

All male strains and chemical stimulants: Two ways to boost

sterile males in SIT programs

Drs. Donald O. McInnis¹, Todd E. Shelly², and Ronald F. L. Mau³
1. USDA/ARS/PBARC, Honolulu, HI, USA, 2. USDA/APHIS/CPHST,
Waimanalo, HI, USA, 3. University of Hawaii at Manoa
Honolulu, HI, USA
Email address of main authors: dmcinnis@pbarc.ras.usda.gov

Abstract

Genetic and chemical means have been developed to significantly improve the effectiveness of the sterile insect technique against tephritid fruit flies in recent years. Beginning with the development of genetic sexing techniques some 25 years ago, all-male strains of several species of fruit flies have greatly improved the SIT. More recently, chemical supplements such as ginger root oil for *Ceratitidis capitata* (medfly) males and methyl eugenol for *Bactrocera dorsalis* males have further increased the field efficiency of released mass-reared sterile males at very limited cost.

KEYWORDS: sterile insect technique, genetic sexing, aromatherapy, chemotherapy

Introduction

Control of tephritid pests has traditionally been carried out by chemical means, such as protein bait sprays or male annihilation (Steiner et al. 1965), but more recently biological control techniques, especially the sterile insect technique (SIT), have gained wider use due to their environmentally benign nature. Large-scale control or eradication programs utilizing the SIT against the melon fly were successful in the Pacific basin, especially in Japan, where the melon fly was eradicated (Kakinohana et al. 1990, Hibino and Iwahashi 1991). Many SIT programs have been initiated in recent decades against the related tephritid pest, the Mediterranean fruit fly, *Ceratitidis capitata* (Wiedemann) around the world. One important factor in promoting the use of the SIT against *C. capitata* has been the development of effective genetic sexing strains that permit the separation of males from females at some developmental stage (Robinson et al. 1999, McInnis et al. 2004, McCombs and Saul 1995). By releasing only sterile males, the efficiency of the technique can be increased several fold, and fruit damage due to stings by sterile females is avoided (McInnis et al. 1994, Rendon et al. 2000, McInnis et al. 2004, McInnis et al. (in press)).

Chemical aromas emitted by plants, insects, or other animals have long been known to affect the behavior of insects. Strictly speaking, the use of aromas to improve the condition of an animal (or plant) is known as aromatherapy, while in general terms, chemotherapy involves the similar use of any type of chemical, whether it be aromatic or not. The use of aromas to affect the behavior of tephritid fruit flies has its origin in the use of certain male attractants, or parapheromones, such as methyl eugenol or alpha-copaene (found in ginger root oil) (Shelly and

Dewire 1994, Shelly and Nishida 2004, Shelly et al. 2002). Natural sources, including plant flowers or stems, have been found to emit such compounds and can benefit males of certain fly species. Artificial sources of these same compounds can be used to improve the quality of male flies exposed to them. The first such cases involved the use of male attractants- methyl eugenol to improve the mating vigor of oriental fruit flies (OFF), *Bactrocera dorsalis*, and trimedlure to improve the mating vigor of the medfly, *Ceratitidis capitata* (Shelly and Dewire 1994, Shelly et al. 1999).

Recently, field demonstrations combining the benefits of all-male strains with the chemotherapeutic effects of ginger root oil (GRO) in medflies and methyl eugenol (ME) in the OFF have been carried out in Hawaii, and later in California, Florida, Guatemala, and several other foreign countries (Shelly et al 2002, Shelly et al. 2005, McInnis (unpub.)). The application of GRO has progressed rapidly in scope, beginning with exposures in small cups, to large holding boxes, and finally to large rooms holding millions of sterile male adults in operational programs. Similarly, the testing areas for evaluating the mating and survival abilities of GRO treated males compared to control males has progressed from small lab cages, to standard outdoor field cages, to much larger field cages, and finally, to the open field where millions of sterile males were released. In all of these cases, exposures of 12 –24 hrs of GRO to young male medflies has resulted in at least a doubling of mating ability against wild or wildish females at a very low cost. Similarly, chemotherapy with ME has been shown to significantly increase the mating ability of treated OFF males against wild or wildish females. Mating tests have increased in scale from laboratory mating tests to outdoor field cages, both small and large, and finally to open field releases of sterile genetic sexing strain males. Wild medfly and OFF populations have been monitored in open field tests, and host fruits collected to monitor egg sterility in treated and control areas (Rendon et al 2000, Shelly et al., in review; McInnis and Shelly, in review). The sperm ID technique, developed for medflies initially (McInnis 1993), has been applied to the OFF in an open field experiment recently conducted on the island of Oahu, Hawaii.

