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## EFFECTS OF PRE-RELEASE CHILLING ON THE FLIGHT ABILITY OF STERILE MALES OF THE MEDITERRANEAN FRUIT FLY (DIPTERA: TEPHRITIDAE)

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### ABSTRACT

The Sterile Insect Technique (SIT) is used to suppress or eradicate infestations of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann). The success of the SIT depends to a large degree on the ability of sterile males to compete successfully against wild males in obtaining matings with wild females. Sterile males are chilled to allow their transfer to and subsequent storage within the aircraft used for the releases. Here, we describe the results of an experiment that investigated the effects of varying chill duration (at 3-8 °C for 2-6 h) on flight ability of sterile males derived from mass-rearing facilities in Hawaii and Guatemala. Flight ability decreased significantly, and at the same rate, with increasing chill duration for flies from both production facilities. However, for any given chill duration, the Hawaii-derived flies displayed greater flight ability than the Guatemala-derived flies. In addition, there was significant variation in flight ability among daily shipments from both facilities. Nevertheless, the present data clearly reveal that limiting chill duration promotes higher flight performance of released sterile males. Implications of our findings for Mediterranean fruit fly SIT programs are discussed.

Key Words: Sterile Insect Technique, mass rearing, chilling, temperature effects

### RESUMEN

Se utiliza La Técnica del Insecto Estéril (TIE) para suprimir o erradicar las infestaciones de la mosca mediterránea de la fruta (mosca de la fruta), *Ceratitis capitata* (Wiedemann). El éxito de la TIE depende en gran medida de la capacidad de los machos estériles para competir con éxito contra los machos silvestres en el apareamiento con las hembras silvestres. Se enfrían los machos estériles para permitir su traslado y su almacenamiento subsiguiente en el avión utilizado para liberarlos. A continuación, describimos los resultados de un experimento que investigó los efectos de la duración variable (frío a 3-8 °C durante 2-6 horas) sobre la capacidad de vuelo de machos estériles criados en las instalaciones de la producción masiva de Hawaii y Guatemala. La capacidad de vuelo disminuyó significativamente, y en la misma proporción, con el aumento en la duración del enfriamiento para las moscas de las dos instalaciones de reproducción. Sin embargo, en todos los períodos de enfriamiento, las moscas de Hawaii mostraron una mayor capacidad de vuelo que las moscas de Guatemala. Además, hubo una variación significativa en la capacidad de vuelo de las moscas entre los envíos diarios de ambas instalaciones. Sin embargo, estos datos muestran claramente que la limitación de la duración del enfriamiento promueve un mayor rendimiento de vuelo de machos estériles liberados. Se comenta sobre las implicaciones de nuestros resultados para los programas de TIE para la mosca mediterránea de la fruta.

Palabras Clave: Técnica del Insecto Estéril, cría en masa, enfriamiento, efectos de temperatura

The Sterile Insect Technique (SIT) is widely used to suppress or eradicate infestations of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), a pest that attacks many

commercially important fruits and vegetables worldwide (Enkerlin 2005). The SIT involves the production, sterilization, and release of large numbers of male Mediterranean fruit flies into

the environment. Matings between sterile males and wild females result in the oviposition of infertile eggs, causing a decline of the wild population. The success of the SIT depends to a large degree on the ability of sterile males to compete successfully against wild males in obtaining matings with wild females (Calkins 1984; Calkins & Ashley 1989).

Unfortunately, there is ample evidence showing that, with respect to procuring copulations, sterile males are competitively inferior to wild males (e.g., Rössler 1975; Lance et al. 2000). Reasons for this trend are not known with certainty, but several studies (Liimatainen et al. 1997; Briceño & Eberhard 1998) identified altered courtship behavior, presumably arising through artificial selection under crowded mass-rearing conditions, as a key factor. Other features of the production and release process may also constrain the performance of sterile male Mediterranean fruit flies. For example, the composition of the adult diet, specifically the presence or absence of protein, may influence the mating success of sterile *C. capitata* males (Yuval et al. 2007). Other studies have examined the potential influence of radiation dose (Wong et al. 1983; Shelly et al. 2005) and atmosphere (Hooper 1971), dietary microbes (Ben-Yosef et al. 2008), and age at release (Liedo et al. 2002) on the sexual competitiveness of sterile *C. capitata* males.

