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Age- and temperature-related pupal eye colour changes in various tephritid fruit fly species with a view to optimizing irradiation timing

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Abstract. The application of the sterile insect technique to fruit flies involves the mass-production of the pest insects using an artificial diet, irradiation during a narrow time window at the late pupal or early imaginal stage to inhibit reproduction without affecting reproductive capacity, and then release into the target area where the sterile insects compete reproductively with their wild counterparts. The timing of irradiation is important to enable the release of males that are sterile but of good quality and exhibit an acceptable sexual performance. In this study, we examined the pupal development of 12 tephritid (Diptera: Tephritidae) species: *Anastrepha fraterculus* (Wiedemann), *A. ludens* (Loew), *A. obliqua* (Macquart), *A. serpentina* (Wiedemann), *Bactrocera cucurbitae* (Coquillett), *B. dorsalis* (Hendel), *B. invadens* (Drew, Tsuruta & White), *B. oleae* (Rossi), *B. philippinensis* (Drew & Hancock), *B. tryoni* (Froggatt), *B. zonata* (Saunders) and *Ceratitis capitata* (Wiedemann). The insects were reared at various temperatures, in the laboratory (15–28 °C) and under fluctuating natural conditions (20–35 °C). The gradual colour changes of the insect eyes during metamorphosis were observed and photographed, measuring the specific eye colour parameters of each species and matching them with the colour scale of the Munsell Soil Color Charts. The duration of pupal development and the time to emergence in *Anastrepha* species were longer than those in *C. capitata* and *Bactrocera* species at all the holding temperatures. The data obtained can be used by mass-rearing facilities to manage pupal holding conditions and as indicators for optimizing the timing of irradiation.

Key words: *Anastrepha* spp., *Bactrocera* spp., *Ceratitis capitata*, tephritids, irradiation timing, sterilization, pupal eye, eye colour, metamorphosis, Munsell Soil Color Chart

Introduction

The sterile insect technique (SIT) is increasingly being applied as a component of area-wide integrated pest management (AW-IPM) programmes (Hendrichs *et al.*, 2007). It has proven to be an effective environmentally friendly tool for the suppression, containment, prevention or eradication of some major insect pests that affect agricultural crops, animal livestock and human lives (Enkerlin, 2005; Hendrichs *et al.*, 2005; Klassen, 2005). The SIT involves mass-producing the pest insect using an artificial diet, careful timing of irradiation to inhibit reproduction without affecting the performance of the sterile insects, and then releasing them into the target area where they compete reproductively with their wild counterparts (Dyck *et al.*, 2005).

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The application of the SIT requires that insects be irradiated at a specific age to maximize the induction of sterility and to minimize the negative effects on the quality and mating competitiveness of the released sterile adults (Bakri *et al.*, 2005). In facilities mass-rearing the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) at standard holding temperatures, pupae are usually irradiated at about 48 h before adult emergence; this ensures the sterility even of females that are advanced in their development (Ohinata *et al.*, 1971; Williamson *et al.*, 1985; Fisher, 1997; Barnes *et al.*, 2007). This pupal irradiation protocol is also commonly applied to the South American fruit fly *Anastrepha fraterculus* (Wiedemann) (Allinghi *et al.*, 2007), the Mexican fruit fly *Anastrepha ludens* (Loew), the West Indian fruit fly *Anastrepha obliqua* (Macquart) (Hernández *et al.*, 2007), the melon fly *Bactrocera cucurbitae* (Coquillett) (Teruya and Zukeyama, 1979; Teruya and Isobe, 1982), the oriental fruit fly *Bactrocera dorsalis* (Hendel) (Sutantawong *et al.*, 2004) and the Philippines fruit fly *Bactrocera philippinensis* Drew & Hancock (Resilva *et al.*, 2007). However, for genetic sexing strains such as the temperature-sensitive lethal (*tsl*) strains of *C. capitata* (Franz, 2005) that allow the elimination of most of the females at the egg stage, the age at irradiation can be delayed to 24 h before emergence; this increases the sexual performance of the sterile males (Bakri *et al.*, 2005; Cáceres *et al.*, 2007).

