# Testing the Efficacy of Aromatherapy at the World's Largest Eclosion Facility for Sterile Males of the Mediterranean Fruit Fly (Diptera: Tephritidae)

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**Abstract.** Exposure to the aroma of ginger root oil (GRO) increases the mating competitiveness of adult sterile males of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann). This effect has been observed under various exposure regimes, ranging from small groups of males (25 individuals) held in small cups to large numbers of males (14 million individuals) held in trailers. Here, we assess the efficacy of GRO treatment at an even larger scale at the world's largest eclosion facility for sterile male medflies located in Retalhuleu, Guatemala. GRO exposure was conducted in several different holding rooms each with a particular dose (0.37–0.91 ml GRO per $m^3$ room volume) and each with a unique number of sterile males (59–127 million adult males per room). Treated sterile males were exposed to GRO at 3 d of age for 24 h and then held 24 h before testing. Control sterile males were tested at the same age as treated males but were held in rooms not receiving GRO application. In field tents, we released 50 sterile males (treated or control), 50 wild males, and 50 wild females and collected copulating pairs. Over all trials, control sterile males obtained 19%–26% of the total matings per replicate, whereas treated sterile males obtained significantly higher proportions of the total matings (34%–41%) for all doses tested. Among treated sterile males, relative mating success did not vary significantly with GRO dose. These findings suggest that the use of GRO aromatherapy in the large holding rooms in the Retalhuleu facility will substantially enhance the mating competitiveness of the sterile male medflies.

**Key words:** *Ceratitis capitata*, sterile insect technique, mating competitiveness, ginger root oil

Exposing adult sterile males of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), to the aroma of ginger root oil (GRO hereafter, *Zingiber officinale* Roscoe) increases their mating competitiveness, and hence implementation of this procedure may improve the effectiveness of the Sterile Insect Technique (SIT) against this serious agricultural pest (Shelly 2008). When competing against wild males for copulations with wild females in field tents, untreated sterile males typically account for only 20%–33% of all matings (1:1 sterile:wild male ratio). However, exposing sterile males to the aroma of GRO boosts mating success dramatically, and exposed males typically obtain 35%–50% of all matings. This general finding derives from a series of experiments that involved GRO exposure to increasing large numbers of sterile males (Shelly 2008). Initial work involved exposure of groups of 25 males in plastic drinking cups. GRO exposure was then applied to individual PARC boxes (Plastic Adult Rearing Containers, ≈ 0.1 $m^3$) holding ≈ 40,000 males and individual eclosion towers (≈ 0.9 $m^3$) holding ≈ 1.25 million males. Both of these exposure regimes resulted in a significant increase in the mating competitiveness of sterile males. Subsequent work conducted at the USDA-CDFA medfly eclosion facility in Los

Alamitos, CA, showed that GRO exposure of entire rooms (≈ 135 $m^3$) holding ≈ 14 million males in PARC boxes likewise resulted in increased mating performance of GRO-exposed sterile males relative to non-exposed sterile males.

The present study expands upon this earlier research and assessed the effectiveness of GRO exposure at the Retalhuleu Packing Center for Chilled Flies, Retalhuleu, Guatemala, the largest eclosion facility for sterile male medflies in the world. The facility typically receives 129 million irradiated pupae each day and, correspondingly, releases this same number of adult sterile males daily into the environment. Here, we describe GRO exposure of entire rooms, holding as many as 127 million adult sterile males, and present the results of mating trials that compared the mating success of sterile males from GRO-exposed versus non-exposed rooms in competition against wild males for copulations with wild females.

## Materials and Methods

**Study insects.** Mass-reared flies were obtained from the El Pino Center for Excellence for the Production and Investigation of Fruit Flies, El Pino, Guatemala, which produces a *tsl* (temperature sensitive lethal) genetic sexing strain (Vienna-7/Tol-99). Owing to a sex-linked mutation, heating eggs allows for selective culling of female embryos and the exclusive production of males (Franz et al. 1996). At the El Pino facility, larvae are reared on standard diet (Tanaka et al. 1969), and pupae are dyed with a pink fluorescent powder (residue remains on the adult fly allowing identification following release) and irradiated (100 Gy) 2 d before adult emergence. Dyed, irradiated pupae (exclusively males) are transported daily by refrigerated truck from the rearing facility to the eclosion facility in Retalhuleu, Guatemala (4 h trip).

