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Documented Pupal Eye Color of the West Indian Fruit Fly, *Anastrepha obliqua* (Maquart), as a Tool for Radiation Sterilization

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Pupal age is critical when sterilizing fruit fly pupae for field releases in the sterile insect technique (SIT) programme. When kept at 26 °C, pupae of the West Indian fruit fly, *Anastrepha obliqua* (Maquart), are irradiated after 12–13 d or 22 d before emergence. At this age, pupal eye color, which is used to determine the optimum stage, is very dark brown and grayish green as classified based on the Munsell® Soil Color Charts. However, it is often necessary to use different pupal holding temperatures in order to manipulate pupal development, especially when unforeseen problems occur during the rearing procedure for *A. obliqua*. Holding pupae at lower temperatures delays pupal development and slows down the progression of eye color changes, but at higher temperatures, the opposite occurs. The pupal eye color of the fruit fly was documented at different ages at different holding temperatures. Using this eye color as the reference guide for timing the irradiation of pupae, the optimum pupal age for irradiation when held at 15, 19, 28°C and natural environment (24–34°C) was 35–39, 28–30, 11 and 12–13 d old, respectively. The results indicate that for the *A. obliqua* used for SIT programs anywhere in the world, pupae destined for radiation sterilization can be maintained at holding temperatures between 15°C and 28°C without affecting their development. Pupal eye colors identified in each holding temperature can be used as baseline information in the mass rearing facility to judge the optimum time for radiation sterilization of pupae kept at the required holding temperature to accelerate or delay pupal development. Pupal eye color is a very useful tool to avoid or solve potential problems that may be encountered in mass rearing operations in the SIT release program.

Key Words: *Anastrepha obliqua*, pupal eye color, pupal development, sterile insect technique, West Indian fruit fly

Abbreviations: FAO – Food and Agriculture Organization, IAEA – International Atomic Energy Agency, SIT – sterile insect technique, USDA – United States Department of Agriculture

INTRODUCTION

The West Indian fruit fly, *Anastrepha obliqua* (Macquart), occurs throughout the Caribbean, south to southern Brazil, and is the most abundant species of *Anastrepha* in the West Indies and Panama (Weems 1980). It is considered a pest of several economically important fruit crops. It is the main fruit fly species that attacks mango (*Mangifera indica* L.) and hog plums (*Spondias* spp.) in commercial orchards situated at lower altitudes (Enkerlin et al. 1989). Occurrence of *A. obliqua* has been recorded in the USA (Florida and Texas), South America, and the Caribbean Islands (Hernández-Ortiz and Aluja 1993).

In Mexico, this fly has the second greatest economic impact next to the Mexican fruit fly among all species belonging to the genus *Anastrepha*. In 1992, the Mexican government launched the National Campaign against Fruit Flies to establish pest-free areas and strengthen the fruit export expectation potential (Reyes et al. 2000). An integrated program utilizing the sterile insect technique (SIT) has been implemented to control this pest in northern Mexico since 2001 (Gabayet et al. 1996).

To achieve these objectives, weekly production of sterile *A. obliqua* in the Moscafrut Fruit fly facility, DGSV -SAGARPA located in Metapa de Dominguez, Chiapas, Mexico was increased up to 65 million pupae per week through improved production processes, automation, use of new diet formulas, and establishment of low adult

densities in reproduction cages (Orozco-Davila et al. 2006, 2014).

The SIT, integrated with other pest management measures, is widely regarded as the most practical and cost-effective means of establishing pest-free areas. It is an environment-friendly approach to insect control which involves mass-rearing, sterilizing by ionizing radiation, and releasing sterile insects in the target area in numbers large enough to outcompete their wild counterparts (Knipling 1955; Dyck et al. 2005). In certain cases, this type of insect pest control can lead to eventual eradication of the target pest population (Hendrichs and Robinson 2009).