In SIT programs against the Mediterranean fruit fly, sterile males are typically chilled to allow their transfer to and subsequent storage within the aircraft used for the releases. This method was adopted over 30 years ago and allows the release of a large volume of sterile flies (FAO/IAEA 2007). Despite its wide use, however, relatively few studies have examined the effects of chilling on the flight performance of sterile males of any pestiferous tephritid species, and these have yielded inconsistent results. For example, Tanahara and Kirihara (1989) found no decline in the flight ability of sterile males of *Bactrocera cucurbitae* (Coquillett) chilled between 1-4.5 h. Similarly, Reynolds and Orchard (2011) found that flight performance of *B. tryoni* (Froggatt) was unaffected by chilling for up to 4 h. Working with the medfly, Salvato et al. (2003) reported no significant decrease in flight ability between unchilled males and those chilled for 2.5 h. In contrast, to these studies, Shelly et al. (2012) observed that chilling negatively affected the flight ability of sterile males of *C. capitata* held at high densities (i.e., those used in eclosion towers, see below) prior to testing. In light of this limited and inconsistent data set, a recent report (FAO/IAEA 2007) cited the need for additional studies on the potential effects of pre-release chilling on the performance of sterile *C. capitata* males.

Here, we describe the results of an experiment that expands upon those described in Shelly et

al. (2012) in 2 important ways. In that previous study, the effects of chilling were investigated at the site of fly production and so did not incorporate the possible impact of shipping (both in terms of time and handling) to the eclosion and release site on fly performance. In the present study, by contrast, the effect of chill duration was assessed for flies following their transport from 2 production facilities to an eclosion/release facility in southern California. In addition, the earlier study included only medflies produced in Hawaii, whereas this study also includes medflies derived from a production facility in Guatemala. While the same strain of *C. capitata* is reared under very similar conditions at both facilities, there nonetheless may be differences between flies from the 2 sources, e.g., the pupae derived from Hawaii are generally larger than those from Guatemala (M. War, unpublished data). Consequently, we investigated the relationship between chill duration and flight ability to examine another potential difference between *C. capitata* males from the 2 production sources. The implications of our findings for Mediterranean fruit fly SIT programs are discussed.

#### MATERIALS AND METHODS

Experiments were conducted at the California Department of Food and Agriculture's (CDFA) David Rumsey Eclosion and Release Facility, Los Alamitos, California, which serves as the administrative and operations headquarters for a Medfly Preventative Release Program covering  $\approx 6,400$  km<sup>2</sup> ( $\approx 2,500$  mi<sup>2</sup>) in southern California. A basic knowledge of the pre-release procedures for handling pupae and adult flies is necessary for understanding the experimental design, and a brief outline of this protocol follows.

Fly pupae are obtained from 2 rearing facilities, the CDFA Fruit Fly Rearing Facility, Waimanalo, Hawaii, and the United States Department of Agriculture (USDA) Medfly Rearing Facility, El Pino, Guatemala. Both facilities produce flies of the strain Vienna 7/Tol-99, a genetic sexing strain containing a temperature sensitive lethal (*tsl*) mutation, which, following heat-induced death of female eggs, allows production of males exclusively (Franz et al. 1996). At both facilities, the pupae are coated with fluorescent dye (3 g per liter of pupae; Schroeder et al. 1972), packed in 4 L plastic bags, and irradiated (using a Co<sub>60</sub> source) under hypoxia (to reduce the deleterious effects on adult quality; Nestel et al. 2007) at a target dose of 145 Gy (Guatemala) or 150 Gy (Hawaii) at 2 d prior to adult emergence. Application of dye is standard protocol for SIT programs, and dye particles retained on the retracted ptilinum or the body of emerged adults allows differentiation between released and wild males. Following irradiation, bags with pupae are

packed along with coolant in insulated shipping boxes and transported via commercial airlines to Los Angeles, where CDFA personnel collect and transport them to Los Alamitos. The time spent under hypoxia during handling and shipping pupae is typically 24-25 h for pupae from both Hawaii and Guatemala.

Upon arrival at the eclosion facility, the pupae are removed from shipping boxes and loaded into eclosion towers. Eclosion towers are composed of stacks of aluminum frame trays (75 cm by 75 cm by 2.5 cm) with lumite mesh bottoms (24 by 24 per sq. inch, or  $\approx 6.45 \text{ cm}^2$ ) placed on wheeled frames that can be moved easily. Each tray has a small ridge on top and a channel on the bottom that interlock with adjacent trays and thus stabilize the entire tower. Pupae are placed in channels along all 4 sides of each tray, and eclosed adults exit the channels to gain access to food and water (provided by 2 blocks of sugar-agar gel per tray, each 10 cm by 7.5 cm by 1.25 cm) and resting space. A volume of 350 ml of pupae ( $\approx 21,000$  pupae) is placed in each tray. An electric fan (blowing upwards) is attached to the top of each tower to provide air circulation and cooling. The number of trays composing individual towers varied daily with shipment size, with towers composed of 52-53 trays for flies from the Hawaii facility and 41-50 trays for flies from the Guatemala facility.