In this paper, we review the recent evidence in favor of using both a genetic sexing strain (melon fly) and a genetic sexing strain plus chemotherapy (oriental fruit fly) to boost the effectiveness of the SIT for each of those species. Genetic sexing or chemotherapy alone can significantly increase sterile male competitiveness, and together they have a cumulative effect of increasing the cost-effectiveness of the method.

Genetic Sexing of the Melon Fly

In 2001, a desirable chromosomal translocation was successfully induced in the melon fly via low dose gamma irradiation. For the first time in this species, a genetic sexing strain based on pupal coloration was isolated and bred true- normal brown pupal color males and white mutant pupal color females. The strain was developed and reared at the USDA/ARS/PBARC fruit fly facility in Honolulu, Hawaii. The strain was expanded as rapidly as possible after its discovery, resulting, in a few months, in the regular production of thousands of individuals per sex. The strain was then intensively evaluated on a small scale (McInnis et al. 2004). These initial tests

determined that the melon fly pupal color sexing strain was fully compatible with wild melon flies, with respect to mating and survival abilities of sterile lab-reared males in field cages.

Following the encouraging initial small-scale rearing and field cage studies (McInnis et al. 2004), the melon fly sexing strain was expanded by mass-production and prepared for sterile fly release into wild fly infested areas. We began a program to integrate the new sexing strain into the ongoing IPM program against the melon fly on the Big Island of Hawaii. Flies were reared to pupation at the USDA/ARS/PBARC laboratory in Honolulu HI, then pupal color sorted using high speed photoelectric sorting machines. At ca. 2 days prior to emergence the all-male pupae were dyed with a standard fluorescent dye to mark emerging adults, then shipped inter-island via air cargo to the field test site, except for Oahu. The pupae were dispensed into 2 gallon 'chicken' buckets containing ca. 1100 pupae/bucket and held with food and water (agar) for ca. 5 days under ambient conditions in large holding rooms prior to ground release in the field. The test site on the Big Island consisted of an 8 X 5 km (40 sq. km) grid and a 4 X 5 km (20 sq. km) grid where sterile flies were released. Flies were initially released at 6 sites on the ground at the top right corner of the grid, mostly in a residential area, then gradually were expanded further into the inner 4 X 5 release grid over ca. 6 months of releases. Approximately 150,000-200,000 males were released once per week into the grid. The important sterile:wild fly overflooding ratios were monitored weekly by standard cuelure bucket traps covering the release area.

Statistical analysis of the correlations between the sterile:wild fly ratios and the observed egg sterility at different time periods (see Table 1) was performed (Snedecor and Cochran 1967). In addition, the numbers of hatched and unhatched eggs for control and fly release sites were compared for each sampling period and the probabilities of obtaining such results by chance were provided by Chi-Square analysis.

The control and release areas overlapped in wild flies per trap-day measures until fly releases began in Feb., 2002. After that time, the control area always had a higher wild fly per trap day catch, and frequently was ca. 5-10 times higher than for the treated area. It should be noted that the control area had other IPM strategies taking place, including bait sprays, male annihilation, and field sanitation, as was true for the treated area, but only the treated area received sterile flies. After August, 2002, the fly releases were expanded to include the former control area so there was no true fly-free control to provide a comparison.

The critical measure of success of an SIT program is the level of induced egg sterility one obtains from released sterile flies. The results of egg dissections in both treated and control areas can be seen in Table 1. Fly releases were conducted for 8 months before the numbers of eggs obtained were so small as to be meaningless, plus the expansion of sterile flies into the former control area, outside the inner 4 X 5 km grid, made the control vs. treated area comparison of dubious value. Based on Chi-Square comparisons of the number of hatched and unhatched eggs for control and release sites, the release area had significantly higher egg sterilities even after

only 1 month of releases, reaching ca. 75% sterility, or higher, at all times during the test ($P < 0.001$). Control egg sterilities were 11% before releases, and averaged ca. 15% during the releases.