Success of a SIT program depends on a number of factors, one of which is that the insects should be sterilized at the age at which the mating competitiveness of the released sterile adults with their wild counterparts is best preserved (Seo et al. 1987; Calkins and Parker 2005). Calkins and Parker (2005) also pointed out that in many insect groups irradiation results in a reduction in competitiveness. For practical reasons, irradiation sterilization is usually carried out in the pupal stage, and for tephritid flies, shortly before irradiation (Bakri et al. 2005). Ruhm and Calkins (1981) found that in the Mediterranean fruit fly, *C. capitata*, pigmentation of the pupal compound eye changes with the advance of physiological development. In all fruit fly mass production facilities, pupae are irradiated 2 d before adult eclosion when held at standard pupal holding temperatures, using pupal eye color to judge the optimal pupal age for irradiation. These pupal sterilization protocols are commonly applied to the Mexican fruit fly, *Anastrepha ludens*, and to the West Indian fruit fly, *A. obliqua*, in Mexico (Hernandez et al. 2007); *C. 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, *A. fraterculus*, in Argentina (Allinhi 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 rearing operations, there are occasional situations that require delaying or accelerating the time of fly emergence, especially when there is inclement weather condition, mechanical failures with irradiation equipment, large differences in cohort sizes, breakdown in the release operations, or a necessity for smaller or larger volumes of released flies (FAO/IAEA/USDA 2014). In these situations, the appropriate time of adult emergence is achieved by manipulating the pupal holding temperature to synchronize pupal development and pupal eye color is a reliable indicator for determining the correct physiological age for pupal irradiation (Ruhm and Calkins 1981; Resilva et al. 2007).

The pupal eye color of *B. philippinensis*, *C. capitata* and *A. ludens* was documented at different holding temperatures and the daily eye color changes were recorded and then matched with Munsel Soil Color Charts (Resilva and Obra 2016; Resilva et al. 2019a; Resilva et al. 2019b). The Munsel® Soil Color Chart system is a means to visually describe and match color using a scientific approach. It enables colors to be accurately described using verbal descriptions and using color codes, making it easy to define and express colors within narrow ranges in a very specific way (Anonymous 2000). The documented pupal eye color can be used as reference guide in the manipulation of pupal holding temperatures during rearing for SIT.

In this investigation, systematically daily pupal eye color changes of *A. obliqua* pupae at different holding temperature regimes were observed and documented from pupation to adult emergence. Specifically, the pupal eye color at 26°C (standard holding temperature) was the calibration point on the day of irradiation. The same pupal eye color served as an indicator of the irradiation time for the other holding temperatures. The results obtained can be used as a guideline for irradiating A. obliqua pupae at the optimum time before adult emergence.

MATERIALS AND METHODS

Insects

A. obliqua used in this study was obtained from the Moscafrut Fruit Fly Mass Rearing Facility. The insects were reared following procedures described by Artiaga-Lopez et al (2004) and Hernandez et al. (2014).

Environmental Conditions for Pupal Development

Adult colonies were maintained in wooden or rectangular aluminum screen cages kept at room temperature at 26 ± °C and 65 ± 10% relative humidity. The flies were provided with a mixture of sugar and yeast hydrolysate (3:1) for food and water provided using "drinking pipes". Female adults laid eggs directly on the screen that fell on metal trays half filled with water. Eggs were seeded on the surface of artificial standard larval diet lined with tissue paper. Larval development is under constant supervision in controlled temperature rooms until maturity.

Samples of mature *A. obliqua* larvae were collected within 1 h after they had left the larval diet to pupate to synchronize pupal development and adult emergence. About five groups of 500 mL of larvae were mixed with 25% moistened vermiculite, subdivided according to four temperature regimes, and placed in plastic pupation trays covered with cheesecloth. Trays were held for pupation in controlled temperature rooms or chilling incubators at 15, 19, 26 (pupal holding standard) and 28°C, and Natural Environment (24–34°C). In case of Natural Environment, larval samples were held under the trees to simulate pupal development under natural conditions where temperature fluctuated between 24°C and 34°C.

Pupal Dissections, Eye Color Determination and Photography

For each holding temperature, about 50–100 pupae samples were collected and dissected daily 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, as described by Ruhm and Calkins (1981). Photographs of the eye color of *A. obliqua* pupae from each of the temperatures were then taken using a Digital Blue QX5 computer microscope (Digital Blue Inc., Atlanta, Georgia, USA) at 60x magnification that was connected to a computer. Each pupa with eyes exposed was positioned under the microscope, focused appropriate illumination and a close-up photograph taken as described in different fruit fly species (Resilva and Pereira 2014); in *B. philippinensis* (Resilva and Obra 2016); in *C. capitata* (Resilva et al. 2019a); and in *A. ludens* (Resilva et al. 2019b).

