place two days before adult eclosion at standard pupal holding temperature based on pupal eye color. This pupal sterilization protocol is commonly applied to Mexican fruit fly, *Anastrepha ludens* and West Indian fruit fly, *A. obliqua* in Mexico (Hernández *et al.* 2007); Mediterranean fruit fly, *Ceratitis capitata* in Hawaii (Ohinata *et al.* 1971, Williamson *et al.* 1985), South Africa (Barnes *et al.* 2007), and Australia (Fisher 1997); Melon fly, *Bactrocera cucurbitae* in Japan (Teruya and Yukeyama-1979, Teruya and Isobe 1982); South American fruit fly, *Anasrepha fraterculus* in Argentina (Allinghi *et al.* 2007); Oriental fruit fly, *Bactrocera dorsalis* in Thailand (Sutantawong *et al.* 2002); and Philippine fruit fly, *B. philippinensis* in the Philippines (Resilva *et al.* 2007).

During SIT operations, there are occasional situations that require delaying or speeding up of fly emergence – especially with inclement weather, mechanical failures with irradiation equipment, large differences in cohort sizes, breakdown in the release operations, and/or desirability of fewer but larger releases of flies (FAO-IAEA-USDA 2003). In this situation, eye color is a reliable indicator of determining the physiological age of pupae (Ruhm and Calkins 1981). This is very useful especially when regulating pupal holding temperature to accelerate or delay pupal development from different ages of pupae for the correct timing of pupal irradiation. In this case, age of pupae for irradiation is determined by examination of the pupal eye color (Resilva *et al.* 2007). This study was conducted to document daily pupal eye color changes of the Mexican fruit fly reared at different temperature regimes. Specifically, the pupal eye color at 26 ºC (standard holding temperature) was the calibration point on the day of irradiation. The same pupal eye color will serve as an indicator of the time to irradiate for the other holding temperatures. The results obtained can be used as a guideline to carry out irradiation at the recommended time of two days before adult emergence.

MATERIALS AND METHODS

Insects

Anastrepha ludens used in this study were obtained from the Moscafrut Fruit Fly Mass Rearing Facility, DGSV-SAGARPA located in Metapa de Domínguez, Chiapas, Mexico. The strain is a mixture of an old colony from Mission, TX, USA and wild material collected from different regions in Chiapas. The strain has been reared for more than 10 years for at least 120 generations (Orozco-Dávila *et al.* 2007) – following procedures described by Stevens (1991), Hernández *et al.* (2014), and Orozco-Dávila *et al.* (2016).

Environmental Conditions for Pupal Development

Mature larval samples of *A. ludens* were collected within 1 h after dry larval separation using separating machines to synchronize pupal development. Five hundred (500) ml of larvae were mixed with 25% moistened vermiculite, subdivided according to temperature regimes, and placed in covered plastic pupation trays (75.5 x 38 x 4 cm). Pupation trays with the larvae were held for pupation in controlled temperature rooms or chilling incubators at 26 (pupal holding standard), 28, 19, and 15 ºC and at environmental temperature (24–34 ºC). The controlled temperature pupation room was kept dark with 70–80 ± 0.52 % relative humidity.