Due to the success of the open field fly releases using the melon fly sexing strain, the USDA/ARS laboratory (Honolulu, HI), in collaboration with the University of Hawaii (Manoa), began releasing sterile melon flies on the island of Maui, again as part of the current IPM program. Fly releases began in March, 2003 in a ca. 10 sq. km area in lower Kula, Maui where a much larger melon fly population existed compared to the earlier Big Island population. This area greatly challenged the SIT capability of the new strain, and required a large expansion of our mass-rearing production in order to succeed. Production of sterile flies increased from ca. 200,000/wk to ca. 800,000/wk. for this test on Maui. In addition, in order to produce large numbers of flies at a consistently high level of purity, we adopted a filter rearing system in which a purified colony is continuously maintained then expanded through 3 cycles of rearing, in order to produce sufficient numbers for color machine sorting of pupae and release of virtually 100% males into the field. Results of the sterile fly releases in Maui between March and September, 2003 are shown in Fig. 1. As can be noted in the figure, egg sterility rose significantly once the sterile:wild (S:W) fly ratio increased significantly in July, 2003, after 4 months of releases. As the S:W ratio increased into August, the egg sterility obtained from egg dissections from host fruit collected in the field test site also continued to increase. Finally, when the fly releases were discontinued in September, 2003, the egg sterility dropped accordingly until the final collections made in November. The overall statistical correlation between the S:W fly ratio and egg sterility was highly significant ($r = 0.850$, $P < 0.01$).

Encouraged by these results, we proceeded to attempt sterile fly releases on the island of Oahu, HI in a large commercial plantation covering ca. 450 ha and many types of melon fly hosts throughout the year. We increased our fly production to 1,500,000 sterile males per week from a total production of ca. 5 million pupae prior to color sorting. During the period from Nov., 2003 to July, 2004 we released flies on the standard weekly basis and trapped flies on the same 2-week interval. Unfortunately, the wild fly population level was so high that the S:W overflooding ratio was less than 1:1 until the 5th month of releases. Egg sterility increased slowly in accordance with the S:W ratio, reaching ca. 45% at a ca. 1.5:1 ratio. Then, after releases stopped in early July, both the S:W ratio and egg sterility dropped until all sterile flies died off in the field some 2 months later. The relationship between the S:W ratio and egg sterility was again statistically significant, $r = 0.934$, $P < 0.05$.

In conclusion, the newly developed melon fly sexing strain, has proven to be a very competitive strain, as evidenced by earlier results indicating high quality laboratory and field cage performances. The following SIT programs for this strain progressed over 3 islands and 3 years from 2001-2004 in increasingly larger test sites and larger wild fly populations. Fly production increased to compensate for the higher wild fly populations, until the Oahu program when the S:W ratio never exceeded 1.5:1, in spite of maximal sterile male production. Nonetheless, the egg sterility obtained in all 3 programs was relatively high, indicating very good

competitiveness for the strain under field conditions. Based on these studies, the strain is ready for both mass production and aerial releases of sterile males into the field. Aerial releases will likely improve fly distribution and, consequently, melon fly control by the SIT.

Chemotherapy of the Oriental fruit Fly

Previous studies of the oriental fruit fly (OFF) have found that male adults that consume a specific chemical, found in certain plants, methyl eugenol (ME), can significantly increase their mating ability in competition with unexposed males (Nishida et al. 1988, 1997). Shelly (2000) discovered that OFF males fed flowers of the golden shower tree, *Cassia fistula*, obtained twice as many matings as did unfed males in the laboratory and that exposure to the aroma of ME alone did not enhance mating ability- i.e. the males had to ingest the chemical to obtain the benefit. Shelly and coworkers (2005) further found that dietary protein was necessary in order for OFF males to mate, and that an adult diet of sugar-only with ME could not 'rescue' or increase mating ability significantly. However, males provided an adult diet of protein supplemented with ME produce a more attractive mating signal and succeed in mating more often (Shelly and Dewire 1994, Tan and Nishida 1996). Additional laboratory studies showed that mass-produced and sterilized (irradiated) OFF males could also benefit as they obtained a 2 to 3-fold mating advantage against wildish males (less than 8 generations in the laboratory and reared on natural host fruit). In a later study, Shelly and co-workers (2000) again demonstrated a significant mating advantage for the mass-produced genetic sexing strain of this species (McCombs and Saul 1995).