The loaded towers are housed inside climate controlled rooms (24-27 °C, 50-80% RH, continuous darkness) for 4 d, with peak adult emergence occurring 2 d after pupal placement. After this 4-d period, the towers are wheeled into a refrigerated trailer (typically maintained at 4 °C but with sporadic fluctuations between 3-8 °C), where they are chilled for 45-80 min to immobilize (or “knock-down”) the flies. Towers are then disassembled tray by tray inside the chilled trailer, and flies are dislodged by striking the trays over chilled release boxes (3-8 °C), which are then taken to aircraft for release. The typical duration from the start of cooling for knockdown until release is 2-3 h.

On a given sampling d,  $\approx 60$  ml of adult flies were taken from the 30th tray (counted from the bottom) from each of 6-8 towers for a given source (i.e., Hawaii or Guatemala). The sample was collected by shaking flies on to a sheet of white paper and then transferring flies to a container, which was placed in an insulated cooler. Upon collection of all samples, the cooler was taken to a separate refrigerated trailer (maintained at the same temperature as the knockdown trailer), where 5 sub-samples each of 100 males were counted per tower. These sub-samples were then held for varying chill durations (2, 3, 4, 5, or 6 h), with 1 sub-sample per tower held for each of the respective chill durations. (We recognize that our study included no control (non-chilled) group, but this was not feasible as the interlocking tower trays prevented

sampling flies without chilling). Thus, on a given sampling d, adult flies from 6-8 different towers were held at each of the different chill durations. Both Hawaii- and Guatemala-produced flies were sampled in this manner on 15 different d, with sampling conducted for only 1 source per d. To reduce verbiage, flies tested on the same d belonged to the same “batch”, meaning flies tested on different d belonged to different batches.

After the appropriate chill duration, flies were transferred to the laboratory (23-25 °C and 45-75% RH) for flight ability testing. The test protocol was based on the standard procedure for measuring flight ability in sterile, mass-reared tephritid fruit flies (FAO/IAEA/USDA 2003). Briefly, the trials involved placing the flies at the bottom of a 10 cm tall, plastic tube (with talc-coated inner surface to prevent escape by walking) and recording the number of flies that successfully exited the tube after 2 h.

Data on flight ability were analyzed using Restricted Maximum Likelihood (REML) to fit a standard least squares model. The model used was: # fliers = chill duration + colony source + batch + sample number (which refers to the chronological order in which samples were taken on a given d) + chill duration\* chill duration+chill duration\* colony source. Chill duration and colony source were fixed effects, while batch and sample number were treated as random effects (with batch treated as a repeated measure nested in colony source). The 2 interaction terms assessed i) the linearity of the model (by examining variation in the level of response among different chill durations, where an insignificant interactions denotes linearity) and ii) the similarity in the relationship between flight ability and chill duration between the 2 sources (where an insignificant interaction denotes similar responses [slopes] for the 2 sources). Effects that were not statistically significant were removed from the model, and the data analyzed using the reduced model to obtain the simplest equation. As shown below, the final model was: # fliers = chill duration + colony source + batch + sample number. Statistical analyses were completed using JMP 9 (2010 SAS Institute).

In addition to the results derived from experimental manipulation of chill duration, we present post-shipment data from standard quality control tests for adult emergence and flight ability for the specific batches used in the experiments. As part of its routine operating procedure, CDFA collects random samples of pupae from daily shipments from both Hawaii and Guatemala, places the pupae in Petri dishes at the bottom of the plastic tubes (prepared as described above), and records the number of flies that i) successfully emerge from puparia and ii) successfully exit the tube after 4 d (see FAO/IAEA/USDA 2003 for details). This measure of flight ability is thus a composite

index, which includes adult emergence and subsequent flight from the tubes. Here, for comparative purposes, we also computed an "adjusted" flight ability, which presents the relative number of males escaping the tubes based on the number of successfully emerged adults (and not the number of pupae tested as in the standard FAO/IAEA/USDA [2003] index). Pre-shipment estimates of flight ability were not included in the study, because factors potentially affecting fly quality during transit were beyond our control, and so emphasis was placed on measuring the performance of flies following their arrival in Los Alamitos (the routine quality testing using pupae) and their eclosion and chilling (our experimental testing using adults). In addition, following standard FAO/IAEA/USDA (2003) procedures, we computed the average pupal weight for each batch based on measurements of 5 lots of 100 pupae per batch.