At the eclosion facility, the pupae are placed in PARC boxes (≈ 75,000 pupae per box at the time of our study). A slab of sugar agar is placed on the screened opening on top of each box as a source of food and water for the emerging adults. The boxes are then moved into holding rooms maintained at 21–23°C and 65%–70% RH. Peak emergence occurs 2 d after pupal arrival, and adult males are chilled (40–50 min at 0–3°C) and released when 3 d old. Chilling (or knock-down) occurs in the early morning, and cooled flies are immediately transported to airplanes for release over western Guatemala and southern Mexico.

Wild flies were reared from coffee fruits collected in the vicinity of Antigua, Guatemala. Fruits were returned to a laboratory in Petapa, near Guatemala City, and the wild flies completed their development in situ and pupated in sawdust placed beneath the fruits. Emerging adults were separated by sex within 1 d of eclosion and were given water and a sugar-protein diet (yeast hydrolysate and sucrose in a 3:1 ratio by weight). Several days before starting the mating trials, the adult wild flies were transported by air-conditioned car from Petapa to Retalhuleu (4 h trip). At both sites, wild flies were held at 24–26°C and 65–75% RH and received artificial light under a photoperiod of 12:12 (L:D) h. At Retalhuleu, wild flies were held in a room isolated from sterile males. Wild flies of both sexes were 9 d old when tested (sexual maturity in wild flies is attained at a later age than in the mass-reared strain).

**Ginger root oil exposure and mating trials—2006.** Experiments were performed in March, 2006, and April, 2008. Because the protocols used for both GRO exposure and the mating trials differed between years, we describe them separately. In both years, the GRO used was obtained from Citrus & Allied Essences Ltd., Lake Success, NY.

In 2006, we exposed sterile males to GRO on three different days, each involving a different holding room containing a unique number of sterile males exposed to a unique GRO dose. Previous work (Shelly et al. 2007) conducted in California indicated that an application of approximately 0.3 ml GRO per $m^3$ of room volume resulted in a significant

**Table 1.** Parameters for the individual holding rooms subject to ginger root oil (GRO) exposure at the eclosion facility in Retalhuleu, Guatemala, in 2006 and 2008.

| **2006** | **Holding room** | | |
|---|---|---|---|
| **Parameter** | **A** | **B** | **C** |
| Room volume ($m^3$)[a] | 432 | 432 | 792 |
| PARC boxes present | 1,332 | 1,110 | 2,389 |
| Pupae loaded (millions) | 100 | 83 | 179 |
| Flight-able males (millions)[b] | 71 | 59 | 127 |
| Total dose GRO (ml) | 160 | 200 | 400 |
| GRO/room volume (ml/$m^3$) | 0.37 | 0.46 | 0.50 |
| GRO/flight-able male (ml/million) | 2.25 | 3.39 | 3.15 |

| **2008** | **Holding room** | | |
|---|---|---|---|
| **Parameter** | **E/F[c]** | **G/H** | **I/J** |
| Room volume ($m^3$)[a] | 504 | 432 | 504 |
| Number PARC boxes present | 1,710 | 1,320 | 1,775 |
| Pupae loaded (millions) | 128 | 99 | 133 |
| Flight-able males (millions)[b] | 91 | 70 | 94 |
| Total dose GRO (ml) | 216 | 252 | 459 |
| GRO/room volume (ml/$m^3$) | 0.43 | 0.58 | 0.91 |
| GRO/flight-able male (ml/million) | 2.37 | 3.60 | 4.88 |

[a]Rooms were square or rectangular with ceiling height of 3 m.
[b]Values represent the estimated total number of flight-able, adult males, where an estimated 71% of pupae yield adult males capable of flight in standardized flight-ability tests (FAO/IAEA/USDA 2003).
[c]Pairs of rooms, each having same values for all parameters.

increase in the mating success of the GRO-exposed (treated) sterile males. In Retalhuleu, we tested three different doses (i.e., one dose per holding room used), ranging from 0.37 to 0.50 ml GRO/$m^3$ of room volume (Table 1).