In order to determine if the mating advantage for ME treated OFF males, as seen in the laboratory, would benefit an SIT program, the test arena needed to move to the field. Shelly and McInnis (unpub. data) have recently completed a replicated large field cage study in which sterile males interacted with wildish males for matings with wild females. Induced sterility was measured from eggs dissected from host fruit placed inside the cages on 3 1-day periods during the test. OFF males were exposed to ME in 2 different ways: 1) to 2 gm standard plastic trapping plugs containing ME for 1-3 day old adults; and 2) to liquid ME placed on blotter paper exposed to 5-7 day-old adults. Sexually mature and virgin sterile males (1,000) and normal wildish flies (200/sex) were released into the large cages (6M x 16 M X 3M high) containing 15-20 guava trees, *Psidium guajava*, and 150-200/sex mature wildish flies were released into a nearby small outdoor cage to serve as the control. On days 3, 7 and 10, 15 host apples in each large cage (2 apples in the control cage) were hung to collect eggs and determine egg sterility 3 days later. From these egg hatches, Fried mating competitiveness values, C, were calculated for sterile males based on both the plug exposure to young adults, and the blotter paper exposure to older adults (see Table 2). As can be noted, over 6 replications with the 2 gm plugs, there was no effect seen for the exposure to 1-3 day old males using the plastic plug (C values, 0.22 without ME, 0.23 with ME). It is not known exactly why the exposure failed to enhance mating but the relatively young age of the males, or the need to for males to obtain the ME from a small number of plugs (5) in competition with ca. 15,000 males in a standard release box, are surely likely factors.

In contrast to the plug, the use of the previous standard method of adult exposure to liquid ME on blotter papers gave excellent results, with C values of 0.18 and 0.68 for untreated and ME treated, respectively. With those encouraging results, further refinement of the adult exposure method led to an effective method to expose large numbers of OFF males in the standard release plastic boxes (as above) at 5-6 days of age, overnight just prior to fly release in the field.

Currently, a larger scale open field study is near completion on the Hawaiian island of Oahu in which two fruit orchards are being used to compare ME exposed sterile OFF males with unexposed sterile males against wild flies in a natural setting. Males at 5-7 days old (close to sexual maturity for the laboratory strain) were exposed overnight to 15 mls of ME placed on 5 cotton wicks in boxes containing ca. 15,000 males. Since Jan. 27, 2006, approximately 50-150,000 sterile males were released weekly from several points at each site. Several standard male traps using the powerful ME lure, plus 10 standard liquid protein traps to catch females were set up in each test site. Wild and sterile male populations were monitored on a weekly basis in each test area, while wild female populations were also monitored every 2 weeks and fly samples were saved for later dissection and determination of mating status. Male sterile:wild fly numbers were calculated each week and used to adjust the numbers of flies released at each site. Sterile:wild ratios of 20-50:1 were desired for each area. If females were mated, the type of mating involved (sterile, wild, or both) was assessed by application of the sperm ID method after McInnis (1993). Results to date, following over 6 months of fly releases, indicate that both the ME exposed and unexposed sterile males have induced significant egg sterility (Figure 2) and sperm ID sterility into the wild population. ME exposed males have recently surpassed (80-90% sterility) the levels attained by the non-ME exposed males 50-80%, where the levels reached for the sperm ID method rose earlier than did the egg sterility data. This is likely because the sperm ID method information comes from actual matings by sterile males, rather than oviposition by the wild females that can take place days, weeks or even months after the matings take place. Plans are to have the fly releases continue until the end of September, and sterile and wild fly monitoring continue for ca. 2 months after that.

To date, the significant sterility induced by the sterile genetic sexing strain of OFF has been attained at the relatively low sterile:wild fly ratio of ca. 10-20:1 for most of the time period of the test, indicating that the sterile flies released, especially the males exposed to methyl eugenol, have been very competitive with wild males in an open field setting. These results suggest that the genetic sexing strain, with males exposed to ME as adults, could be used as an effective weapon to control or eradicate the oriental fruit fly in SIT programs worldwide.

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Table 1: Melon fly egg sterility data was shown for periods before and after SIT releases of the genetic sexing strain in control and treated areas on the Big Island of Hawaii, Hawaii (2002)

	<u>Area</u>	<u>Total # Eggs</u>	<u># Hatched</u>	<u># Unhatched</u>	<u>% Sterility</u>	<u>Chi-Sq.</u>
Pre-Release		2428	2171	257	10.6	
Oct. '01- Jan.'02)						
Months						
Post-Release						
1	Control	75	54	21	28.0	93.8
	(P<0.001)					
	Treated	131	28	103	78.6	
2	Control	39	35	4	10.3	63.4
	(P<0.001)					
	Treated	281	69	212	75.4	
3 and 4	Control	72	65	7	9.7	79.5
	(P<0.001)					
	Treated	108	23	85	78.7	
5 and 6	Control	164	112	52	31.7	44.8
	(P<0.001)					
	Treated	128	36	92	71.9	
7 and 8	Control	113	98	15	13.3	152.1
	(P<0.001)					
	Treated					
8 Months	Control	463	364	99	21.3	403.4
	p<0.01)					
Cum. Total	Treated	802	172	630	78.6	

Chi-Square analysis comparing the numbers of hatched and unhatched eggs for Control and Treated (release) sites for each sampling period. The probabilities of obtaining such results by chance are provided (these are highly significant for all sampling periods, P< 0.001).