### RESULTS

Chill duration ( $F = 301.5$ ,  $df = 1$ ,  $P < 0.0001$ ) had a significant negative effect of flight ability that was linear (chill duration \* chill duration,  $F = 0.002$ ,  $df = 1$ ,  $P = 0.96$ ; Fig. 1). Colony source ( $F = 87.1$ ,  $df = 1$ ,  $P < 0.0001$ ) also had a significant effect on post-chill flight ability. Collectively, these 2 fixed effects accounted for approximately 2/3 of the total variation observed ( $r^2 = 0.67$ ). Although colony source did not alter the slope resulting from chill duration effects (chill duration\*colony source,  $F = 0.38$ ,  $df = 1$ ,  $P = 0.54$ ), the large colony source effect indicates a difference between the intercepts for

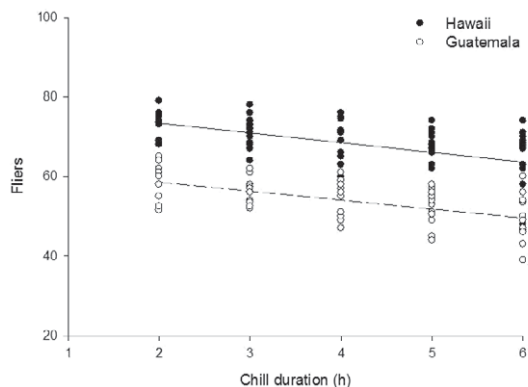


Fig. 1. Relationship between flight ability and chill duration for *Ceratitits capitata* sterile males from rearing facilities in Hawaii and Guatemala. For a particular chill duration, each symbol represents the average number of males in groups of 100 males ( $n = 6-8$  groups, each derived from a different eclosion tower) that escaped flight tubes on a particular test date ( $n = 15$  d for both Hawaii and Guatemala). Regression equations: Hawaii  $Y = 78.10 - 2.33 \cdot \text{chill duration}$ ; Guatemala:  $Y = 63.44 - 2.33 \cdot \text{chill duration}$ .

the Hawaii and Guatemala colonies as clearly illustrated in plots of flight ability versus chill duration (Fig. 1).

The 2 random effects, sample number and batch, accounted for about 3% and 29% of the variance unexplained by the 2 fixed parameters, respectively (the remaining 68% was unexplained error). As batch appeared to have a large effect on flight ability, we subsequently analyzed data separately for the 2 colonies considering chill duration and batch as fixed effects in generalized linear models. For both colonies, both chill duration (Hawaii: Likelihood Ratio  $\chi^2 = 288.2$ ,  $df = 1$ ,  $P < 0.0001$ ; Guatemala:  $\chi^2 = 245.0$ ,  $df = 1$ ,  $P < 0.0001$ ) and batch (Hawaii:  $\chi^2 = 482.9$ ,  $df = 14$ ,  $P < 0.0001$ ; Guatemala:  $\chi^2 = 333.1$ ,  $df = 14$ ,  $P < 0.0001$ ) had significant effects on flight ability. Data from the 2 h chill duration illustrate the high level of inter-batch variation observed. Under this treatment, the average number of fliers ( $n = 6-8$  samples [towers] each of 100 males) ranged from 64-79 among the 15 batches of Hawaii flies and between 52-65 fliers among the 15 batches of Guatemala flies.

The standard quality control measures of emergence and flight ability differed between Hawaii- and Guatemala-derived flies for the 15 batches sampled from each production facility. Males from Guatemala displayed significantly higher emergence ( $91 + 1\%$  vs.  $85 + 1\%$ ;  $t = 4.7$ ,  $df = 28$ ,  $P < 0.001$ ) and flight ability ( $86 + 1\%$  vs.  $80 + 2\%$ ;  $t = 3.9$ ,  $df = 28$ ,  $P < 0.001$ ) than males from Hawaii. However, adjusted flight ability (which effectively eliminates the difference in emergence success) was the same ( $t = 0.1$ ,  $df = 28$ ,  $P = 0.94$ ) between males from Guatemala ( $94 + 1\%$ ,  $n = 15$ ) and Hawaii ( $94 + 2\%$ ,  $n = 15$ ). This latter result indicates that, among flies that emerged from puparia, there was no significant difference in the flight ability of flies from Guatemala and Hawaii before placement in the eclosion towers. For the batches used in this study, there was a significant size difference between pupae from Hawaii ( $8.38 + 0.08$  mg,  $n = 15$ ) and Guatemala ( $8.14 + 0.03$  mg,  $n = 15$ ;  $t = 2.8$ ,  $df = 28$ ,  $P = 0.01$ ).