Occasionally during SIT operations, there are situations that require fly emergence to be delayed or accelerated, e.g. inclement weather, mechanical failure of irradiation equipment, large differences in cohort size, breakdown in the release operations, and when there is a need to have fewer but larger releases of flies (Enkerlin, 2007). These situations require a non-routine management of the temperature conditions of the pupal rooms to accelerate or delay pupal development and careful monitoring of pupal age for the correct timing of pupal irradiation. For *C. capitata*, pupal eye colour has been used routinely as the most practical and reliable indicator of pupal development and to determine the correct age for irradiation (Ruhm and Calkins, 1981). However, in the various tephritid fruit fly species, pupal development rates differ significantly in different temperature regimens.

Based on the approach developed by Ruhm and Calkins (1981) for *C. capitata*, in this study, we observed systematically the eye colour changes during metamorphosis (from pupation to adult emergence) of 12 tephritid fruit fly species held at a wide range of temperatures. The results obtained can be used as a guide or baseline information to determine pupal age and to properly time irradiation.

Table 1. Fruit fly species and strains and locations at which pupal eye colour changes were recorded at various holding temperatures

Species	Location of recording
Anastrepha fraterculus (ARG)	Austria[1]
Anastrepha ludens	Mexico[2]
Anastrepha obliqua	Mexico[2]
Anastrepha serpentina	Mexico[2]
Bactrocera cucurbitae (bisexual strain)	Mauritius[3]
B. cucurbitae (*wp*-GSS MAR)	Mauritius[3]
B. cucurbitae (*wp*-GSS)	Austria[1]
Bactrocera dorsalis (bisexual strain)	Austria[1]
B. dorsalis (*wp*-GSS)	Austria[1]
Bactrocera invadens	Austria[1]
Bactrocera oleae	Austria[1]
Bactrocera philippinensis	Philippines[4]
Bactrocera tryoni	Austria[1]
Bactrocera zonata	Mauritius[3]
Ceratitis capitata (bisexual strain)	Austria[1]
C. capitata (VIENNA-8)	Austria[1]
C. capitata (VIENNA-8 SAF)	South Africa[5]

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Materials and methods

Insects

The 12 tephritid fruit fly species studied were *A. fraterculus*, *A. ludens*, *A. obliqua*, *A. serpentina* (Wiedemann), *B. cucurbitae*, *B. dorsalis*, *B. invadens* (Drew, Tsuruta & White), *B. oleae* (Rossi), *B. philippinensis*, *B. tryoni* (Froggatt), *B. zonata* (Saunders) and *C. capitata*. In addition, sexing strains of *B. cucurbitae* and *B. dorsalis*, developed in Hawaii (McCombs and Saul, 1995; McInnis *et al.*, 2004), were included and sexing strains of *C. capitata* developed at the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf, Austria, were also included (Franz, 2005). The flies had been reared for several generations. The pupal eye colour recordings were made at research laboratories or at various mass-rearing facilities used in SIT programmes (Table 1). The studies were conducted over several years at various research laboratories and mass-rearing facilities, where experimental conditions were not identical.

Rearing conditions

Adult fruit flies were held in rectangular aluminium and nylon screen cages kept in rooms

maintained at 23 °C and 55–60% relative humidity. Adult flies were provided with water and a mixture of sugar and yeast hydrolysate (3:1 ratio). Females laid their eggs in egging bottles impregnated with guava juice or directly through the screen (eggs fell into metal trays partly filled with water). Eggs were seeded onto the surface of larval diet lined with tissue paper. The larval diet used for each fruit fly species was the one used in the various laboratories and mass-rearing facilities. The larval trays were held in controlled temperature rooms until the larvae left the diet. At all the locations, mature larvae were collected within 1 h of their leaving the larval diet to permit synchronization of the pupal development of the various cohorts studied.

Cohorts of 200–500 ml of larvae of each fruit fly species were mixed with vermiculite or sawdust, subdivided according to temperature regimens, and placed into covered plastic pupation trays. Trays with the larvae were held for pupation in controlled temperature rooms or chilling incubators at temperatures of 15–28 °C, according to the various treatment regimens. Most of the species and strains were also held under trees to simulate pupal development under natural conditions, where the temperatures fluctuated between 20 and 35 °C.