Pupal Dissections, Eye Color Determination, and Taking Photographs

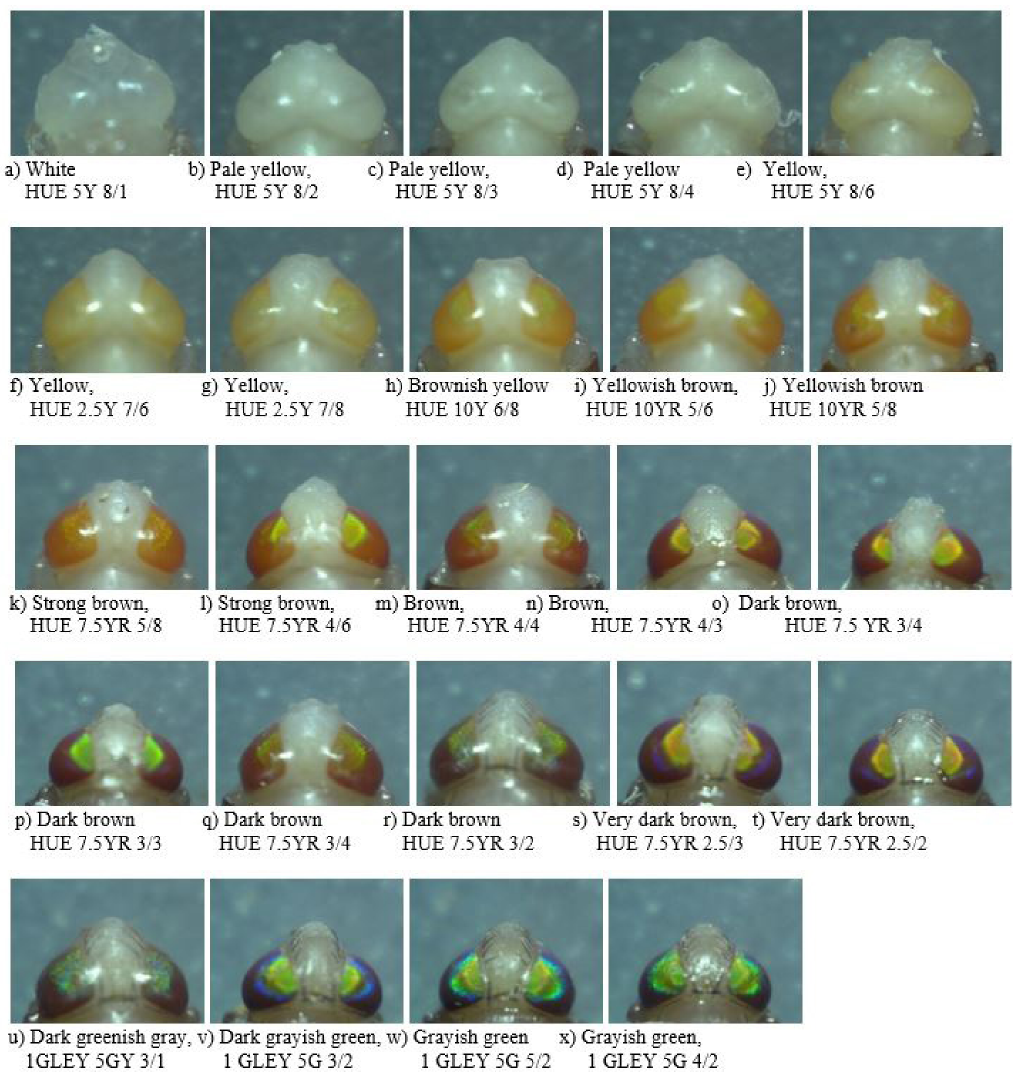
About 50–100 pupal samples were collected and dissected daily from different holding temperatures to observe pupal eye color changes from the day of pupation to the day of emergence. During dissection, the shell of the anterior part of the puparium was carefully removed to expose the eyes of the developing imago (Ruhm and Calkins 1981). At the same time, photographs of the eye color of *A. ludens* held at different temperatures were taken using the QX5 computer microscope at 60x magnification (2007 Digital Blue, Inc., Microsoft Corporation, Atlanta, GA, USA) that was connected to a computer monitor. Each pupal eye was positioned under the microscope, focused with proper illumination and had a close-up photograph taken (Resilva and Obra 2016, Resilva and Pereira 2014). The daily eye color data at each temperature were recorded and matched with the color scale of the Munsell® Soil Color Charts (Anonymous 2000). The calibration point at standard holding temperature was the pupal eye color on the day of irradiation. Then, the same pupal eye color was used as the indicator for the age of pupae to be irradiated for the other holding temperatures.

Determination of Adult Emergence and Flight Ability

Three days before adult emergence, samples of 100 pupae in five replications were placed in a petri dish and set in black plexiglass tubes (10 cm high, 8.8 cm diameter) to determine adult emergence and flight ability for all pupal holding temperatures – following the standard quality control test procedure (FAO-AEA-USDA 2003). The black plexiglass tube was coated with unscented talcum powder to prevent the flies from walking up. A 1 x 10 cm strip of paper folded accordion-wise was placed at the bottom of the flight tube as resting place for the emerging flies. As the flies emerged, their only access to food and water is to fly out of the tube. The test was allowed to run for 2–3 d until all the flies have escaped or died. These tests were conducted for daily eye color changes to determine if different holding temperature tested effected the quality of flies.

RESULTS AND DISCUSSIONS

The method for estimation of the pupal age of *A. ludens* was based on eye color changes and compared with the color scale of the Munsel Soil Color Charts (2000). Pupal development times until adult emergence at different holding temperatures of 15, 19, 26 (pupal holding standard), and 28 °C and at environmental temperature (24–34 °C) with corresponding color codes are given on Table 1. The fastest pupal development of 15 d was observed at 28 °C. Similar pupal development time was noted when held at 26 ºC (pupal holding standard) and at environmental temperature (24–34 ºC) (16 d). Duration of pupal development was longer when held at 19 ºC and 15 ºC (33 and 49 days, respectively). Figure 1 shows the daily matching of color codes of the Munsel Soil Color Charts with the eye colors that were observed in the dissected pupae in all holding temperatures. The recommended timing of pupal irradiation for *A. ludens* at 26 ºC (pupal holding standard) is at 2 d before adult emergence, where the pupae are 12–14 d old and the eye colors are dark brown (HUE 7.5YR 3/4), very dark brown (HUE YR 2.5/2), and dark grayish green (1GLEY 5G 3/2). Using these eye colors as the reference guide for irradiation of pupae, the right age when held at 15, 19, and 28 ºC and at environmental temperature (24–34 ºC) was 41–45, 28–31, 11–13, and 12–14 d old, respectively. All data on the percentage adult emergence and percentage



Figures 1a–x. Photographs of eye color changes of *Anastrepha ludens* during pupal development.