Sterile males were exposed to GRO for the 24 h period immediately preceding knock-down. For all exposure periods, we placed 10 ml of GRO on individual cotton wicks (15 cm long, 1 cm diameter) and placed the wicks evenly around the holding room. The number of wicks used per room varied with room size and desired dose from 16 (Room A) to 40 (Room C). Wicks were suspended from metal rods with paper clips, and the rods, in turn, were suspended between adjacent rows of PARC boxes about 1.5 m above the floor. All rooms were ventilated with three large fans placed equidistant from one another along the walls and oriented toward the center of the room. Following placement of the GRO-laden wicks, we randomly selected and marked (with flagging tape) eight PARC boxes.

During knock-down the following morning, we collected a sample of 100–200 males from each of the marked boxes for subsequent use in the mating trials. During the same knock-down, we also collected sterile males (100–200 individuals from each of eight randomly chosen PARC boxes) that had been held in a separate holding room not exposed to GRO. These non-exposed (control) males were the same lot (and thus the same age) as the

GRO-exposed males. The sterile males were held in screen cages (30 cm cubes with sugar agar provided) until the following day when they were used in mating trials (i.e., sterile males were 4 d old when tested). Treated and control sterile males were kept in separate rooms (under the same conditions described above for the wild flies). Wild flies were neither chilled nor exposed to GRO prior to testing.

Mating trials were performed in a coffee field near Filadelfia (elevation 550 m), 6 km north of Retalhuleu, using nylon-mesh, tents (3 m high, 3 m diameter) placed over individual coffee bushes planted beneath large shade trees. Sixteen tents were used each of the three test days, with eight tents involving treated sterile males and eight tents involving control sterile males. We released 50 sterile males (treated or control), 50 wild males, and 50 wild females into each tent, with males being released at 0645 hrs and females released 15 min later. Treated and control sterile males were randomly assigned to individual tents on each test day, and all sterile males released in a given tent derived from a "unique" (non-shared) PARC box. Mating pairs were collected continuously until 1200 hrs by gently coaxing individual pairs into plastic vials. Based on hourly measurements averaged over the three test days, air temperature increased from approximately 21°C to 28°C and relative humidity decreased from 73% to 65 % between 0700–1200 hrs. At the end of the trial, the remaining (unmated) flies were captured and removed from the tents, the mated pairs were returned to the laboratory, and the males were identified using a black (UV) light.

**Ginger root oil exposure and mating trials—2008.** As noted below, the GRO doses tested in 2006 had a significant effect on the mating success of sterile males, and to confirm these results, in 2008 we re-tested two similar GRO doses and also tested a third, higher dose (Table 1). In 2008, we exposed three pairs of holding rooms to GRO, with paired rooms (1) having the same volume, male "population," and GRO dose, and (2) being exposed to GRO on different (consecutive) days.

As in 2006, GRO exposure lasted 24 h and commenced 24 h before knock-down. Unlike 2006, however, in 2008 GRO was applied to thin sponges (7.5 by 15 cm, 3 mm thick), which were then placed directly on the fans (specifically, on the metal screen covering the blades). For all three doses tested, we placed three sponges on each fan in a given room (i.e., 9 sponges total per room) and divided the total GRO dose equally among the sponges. Collection and holding of treated and control sterile males followed the same procedures used in 2006, and mating tests were conducted the day following GRO exposure. The handling procedures for wild flies were likewise identical to those described above.