Determination of Adult Emergence and Flight Ability

Three days before adult emergence, samples of 100 pupae from the *A. obliqua* colony, in five replications from each of the five pupal holding temperatures, were placed in black plexiglass tubes (10 cm high, 8 cm diam). The flight tubes were coated internally with unscented talcum powder to prevent poor quality adults from crawling out of the tubes. 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 fruit flies. As the flies emerge, their only access to food and water is to fly out of the tube. The percentage adult emergence and flight ability was determined following the standard quality control test procedure (FAO/IAEA/USDA 2014).

RESULTS AND DISCUSSION

As in the case of *B. philippinensis* (Resilva and Obra 2016), the results of this study showed that the pupal duration and corresponding eye color in *A. obliqua* changes during pupal development when pupae are held at different holding temperatures from the day of pupation to the day of emergence. The fastest pupal development was observed at 28°C (12 d) followed by 26°C (pupal holding standard) and Natural Environment (24–34°C); 14 d. Duration of pupal development was longer when held at 19°C and 15°C, (31 and 40 d, respectively). Descriptions of the eye color changes for pupae held at the five different holding temperatures are shown in Fig. 1–5.

a. 15°C

At 15°C the pupal duration was 40 d. Dissection of the puparium only became possible 6 d after pupation. Based on the Munsell® Soil Color Chart, the sequence of eye color changes between 6 and 39 d were white, pale yellow, yellow, brownish yellow, yellowish brown, strong brown, brown, dark brown, very dark brown and grayish green (Fig. 1). Holding pupae at this temperature caused

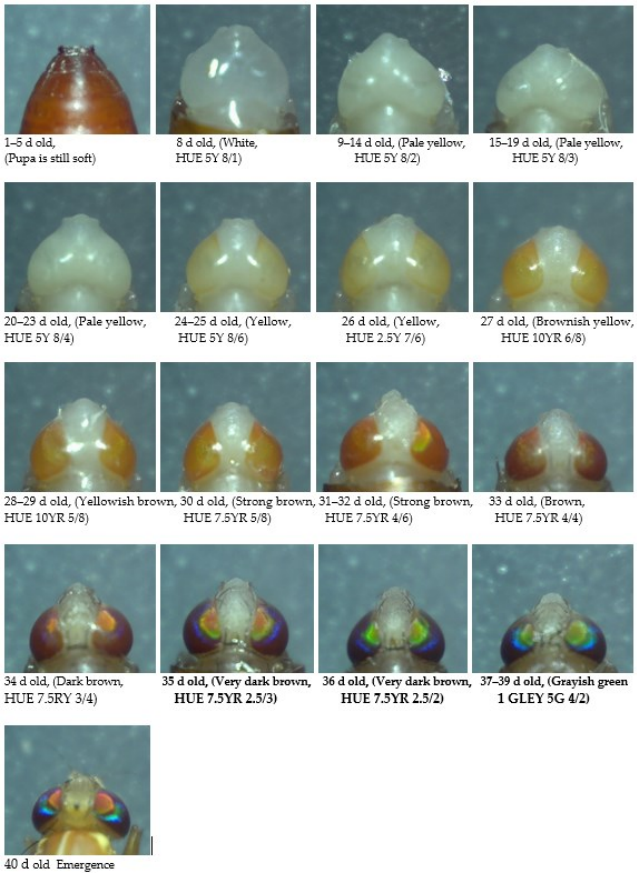

Fig. 1. Photographs of the daily eye color changes of the West Indian fruit fly (*Anastrepha obliqua*) taken during pupal development at 15 °C (Pupal age and related color for optimum irradiation is given in bold font).

retardation in development which was manifested by slow progression in eye color changes particularly in the early days of pupation. At 6–8 d old, the eye color was white (Color Code: HUE 5Y 8/1). At days 9–14, 15–19 and 20–23, eye color was pale yellow (HUE 5Y 8/2, HUE 5 Y 8/3 and HUE 5 Y 8/4, respectively), becoming yellow (HUE 5Y 8/6) at 24–25 and (HUE 2.5Y 7/6) 26 d. At 27 d, eye color changed to brownish yellow (HUE 10YR 6/8); at 28–29 d to yellowish brown (HUE 10YR 5/8); to strong brown at 30 (HUE 7.5YR 5/8) and 31–32 d (HUE 7.5YR 4/6); and to brown at 33 d old (HUE 7.5YR 4/4). The pupae could be irradiated at 35, 36 and 37–39 d old when the eye colors are very dark brown and grayish green (HUE 7.5YR 2.5/3, HUE 7.5YR 2.5/2 and 1 GLEY 5G4/2, respectively) similar to the pupal eye color at irradiation time at 26°C (standard holding temperature). Adults emerged on the 40th day; average emergence was 85.0 ± 1.58% and average flight ability was 81.0 ± 1.58%.