Table 1. Pupal eye color changes of Mexican fruit fly, *Anastrepha ludens* at different holding temperatures.

Age of Pupa (Days)	Temperature / Eye Color Changes									
	15°C		19°C		26°C		28°C		Environmental (24-34°C)	
	Eye Color	Color Code	Eye Color	Color Code	Eye Color	Color Code	Eye Color	Color Code	Eye Color	Color Code
1	Young	-	Young	-	Young	-	Young	Young	Young	-
2	Young	-	Young	-	Young	-	Young	Young	W	HUE 5Y 8/1
3	Young	-	Young	-	W	HUE 5Y 8/1	PY	HUE 5Y8 /2	W	HUE 5Y 8/1
4	Young	-	Young	-	PY	HUE 5Y 8/2	PY	HUE 5Y 8/3	PY	HUE 5Y 8/2
5	Young	-	W	HUE 5Y 8/1	PY	HUE 5Y 8/3	PY	HUE 5Y 8/3	PY	HUE 5Y 8/3
6	W	HUE 5Y 8/1	PY	HUE 5Y8 /2	PY	HUE 5Y 8/4	PY	HUE 5Y 8/4	PY	HUE 5Y 8/3
7	W	HUE 5Y 8/1	PY	HUE 5Y 8/2	PY	HUE 5Y 8/4	PY	HUE 5Y 8/4	PY	HUE 5Y 8/4
8	W	HUE 5Y 8/1	PY	HUE 5Y 8/2	PY	HUE 5Y 8/4	Y	HUE 5Y 8/6	PY	HUE 5Y 8/4
9	PY	HUE 5Y 8 /2	PY	HUE 5Y 8/2	PY	HUE 5Y 8/4	Y	HUE 2.5Y 7/6	Y	HUE 5Y 8/6
10	PY	HUE 5Y 8/2	PY	HUE 5Y 8/3	Y	HUE 5Y 8/6	BY	HUE 10YR 6/8	BY	HUE 10YR 6/8
11	PY	HUE 5Y 8/2	PY	HUE 5Y 8/3	YB	HUE 10YR 5/8	DB	HUE 7.5YR 3/4	SB	HUE 7.5YR 5/8
12	PY	HUE 5Y 8/2	PY	HUE 5Y 8/3	DB	HUE 7.5YR 3/4	DB	HUE 7.5YR 3/3	DB	HUE 7.5YR 3/4
13	PY	HUE 5Y 8/2	PY	HUE 5Y 8/3	VDB	HUE 7.5YR 2.5/2	GG	1GLEY 5G 5/2	VDB	HUE 7.5YR 2.5/3
14	PY	HUE 5Y 8/3	PY	HUE 5Y 8/4	DGG	1GLEY 5G 3/2	GG	1GLEY 5G 4/2	DGG	1GLEY 5G 3/2
15	PY	HUE 5Y 8/3	PY	HUE 5Y 8/4	GG	1GLEY 5G 4/2	Emergence		GG	1GLEY 5G 4/2
16	PY	HUE 5Y 8/3	PY	HUE 5Y 8/4	Emergence				Emergence	
17	PY	HUE 5Y 8/3	PY	HUE 5Y 8/4						
18	PY	HUE 5Y 8/3	PY	HUE 5Y 8/4						
19	PY	HUE 5Y 8/3	PY	HUE 5Y 8/4						
20	PY	HUE 5Y 8/3	Y	HUE 5Y 8/6						
21	PY	HUE 5Y 8/4	Y	HUE 2.5Y 7/6						
22	PY	HUE 5Y 8/4	Y	HUE 2.5Y 7/8						
23	PY	HUE 5Y 8/4	YB	HUE 10YR 5/6						
24	PY	HUE 5Y 8/4	YB	HUE 10YR 5/8						
25	PY	HUE 5Y 8/4	YB	HUE 10YR 5/8						
26	PY	HUE 5Y 8/4	SB	HUE 7.5YR 4/6						
27	PY	HUE 5Y 8/4	B	HUE 7.5YR 4/4						
28	Y	HUE 5Y 8/6	DB	HUE 7.5YR 3/4						
29	Y	HUE 5Y 8/6	DB	HUE 7.5YR 3/2						
30	Y	HUE 2.5Y 7/6	VDB	HUE 7.5YR 2.5/3						
31	Y	HUE 2.5Y 7/8	DGG	1GLEY 5G 3/1						
32	BY	HUE 10YR 6/8	GG	1GLEY 5G 4/2						
33	BY	HUE 10YR 6/8	Emergence							
34	BY	HUE 10YR 6/8								
35	YB	HUE 10YR 5/8								
36	SB	HUE 7.5YR 5/8								
37	SB	HUE 7.5YR 4/6								
38	B	HUE 7.5YR 4/4								
39	B	HUE 7.5YR 4/3								
40	DB	HUE 7.5YR 3/4								
41	DB	HUE 7.5YR 3/3								
42	VDB	HUE 7.5YR 2.5/3								
43	VDB	HUE 7.5YR 2.5/2								
44	DGG	1GLEY 5G 3/1								
45	DGG	1GLEY 5G 3/2								
46	GG	1GLEY 5G 5/2								
47	GG	1GLEY 5G 4/2								
48	GG	1GLEY 5G 4/2								
49	Emergence									