Mating trials in 2008 were conducted on the grounds of the eclosion facility (230 m elevation) in the shade of a large tree in 20 nylon-mesh cages (1.4 by 1.0 by 0.9 m) placed over individual potted coffee plants. The cages enclosed the leaf-bearing portion of the plants and were 0.9 m off the ground. The cage floor contained a zipper to accommodate the plant stem as well as two vertical zippers on opposite sides to allow access to the cage interior. On the two test days, 50 treated or control sterile males, 50 wild males, and 50 wild females were introduced into the cages. On each test day, treated sterile males from a particular dose were placed in five cages (i.e., three doses x five tents/dose = 15 tents), and control sterile males were released in the remaining five cages. Sterile male treatments were assigned randomly to the tents on both test days. The procedures used to perform the mating tests were identical to those used in 2006. Based on hourly measurements averaged over the two test days, air temperature increased from approximately 25°C to 35°C and relative humidity decreased from 79% to 46% between 0700–1200 hrs.

**Statistical analyses.** Pair wise and multi-sample comparisons were made using Students t test and 1-way ANOVA, respectively, as parametric assumptions were always met. Proportions were arcsine transformed for analysis.

**Table 2.** Results of mating trials conducted in field tents on three test days near Filadelfia, Guatemala. Fifty sterile males, 50 wild males, and 50 wild females were released per tent. Values represent means ± SE (n = 8).

| Test day | GRO dose[a] | Male type | Matings per tent | % total matings by sterile males |
|---|---|---|---|---|
| 1 | 0.37 | Sterile: GRO | 14.6 (1.4) | 35.6 (3.6) |
| | | Wild | 26.9 (1.8) | |
| | | Sterile: No GRO | 10.5 (1.3) | 24.8 (3.4) |
| | | Wild | 32.9 (2.2) | |
| 2 | 0.46 | Sterile: GRO | 18.2 (1.4) | 41.4 (2.7) |
| | | Wild | 25.5 (1.2) | |
| | | Sterile: No GRO | 9.0 (1.2) | 20.5 (2.4) |
| | | Wild | 34.3 (1.0) | |
| 3 | 0.50 | Sterile: GRO | 15.4 (1.0) | 37.5 (2.6) |
| | | Wild | 26.0 (1.6) | |
| | | Sterile: No GRO | 10.0 (1.4) | 26.3 (2.8) |
| | | Wild | 26.9 (1.1) | |

[a]Ginger root oil dose, presented as ml/m$^3$ of room volume, see Table 1.

## Results

**Mating trials—2006.** Comparisons among the three test days revealed no significant difference in either the number of matings or the proportion of total matings obtained per replicate by treated ($F_{number} = 2.1$, $P = 0.14$; $F_{proportion} = 0.95$, $P = 0.40$) or control sterile males ($F_{number} = 0.35$, $P = 0.71$; $F_{proportion} = 1.1$, $P = 0.35$; df = 2, 21 in all tests; Table 2). Because different GRO doses were used on different days, the lack of significant between-day variation for the GRO-exposed males indicates that (over the range of doses tested) their mating performance was dose-independent. Given the between-day similarity in the mating success of sterile males, data were pooled across test days for subsequent analysis.

The pooled data revealed two clear trends, namely (1) wild males obtained more matings per tent than treated (26.1 ± 0.9 vs. 15.9 ± 0.8, respectively, $t = 8.5$, $P < 0.001$) or control (31.3 ± 1.1 vs. 9.8 ± 0.7, respectively, $t = 16.5$, $P < 0.001$) sterile males, and (2) treated sterile males obtained significantly more matings per tent and a greater proportion of the total matings per tent than control sterile males (number of matings: 15.9 ± 0.8 vs. 9.8 ± 0.7, respectively, $t = 5.8$, $P < 0.01$; per cent total matings: 38% ± 1.7 vs. 24% ± 1.7, respectively, $t = 5.9$, $P < 0.001$; $n_1 = n_2 = 24$ for all tests).