Dissection of pupae, taking of photos and determination of eye colour

From the day of pupation to the day of emergence, about 50–100 pupae of each species at each of the holding temperatures were sampled daily and dissected to observe changes in eye colour. 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). The eyes of the dissected pupae were photographed at 60 × magnification using an Intel QX5 computer microscope (2007 Digital Blue, Inc., Microsoft Corporation, Atlanta, GA, USA). Whole pupal eyes were positioned under the microscope, focused with proper illumination and photographed close-up. The daily eye colour data of each species at each temperature were recorded and then matched and tabulated with the colour scale of the Munsell Soil Color Charts (Anonymous, 2000) (note that the colour chart used by Ruhm and Calkins (1981) was DuMont's Farben-Atlas).

The calibration point at standard holding temperatures for each species was the pupal eye colour on the day of irradiation. Then, for each species and strain, the same pupal eye colour was used as the indicator of the irradiation time for the other holding temperatures. For *A. fraterculus*, no facility that routinely irradiates this species was visited, and therefore no calibration point was available so as to recommend irradiation ages for the different holding temperatures.

Determination of adult emergence and flight ability

Before (1–3 days) adult emergence, samples of 100 pupae in five replications were placed in black Plexiglas tubes to determine adult emergence and flight ability (adult flies that are capable of flight). This was done for all species and strains at all pupal holding temperatures following the standard quality control procedures (FAO/IAEA/USDA, 2003).

Results

Pupal development times until adult emergence for various species and strains at various holding temperatures are given in Table 2. The shortest pupal development times (8–10 days) occurred in *Bactrocera* spp. and *C. capitata* at 28 °C and the longest in *Anastrepha* spp. at 15 °C. At the

Table 2. Pupal development times (days) until adult emergence of various tephritid fruit fly species and strains held at various temperatures

	Pupal holding temperature (°C)					
Species	15	17	20	26	28	23–35[1]
Anastrepha fraterculus (ARG)	57	25	22	14	13	21
Anastrepha ludens	49	–	33[2]	16	15	16
Anastrepha obliqua	40	–	31[2]	14	12	14
Anastrepha serpentina	49	–	31[2]	16	14	15
Bactrocera cucurbitae (bisexual strain)	45	17	15	10	9	11
B. cucurbitae (*wp*-GSS MAR)	–	–	21[2]	11[3]	9	10
B. cucurbitae (*wp*-GSS)	–	19	15	9	8	12
Bactrocera dorsalis (bisexual strain)	–	19	16	10	9	12
B. dorsalis (*wp*-GSS)	–	19	16	10	9	16
Bactrocera invadens	–	21	16	11	9	13
Bactrocera oleae	44	19	15	11	10	17
Bactrocera philippinensis	37	–	22[2]	13[3]	10	10
Bactrocera tryoni	–	–	18	11	9	13
Bactrocera zonata	–	–	21[2]	11[3]	9	10
Ceratitis capitata (bisexual strain)	–	17	16	10	9	11
C. capitata (VIENNA-8)	–	17	14	9	8	15
C. capitata (VIENNA-8 SAF)	29	–	18[2]	11[3]	9	–

[1] Natural environment (temperature fluctuated between 20 and 35 °C).
[2] 19 °C.
[3] 25 °C.