There were no significant relationships between the standard post-shipment/pre-chilling quality control measurements and post-chill flight ability for batches from either Hawaii or Guatemala. Among the 15 batches sampled from each source, we tested for correlations (using mean values) between pupal weight and i) emergence, ii) adjusted flight ability, and iii) flight ability after 2 h of chilling. We also examined possible relationships between iv) emergence and flight ability after 2 h of chilling and v) adjusted flight ability and flight ability after 2 h of chilling. In no instance, however, was a significant correlation detected in any of these tests for either Hawaii- or Guatemala-derived flies.

## DISCUSSION

The present study demonstrated a decline in flight ability of sterile male medflies with increasing chill duration for flies from both the Hawaii and Guatemala rearing facilities. The rate of decline was similar between flies from the 2 facilities, however, for any given chill duration, flies from Hawaii facility displayed greater flight ability than flies from the Guatemala facility. As noted, the adjusted flight ability (computed using data from the standard tests) did not differ significantly between Hawaii- and Guatemala-derived flies, with the average adjusted flight ability (over 15 batches per production source) being 94% for flies from both 2 facilities. This finding indicates that the difference observed in post-chill flight ability derived not from transit-related factors, but from subsequent handling after pupal arrival at the Los Alamitos facility. In addition, although pupae from Hawaii were, on average, larger than those from Guatemala, we found no association between pupal size and various performance indices, namely emergence, routine flight ability, adjusted flight ability, or flight ability after 2 h of chilling. We therefore conclude that the pupal size difference was not responsible for the difference in post-chilling flight ability observed between flies from Hawaii and Guatemala.

Without a non-chilled control group, we are unable to assess the relative importance of chilling and pre-knockdown holding conditions in adversely affecting the flight ability of emerged adult males. That is, the lower flight performance of the Guatemala flies could have reflected a larger, negative response to the chilling per se, the fly density and food availability in the holding trays, or a combination of both. As noted above, the interlocking trays comprising an eclosion tower prevented access to the emerged adults, precluding this control. However, by drilling a hole in a tray, it may be possible to extract flies using an aspirator just prior to chilling, and we are exploring use of such modified trays with program officials. In fact, earlier results (Shelly et al. 2012) based on flies from the Hawaii production facility suggest the effects of chilling may depend on the density under which flies were held before chilling: chilling had no effect on the flight ability of males held at low density in small cages but resulted in decreased flight for flies held in eclosion tower trays at the same density employed in this present study. High density is known to promote frequent male-male aggression and reduced vigor (Gaskin et al. 2002), and this stress, when combined with chilling, may act to lessen flight ability. While this explanation appears reasonable, Salvato et al. (2003), working with medflies from the Guatemala facility, reported no effect of chilling on flight performance even though flies were held at a higher pre-release density than used in

the present study. Clearly, additional manipulations of chill duration and holding density appear necessary to elucidate general patterns and subsequently assess possible changes to routine handling practices.

Interestingly, significant variation in flight ability was detected among daily shipments (batches) for both rearing facilities. As rearing procedures are relatively constant across d, daily variation in fly quality may reflect variation in other factors, such as environmental conditions (temperature, RH, etc.), the quality and composition of larval diet ingredients, or the microbial community existing in the larval diet. The effect of these factors on the quality of medflies is poorly known for any fruit fly mass-rearing facility (but see Barnes et al. 2007), and so this explanation remains conjectural.

In conclusion, sterile male medflies must overcome various hurdles in order to compete successfully against wild males for wild females, including locating and joining natural mating (lek) sites (Prokopy & Hendrichs 1979). Reduced flight activity could obviously interfere with this requirement and so constrain the effectiveness of an SIT program. While certain barriers are difficult to mitigate (e.g., limiting artificial selection for shorter male courtship under the crowded conditions necessary for high production volume; Briceño & Eberhard 1998), adjusting tower tray densities or/and chill duration to maximize the dispersal of released flies appears a feasible option. As noted above, however, more data on the individual and collective effects of chilling and density are required to identify the necessary adjustments. Nonetheless, the present data clearly reveal that limiting chill duration will promote higher flight performance among released sterile males.

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