Notes:
Color Table was compared from the Munsell® Soil Color Charts (Year 2000 Revised Washable Edition).
Young – pupa still soft; W – white; PY – pale yellow; Y – yellow; BY – brownish yellow; YB – yellowish brown; SB – strong brown; B – brown; DB – dark brown; VDB – very dark brown; DGG – dark grayish green; GG – grayish green

flight ability observed at all pupal holding temperatures exceeded the minimum specification set for *A. ludens* in the FAO-IAEA-USDA Quality Control Manual (2003), which ranged 93.6–99.2% and 90.6–96.0% (Table 2). The high percentage of adult emergence and flight ability observed at all holding temperatures tested suggests that development of *A. ludens* can adequately be manipulated by delaying or speeding up pupal growth by holding pupae from as low as 15 °C to as high as 28 °C. In addition, pupae can be irradiated using eye color as a reference guide to achieve irradiation sterilization without affecting adult emergence and flight ability. This is very useful when there are failures in the rearing operations in the facility or problems with release operations in the field and pupal development needs to be manipulated with temperature.

Table 2. Pupal development, recommended age of pupal irradiation, percent adult emergence, and percent fliers for *A. ludens* at different pupae holding temperatures*.

Holding Temperature (°C)	Pupal Development (days)	Recommended Age of Pupa Irradiation (days)	Adult Emergence (%)	Adult Fliers (%)
15	49	40 – 45	93.6 ± 0.81	93.0 ± 0.63
19	33	28 – 31	94.8 ± 1.16	94.4 ± 0.98
26	16	12 – 14	97.4 ± 0.68	95.0 ± 0.71
28	15	11 – 13	95.4 ± 0.81	90.4 ± 0.81
[1]ET	16	12 – 14	99.2 ± 0.37	96.0 ± 0.55

Note:
*Mean of 5 replicates, [1]environmental (24 – 34 °C), ET environmental temperature

SUMMARY AND CONCLUSIONS

Study on pupation process and recording eye color changes in Mexican fruit fly, *Anastrepha ludens* at different holding temperatures of 15, 19, 26, and 28 ºC and at environmental temperature (24–34 ºC) was conducted at the Moscafrut Fruit Fly Mass Rearing Facility at Metapa de Domínguez, Chiapas, Mexico. For radiation sterilization, maturity and eye color of different holding temperature differ from one another (Table 2). Irradiation of pupae were done 2 d before emergence at standard holding temperature (26 ºC). The age of pupae fall at 12–14 d old where the eye colors are dark brown (HUE 7.5YR 3/4), very dark brown (HUE YR 2.5/2), and dark grayish green (1GLEY 5G 3/2). Using these eye colors as the reference guide for radiation sterilization, the right age of pupae for irradiation timing when held at 15, 19, and 28 ºC and at environmental temperature (24–34 ºC) were 40–45, 28–31, 11–13, and 12–14 d old, respectively.

Quality control data on adult emergence and flight ability on holding temperatures studied – at 15, 19, 26, and 28 ºC and at environmental temperature (24– 4 ºC) passed the minimum specifications set on the FAO-IAEA-USDA QFC Manual (2003) ranging 93.6–99.2% and 90.6–96.0%, respectively (Table 2). The results of this study indicate that for Mexican fruit fly used for SIT programs anywhere in the world, pupae destined for radiation sterilization can be maintained at holding temperatures between 15 and 28 ºC without affecting quality control. The dark brown, very dark brown, and dark grayish green pupal eye colors identified for each holding temperature can be used as baseline information in the mass rearing facility for radiation sterilization of pupae at desired holding temperature to speed up or delay pupal development. The recommended pupal eye color is very useful to avoid or solve anticipated problems that may be encountered in mass rearing operations in SIT release program.

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