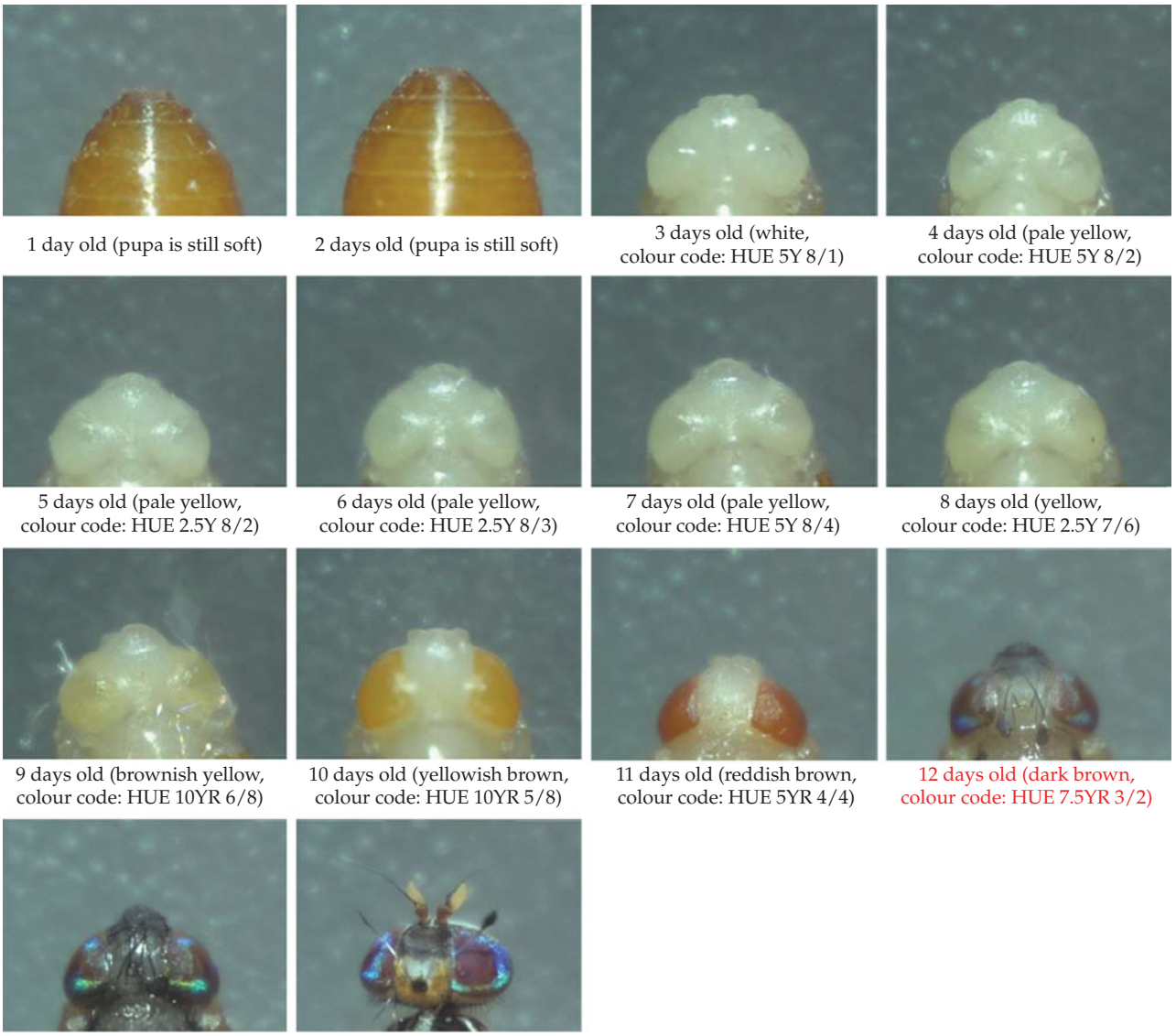

Fig. 1. (colour online) Eye colour change inside the puparium between pupation and adult emergence of *Ceratitis capitata* flies (VIENNA-8 genetic sexing strain) at 20 °C (for the denomination of colour and code refer to the Munsell Soil Color Charts (Anonymous, 2000))

fluctuating temperature regimen (23–35 °C), pupal development times for all species and strains were between 10 and 21 days.

Figure 1 shows an example of the daily matching of colour codes of the Munsell Soil Color Charts with the eye colours that were observed in the dissected pupae. The example shows pupal eye colour change over time in the *C. capitata* VIENNA-8 genetic sexing strain (Franz, 2005) maintained at 20 °C. At this temperature, fly emergence occurs after 14 days in the pupal stage, while 48 h before adult emergence (on day 12 of pupation), the dark brown eye colour is indicative of the age when pupae should be irradiated.