b. 19°C

Pupal duration at 19°C was 31 d. Dissection of the anterior part of the puparium was possible only on the 5th day of pupation. The color changes in the eyes during the 5 to 29-d period were white, pale yellow, yellow, brownish yellow, yellowish brown, strong brown, brown, dark brown, very dark brown and grayish green (Fig. 2). At 5–6 d old, the eyes appeared white (HUE 5Y 8/1), turning pale yellow (HUE 5Y 8/2, HUE 5Y 8/3 and HUE 5Y 8/4) at days 7–9, 10–14 and 15–17. At days 18–19, 20 and 21, they became yellow (HUE 5Y 8/6, HUE 2.5Y 7/6 and HUE 2.5Y 7/8). At 22 d, eye color was brownish yellow (HUE 10YR 6/8); at 23 and 24 d, yellowish brown (HUE 10YR 5/8 and HUE 10YR 5/6); at 25 d, strong brown (HUE 7.5YR 5/8); at 26 d, brown (HUE 7.5YR/4/4); and at 27 d, dark brown (HUE 7.5YR 3/2). The pupae are ready for irradiation at 28 and 29–30 d when the eye colors were very dark brown (HUE 7.5YR 2.5/1 and grayish green (1 GLEY 5G 4/2). Adult emergence was on the 31st day with an average emergence of 90.2 ± 1.48% and average flight ability of 86.0± 2.55%.

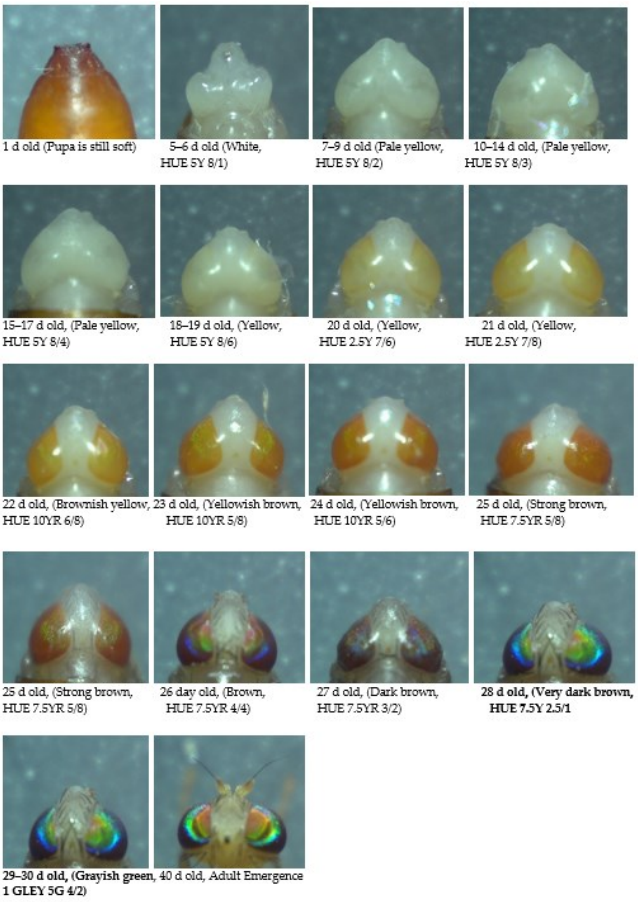

Fig. 2. Photographs of the daily eye color changes of the West Indian fruit fly (*Anastrepha obliqua*) taken during pupal development at 19°C (Pupal age and related color for optimum irradiation is shown in bold font).

c. 26 °C (Pupal Holding Standard)