**Mating trials—2008.** We initially compared between-day mating performance for each treatment to determine whether data could be pooled across days. These analyses revealed no significant difference between the two test days in either the number of matings or the proportion of total matings obtained per replicate by treated sterile males for any of the GRO doses tested (0.43 ml/m$^3$: $t_{number} = 0.1$, $P = 0.91$; $t_{proportion} = 0.2$, $P = 0.85$; 0.58 ml/m$^3$: $t_{number} = 1.1$, $P = 0.31$; $t_{proportion} = 1.1$, $P = 0.31$; 0.91 ml/m$^3$: $t_{number} = 0.9$, $P = 0.37$; $t_{proportion} = 1.0$, $P = 0.34$; $n_1 = n_2 = 5$ for all tests) or control sterile males ($t_{number} = 0.5$, $P = 0.61$; $t_{proportion} = 0.9$,

**Table 3.** Results of mating trials conducted in 2008, Retalhuleu, Guatemala. Fifty sterile males, 50 wild males, and 50 wild females were released per cage. Values represent means ± SE (n = 10 cages over 2 days).

| GRO dose[a] | Male type | Matings per tent | % total matings by sterile males |
|---|---|---|---|
| 0.43 | Sterile: GRO | 10.1 (0.8) | 33.6 (2.9) |
| | Wild | | 20.3 (1.4) |
| 0.58 | Sterile: GRO | 9.8 (0.9) | 35.4 (1.9) |
| | Wild | | 17.6 (0.5) |
| 0.90 | Sterile: GRO | 11.2 (1.3) | 37.8 (3.5) |
| | Wild | | 18.3 (1.3) |
| none | Sterile: no GRO | 4.9 (0.5) | 18.8 (1.8) |
| | Wild | | 21.3 (1.1) |

[a]Ginger root oil dose, presented as ml/m$^3$ of room volume, see Table 1

P = 0.40; $n_1 = n_2 = 5$). Given these results, data were pooled across test days for subsequent analysis (Table 3).

Using these pooled data, we next investigated whether there was significant variation in mating performance among sterile males exposed to the different GRO doses. We found no significant variation among the different GRO treatments for either the number of matings achieved per tent ($F_{2, 27} = 0.52$, P = 0.60) or the proportion of total matings accounted for per replicate ($F_{2,27} = 0.50$, P = 0.61). These results indicate that, over the range of GRO doses tested, the mating performance of the GRO-exposed sterile males was dose-independent.

Consequently, we pooled data across the GRO-treated groups for a final comparison of mating success among wild males and GRO-exposed and non-exposed sterile males. This analysis revealed the same two trends noted above for 2006, namely i) wild males obtained more matings per tent than treated (18.7 ± 0.7 vs. 10.4 ± 0.6, respectively, $n_1 = n_2 = 30$, t = 9.4, P < 0.001) or control (21.3 ± 1.6 vs. 4.9 ± 0.6, respectively, $n_1 = n_2 = 10$, t = 9.9, P < 0.001) sterile males and ii) treated sterile males obtained significantly more matings per tent than control sterile males (18.7 ± 0.7 vs. 4.9 ± 0.6, respectively, t = 5.2, P < 0.001, $n_1 = 30$, $n_2 = 10$) and a greater proportion of the total matings per cage than control sterile males (35.6% ± 1.6% vs. 18.8% ± 1.8%, respectively, t = 5.7, P < 0.001, $n_1 = 30$, $n_2 = 10$).