Similar observations on eye colour changes were made for the other fruit fly species and strains that were studied at various temperatures. However, because of the large volume of data obtained in these observations, only one case is presented herein. Based on these daily observations and eye colour matches corresponding to pupal development phases, recommendations can be made for the specific eye colour and corresponding age when irradiation of each fruit fly species held at a given

Table 3. Recommended age after pupation (days) and pupal eye colour to irradiate various tephritid fruit fly species held at various temperatures

	Pupal holding temperature (°C)						
Species	15	17	20	26	28	23–35[1]	Colour code (hue) (reared at °C)
Anastrepha ludens	45	–	30[2]	14	13	14	
Anastrepha obliqua	39	–	30[2]	13	11	13	
Anastrepha serpentina	46	–	29[2]	15	13	14	
Bactrocera cucurbitae (bisexual strain)	41	15	13	9	8	10	
B. cucurbitae (*wp*-GSS MAR)	–	–	19[2]	9[3]	7	8	
B. cucurbitae (*wp*-GSS)	–	17	13	8	7	11	
Bactrocera dorsalis (bisexual strain)	–	17	14	9	8	10	
B. dorsalis (*wp*-GSS)	–	17	14	9	8	14	
Bactrocera invadens	–	18	14	10	8	12	
Bactrocera oleae	40	16	13	9	8	12	
Bactrocera philippinensis	33	–	20[2]	11[3]	9	15	
Bactrocera tryoni	–	–	16	9	8	11	
Bactrocera zonata	–	–	19[2]	9[3]	7	8	
Ceratitis capitata (bisexual strain)	–	15	14	9	8	10	?
C. capitata (VIENNA-8)	–	15	12	8	7	13	7.5YR 3/2 [20]
C. capitata (VIENNA-8 SAF)	26	–	12	8	7	–	?

[1] Natural environment (temperature fluctuated between 20 and 35 °C).
[2] 19 °C.
[3] 25 °C.

holding temperature should be done. Therefore, we compiled a list of the recommended times (pupal ages) to irradiate each species at a particular holding temperature (Table 3). The eye colours at these times were also observed. For the lower holding temperatures, where the correct pupal eye colour lasts for several days, the latest day is the recommended irradiation day.

With reference to the quality control data, adult emergence ranged from 83.2 to 98.6% and flight ability from 81.0 to 98.0% for all species and strains at temperatures of 17 °C and above. All these results were above the standard minimum specifications (FAO/IAEA/USDA, 2003).

Discussion

The study carried out by Ruhm and Calkins (1981) on eye colour changes of the imago inside the puparium during pupal development was the first attempt to use eye colour as an indicator of the stage of pupal development and then to link it to the optimal age for irradiation and the level of sterility achieved as confirmed by bioassays. This linkage has been the basis for managing the timing of pupal irradiation in *C. capitata* mass-rearing facilities. Their study also provided information to operational programmes on how to manage pupal holding temperatures under adverse conditions. However, their study was conducted using a colour standard based on DuMont's Farben-Atlas.

Our study, using the international standard Munsell Soil Color Charts (Anonymous, 2000), extended this initial work to other species of fruit flies that are the target of SIT programmes. We also observed pupal development at various temperatures in the laboratory and under fluctuating natural conditions. We found that *Anastrepha* species have longer pupal development times and correspondingly slower pupal eye colour changes than the other species.

The pupal eye colour changes differed among the species. The pupal eye colour at 48 h before adult emergence of *B. dorsalis*, *B. oleae* and *C. capitata* is dark brown; this indicates the correct timing of irradiation at all holding temperatures. For *B. cucurbitae* and *B. invadens* pupae, the symptomatic eye colours are dusky red and dark reddish brown, respectively. *Anastrepha ludens* and *A. obliqua* pupae should be irradiated when the eye colour is dark brown, very dark brown and greyish grey, while for *A. serpentina* the indicative colour is dark reddish brown. As no routine irradiation of *A. fraterculus* is conducted, no recommended eye colour for radiation sterilization of this species is available.

The progression of pupal eye colour changes determined for each species can be used as baseline information at mass-rearing facilities for timing the sterilization of pupae at the desired holding temperatures and also for managing pupal development according to programme needs for sterile flies. The results of this study indicate that pupae

of all tephritid fruit fly species used in SIT programmes can be maintained at holding temperatures between 17 and 28 °C without affecting adult emergence and flight ability.

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