The developmental duration from pupa to adult at 26°C was 14 d. Dissection of the anterior part of the puparium only became possible on the 3rd day. The progressive color changes in the eyes during the period 3–13 d were white, pale yellow, yellow, brownish yellow, strong brown, brown and very dark brown and grayish green (Fig. 3). At 3 d old, pupal eye color was white (Color Code: HUE 5Y 8/1) turning pale yellow at 4, 5 and 6–7 d old, (HUE 5Y 8/2, HUE 5Y 8/3, and HUE 5Y 8/4, respectively). At day 8, eye color changed to yellow (HUE 5Y 8/6), turning brownish yellow (HUE 10YR 6/8) on the 9th day. Rapid pupal development was observed on the 10th day and eye color changed abruptly from strong brown (HUE 7.5YR 5/8) to brown (HUE 7.5YR 4/4) on the 11th day. The pupae were ready for radiation sterilization at 12 and 13 d old when the eye colors became very dark brown (HUE 7.5YR 2.5/2) and grayish green (1 GLEY 5G 4/2). Adult emergence on the 14th day was an average of 94.2 ± 4.38% and flight ability was 92.2 ± 3.19%.

d. 28°C

The pupal duration at 28°C was 12 d. Pupae could not be dissected on the 1st and 2nd days of pupation because they were still soft. Dissection of the anterior part of the puparium was possible on the 3rd day. The sequence of color changes of the eyes from the 3rd to 11th days were pale yellow, yellow, brownish yellow, dark brown and very dark brown (Fig. 4). At 3–4, 5–6 and 7 d old, the eye colors were pale yellow (HUE 5Y 8/2, HUE 5Y 8/3 and HUE 5Y 8/4, respectively). After 8 d, pupal eye color was yellow (HUE 2.5Y 7/6), turning brownish yellow (HUE 10YR 6/8) on the 9th day after pupation. Pupae continued

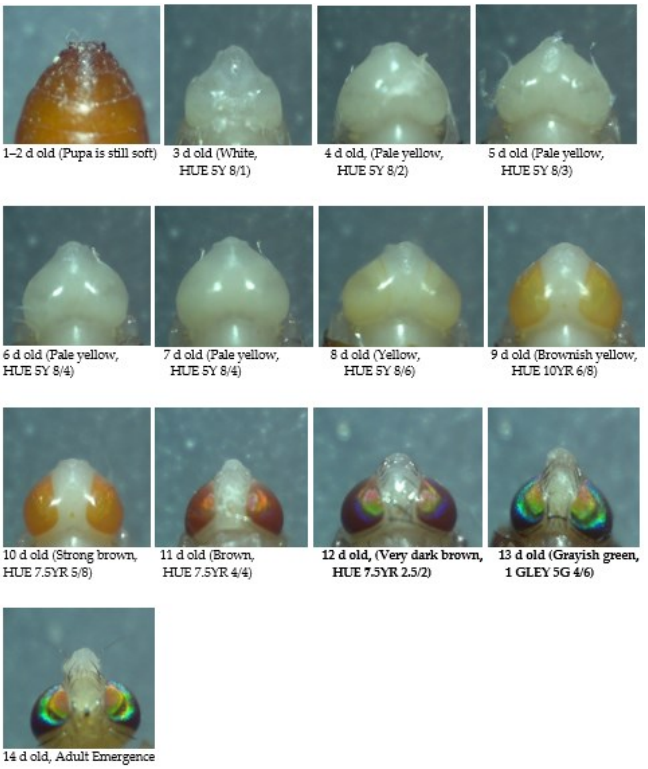

Fig. 3. Photographs of the daily eye color changes of the West Indian fruit fly (*Anastrepha obliqua*) taken during pupal development at 26°C (pupal holding standard). (Pupal age and related color for optimum irradiation is shown in bold font).