**Mating trials—between-year comparisons.** The total number of matings recorded per replicate was, on average, significantly higher at Filadelfia than Retalhuleu (41.7 ± 0.7 vs. 28.4 ± 0.7, respectively, t = 13.6, P < 0.001, $n_1 = 48$, $n_2 = 40$). Consistent with this trend, the mean number of matings obtained per replicate by treated (data combined over all days and doses per site) and control (data pooled over all days) sterile males was significantly higher at Filadelfia than Retalhuleu (treated: 16.1 ± 0.8 vs. 10.4 ± 0.6, respectively, t = 6.0, P < 0.001, $n_1 = 24$, $n_2 = 30$; control: 9.8 ± 0.7 vs. 4.9 ± 0.5, respectively, t = 4.1, P < 0.001, $n_1 = 24$, $n_2 = 10$). Despite this difference in mating activity, the mean proportion of matings achieved by treated sterile males was similar at Filadelfia and Retalhuleu (38.1% ± 1.7 vs. 35.6% ± 1.6, respectively, t = 1.1, P = 0.30, $n_1 = 24$, $n_2 = 30$). The same result was found for control sterile males (23.8% ± 1.7 vs. 18.8% ± 1.7 for Filadelfia and Retalhuleu, respectively, t = 1.8, P = 0.08, $n_1 = 24$, $n_2 = 10$).

## Discussion

The present study shows that GRO exposure in the world's largest holding rooms (containing as many as 127 million flight-able males) enhanced the mating competitiveness of sterile *C. capitata* males. Based on average relative mating success (% total matings), GRO exposure (data pooled over all doses) increased the mating performance of sterile males by 58% (38%/24%) over non-exposed sterile males. In the only other published account of "whole room" exposure to GRO (Shelly et al. 2007), GRO enhanced the relative mating success of mass-reared, sterile males by 53% (58%/38%) in trials conducted in southern California, a value similar to those reported here.

Independent of GRO exposure, wild males had a competitive advantage over mass-reared, sterile males in obtaining matings with wild females. Many studies (e.g., Roessler 1975, Lance et al. 2000) have shown that untreated sterile males are inferior to wild males in sexual competition, and the present results provide further evidence of this pattern. The finding that GRO exposure, while boosting the performance of sterile males, did not result in competitive equivalence between wild and sterile males has been reported in other studies on GRO aromatherapy (e.g., Shelly et al. 2006). In other instances, however, GRO-exposed sterile males had similar (Barry et al. 2003) or even higher mating success (McInnis et al. 2002) compared to wild males.

At the Retalhuleu facility, the mating performance of GRO-exposed sterile males was similar under the entire range of GRO doses tested (0.37–0.91 ml/$m^3$) even though the number and spacing of GRO sources varied greatly from three sources [fans] located along the walls in 2008 to 40 sources [wicks] spaced evenly within the room in 2006. This observation suggests that, if the absolute GRO dose is sufficiently high, the manner in which the odor is dispersed is of secondary importance. The minimum effective dose per room for Retalhuleu has not yet been identified, and additional tests using lower doses distributed among varying numbers of sources may help in reducing the amount of GRO used (thus reducing costs) while maintaining a similar level of mating enhancement as reported here. As noted previously (Shelly 2008), applying GRO is inexpensive relative to other costs associated with SIT programs. At present, GRO costs approximately $79 per kilogram (≈ 1 liter) (L. Milack, personal communication). Thus, in Retalhuleu exposing sterile males to the doses examined here would cost approximately $13–$36 per room or approximately $0.18–$0.38 per million flight-able sterile males.

In conclusion, and apart from GRO treatment, the present study provides additional data on the possible association between elevation and the mating performance of sterile medfly males. In field-cage trials, Shelly et al. (2003) found that sterile males (both GRO-exposed and non-exposed) had much lower mating success at a high-elevation site (Antigua, 1,500 m) than a moderate-elevation site (Sabana Grande, 700 m). For example, non-exposed sterile males accounted for only 11% of the total matings at the high site compared to 47% at the moderate-elevation site. Based on this result, Shelly et al. (2003) suggested that the mating ability of sterile males may vary inversely with elevation in Guatemala. Here, however, the performance of non-exposed sterile males at Retalhuleu (19% total matings, 200 m elevation) or Filadelfia (24% total matings, 550 m elevation) more closely resembled that described for Antigua than Sabana Grande. This result obviously invalidates the simple altitude-dependent relationship proposed earlier and suggests that the mating competitiveness of sterile males expected at a particular site may be affected by multiple factors interacting in a complex manner.

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