Table 2: Fly Competitiveness values, C, for ME treated and untreated OFF sterile males in large field cages (Oahu, Hawaii, USA, 2005-2006).

I. ME exposure with 2 GM plugs to 1-3 day old adults :		
<u>Replicate</u>	<u>No-ME Field Cage</u>	<u>ME Field Cage</u>
1.	0.22 ¹	0.32
2.	0.35	0.52
3.	0.24	0.34
4.	0.26	0.06
5.	0.06	0.04
6.	<u>0.21</u>	<u>0.11</u>
	AVG. 0.22	0.23
II. ME exposure with ME liquid on blotter paper to 5-7 da old adults:		
1.	0.11	0.68
2.	0.23	0.53
3.	0.12	0.29
4.	0.16	0.39
5.	0.10	0.21
6.	<u>0.34</u>	<u>1.95</u>
	AVG. 0.18	0.68

Competitiveness value, C, equal to the value of the sterile male in relation to the wild male value, where the latter is arbitrarily set equal to 1.0.

Figure 1: Egg sterility vs. sterile:wild fly ratios for the melon fly genetic sexing strain on Maui, HI during March- September, 2003.

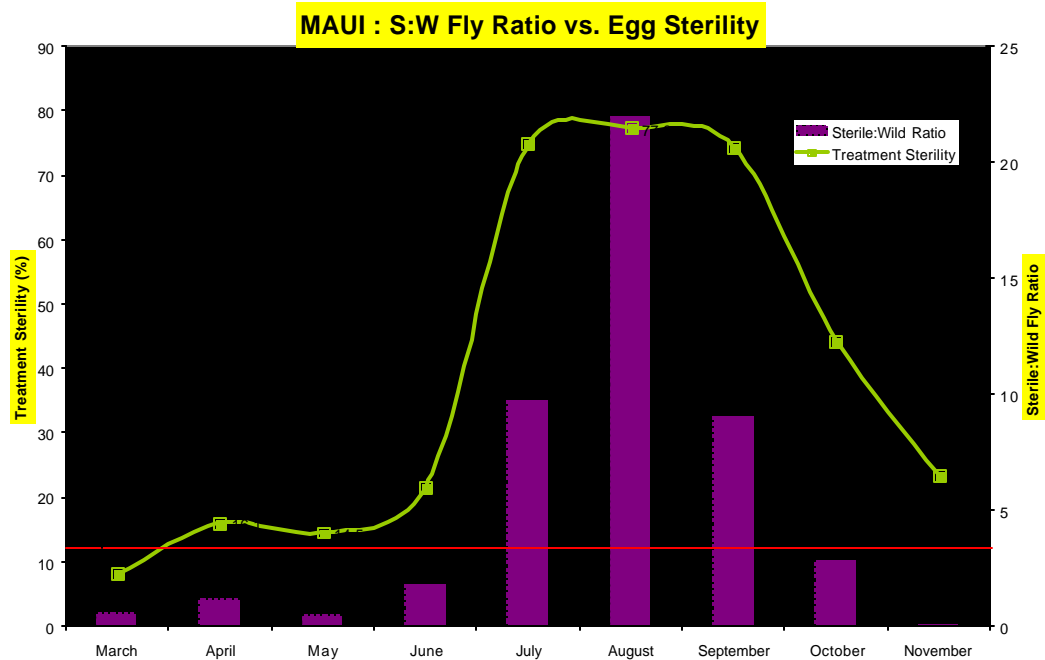


Figure 2: Egg sterility from host fruit in orchards with released ME treated sterile OFF males (Aloun) and Non-ME treated males in 2006 (Oahu, HI, USA). Pre-release sampling dates (until 1/27/2006) and post-release sampling dates are shown. (Aloun : bar shown on left; P. City : bar shown on right in each pair).