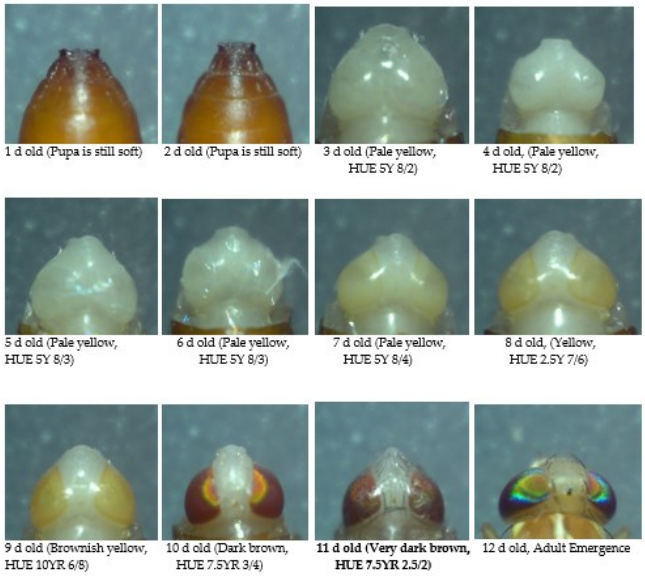

Fig. 4. Photographs of the daily eye color changes of the West Indian fruit fly (*Anastrepha obliqua*) taken during pupal development at 28°C. (Pupal age and related color for optimum irradiation is shown in bold font).

to develop until the eye color became dark brown (HUE 7.5YR 3/4) after 10 d. At this holding temperature, pupae are ready for irradiation on the 11th day of pupation when the eye color is very dark brown (HUE 7.5YR 2.5/2). Average adult emergence on the 12th day was 96.2± 2.77% and flight ability was an average of 88.0 ± 3.16%.

e. Natural Environment (22–34°C)

At this holding condition, development of pupae to adult is 14 d. Color changes in the eyes were white, pale yellow, yellow, brownish yellow, strong brown, brown, very dark brown and grayish green (Fig. 5). At 2 d old, the eye color was white (HUE 5Y 8/1), turning pale yellow (HUE 5Y 8/2, HUE 5Y 8/3 and HUE 5Y 8/4) at 3–4, 5 and 6–7 d old, respectively. The compound eyes progressed to yellow (HUE 5Y 8/6) at day 8, turning brownish yellow (HUE 10YR 6/8) at 9 d old; to strong brown (HUE 7.5YR 5/8) at day 10 and to brown (HUE 7.5YR 4/4) after day 11. The pupae are ready to irradiate for sterilization when they are 12 and 13 d old when the eye colors were very dark brown (HUE 7.5YR 2.5/3) and greyish green (1 GLEY 5G 4/2), respectively. On the 14th day of pupation, there was an average of 94.0 ± 2.92% adult emergence and 89.0 ± 2.62% adult fliers.

All results on pupal duration, recommended pupal age for irradiation, and percentage adult emergence and flight ability, from the five holding temperatures are given in Table 1. Irradiation of *A. obliqua* pupae at the Moscafrut Fruit Fly Mass Rearing Facility for the SIT program is done 2 d before emergence at the standard holding temperature (26°C), when the pupae are 12–13 d old, at which stage the eye color is very dark brown (HUE 7.5YR 3/3) and grayish green (1 GLEY 5G 4/2). Using this eye color as the reference guide for optimum radiation sterilization of *A. obliqua*, the optimum pupal age for irradiation when held at 15, 19, 28 and Natural Environment (22–34°C) was 35–39, 28–30, 11 and 12–13 d old, respectively. The percentage adult emergence and percentage flight ability observed at all pupal holding temperatures exceeded the minimum specification set for *A. obliqua* in the FAO/IAEA/USDA Quality Control Manual (2014), and ranged from 85.0–96.2% and 81.0–92.2%, respectively (Table 1). High percentage of emergence and fliers indicate that fly production in the production facility have met the international quality standards through improved automation process and use of new food formulas (Orozco-Davila et al. 2016). The development of *A. obliqua* can adequately be manipulated by delaying or accelerating pupal growth by holding pupae from as low as 15°C to as high as 28°C without affecting the efficiency of the insect. In manipulating pupal development, pupae can be irradiated using eye color as a reference guide to achieve irradiation sterilization. This is very useful when there are failures in the rearing operations in the facility, or with

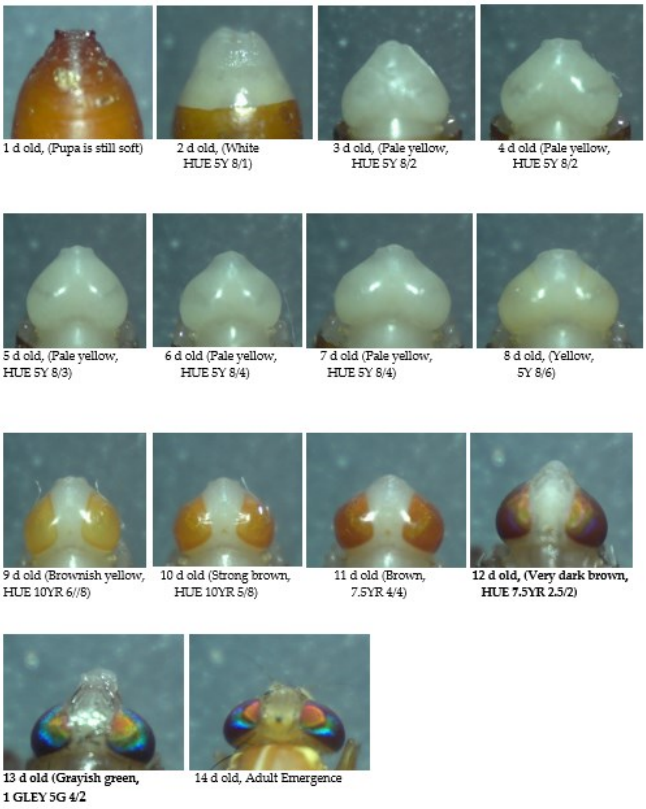

Fig. 5. Photographs of the daily eye color changes of the West Indian fruit fly (*Anastrepha obliqua*) taken during pupal development at Natural Environment (22–34°C). (Pupal age and related color for optimum irradiation is shown in bold font).

release operations in the field, and pupal development needs to be manipulated with temperature.

SUMMARY AND CONCLUSION

We conducted a study on eye color changes during pupal development in the West Indian fruit fly, *Anastrepha obliqua*, at different pupal holding temperatures in order to assess the optimal timing of irradiation sterilization. The recommended age for irradiating *A. obliqua* for optimum sterilization and minimal damage to the pupae and adults is 2 d before adult emergence. At the standard pupal holding temperature of 26°C, pupae should be irradiated when they are 12–13 d old (2 d before emergence), at which stage the pupal eye color should be very dark brown (HUE 7.5YR 3/3) and grayish green (1 GLEY 5G 4/2) as classified by the Munsell® Soil Color Charts. However, problems in the rearing operations in the facility or with release operations in the field can require that pupal development be accelerated or retarded in order to meet sterile fly release schedules, requiring that pupal maturation temperatures be increased or decreased, respectively. Using an eye color of very dark brown (HUE 7.5YR 2.5/2) and grayish green (1 GLEY 5G 4/2) as a reference guide for optimum radiation sterilization, we determined that the optimum age for pupal irradiation when pupae were held at 15, 19, 28 °C and Natural Environment (22–34 °C) was 35–39, 28–30, 11 and 12–13 d old, respectively.

At four holding temperatures, quality control data on adult emergence (85.0–95.2%) and flight ability (81.0–92.2%) using the four holding temperatures exceeded the minimum specifications set in the FAO/IAEA/USDA QC Manual (2003) which ranged from 81.0 ± 1.58 to 96.2 ± 2.77% (Table 1). The results of this study indicate that for the A. obliqua used for SIT programs anywhere in the world, pupae destined for radiation sterilization can be maintained at holding temperatures between 15°C and 28°C without adversely affecting development. The very dark brown (HUE 7.5YR 3/1 to 3/4) and grayish green (1 GLEY 5G 4/2) pupal eye colors as identified in the Munsell® Soil Color Charts for each holding temperature can be used as baseline information in the mass rearing facility to judge the optimum time for sterilizing the pupae when kept at the required holding temperature to accelerate or delay pupal development. This is a very useful tool with the timing or pupal sterilization to avoid or solve potential problems that may be encountered in mass rearing operations in a SIT release program.

Table 1. Pupal duration, recommended pupal age for irradiation, and average adult emergence and flight ability of the West Indian fruit fly, *A. obliqua*, at different holding temperatures*.

Holding Temperature (ºC)	Pupal Duration (d)	Recommended Pupal Age for Irradiation (d)	Adult Emergence (%)	Flight Ability (%)
15	40	35-39	85.0 ± 1.58	81.0 ±1.58
19	31	28-30	90.2 ± 1.48	86.0 ±2.55
26	14	12-13	94.2 ± 4.38	92.2 ±3.19
28	12	11	96.2 ± 2.77	88.0 ±3.16
NE	14	12-13	94.0 ± 2.92	89.0 ±2.62

*Mean of 5 replicates
NE – Natural Environment (22–34°C)

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