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QUALITY CONTROL METHOD TO MEASURE PREDATOR EVASION IN WILD AND MASS-REARED MEDITERRANEAN FRUIT FLIES (DIPTERA: TEPHRITIDAE)

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ABSTRACT

Sterile male insects, mass-reared and released as part of sterile insect technique (SIT) programs, must survive long enough in the field to mature sexually and compete effectively with wild males for wild females. An often reported problem in Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann) SIT programs is that numbers of released sterile males decrease rapidly in the field for various reasons, including losses to different types of predators. This is a serious issue in view that most operational programs release sterile flies at an age when they are still immature. Previous field and field-cage tests have confirmed that flies of laboratory strains are less able to evade predators than wild flies. Such tests involve, however, considerable manipulation and observation of predators and are therefore not suitable for routine measurements of predator evasion. Here we describe a simple quality control method with aspirators to measure agility in medflies and show that this parameter is related to the capacity of flies to evade predators. Although further standardization of the test is necessary to allow more accurate inter-strain comparisons, results confirm the relevance of measuring predator evasion in mass-reared medfly strains. Besides being a measure of this sterile male quality parameter, the described method could be used for the systematic selection of strains with a higher capacity for predator evasion.

Key Words: predator evasion, predation, survival, sterile males, Tephritidae, *Ceratitis capitata*, *Vespula germanica*

RESUMEN

Insectos machos estériles criados en forma masiva para ser liberados en programas que utilizan la técnica del insecto estéril (TIE), tienen que tener la capacidad de sobrevivir en el campo el tiempo necesario para poder madurar sexualmente y competir efectivamente con los machos silvestres por hembras silvestres. Un problema frecuentemente reportado por dichos programas de la mosca del Mediterráneo, *Ceratitis capitata* (Wiedemann), es que el número de machos estériles de laboratorio liberados en el campo, decrecen rápidamente por varias razones, incluyendo pérdidas debidas a diferentes tipos de depredadores. Estudios anteriores conducidos en el campo, y en jaulas de campo, han confirmado que las cepas de machos de laboratorio tienen menos capacidad de evadir depredadores que los machos silvestres. Estos estudios involucran, sin embargo, una considerable cantidad de manipulación y observación de depredadores, por lo que no son adecuados para ser usados como medidas rutinarias en los programas de cría masiva. Aquí describimos un método sencillo de control de calidad usando aspiradores para medir agilidad en la mosca del Mediterráneo y mostramos que este parámetro esta relacionado a la capacidad de la moscas a evadir a depredadores. Aunque aún es necesario refinar la estandarización de éste método para permitir la comparación entre cepas, los resultados confirman la importancia de tener un método rutinario para medir la capacidad de evasión de depredadores en cepas de cría de laboratorio de la mosca del Mediterráneo. Además de medir este parámetro de control de calidad de los machos estériles, el método descrito podría también ser usado para la selección sistemática de cepas con una mayor capacidad de evasión de depredadores.

Translation provided by the authors.

Sterile male insects, mass-reared and released as part of a sterile insect technique (SIT) program, must survive long enough in the field to compete effectively with wild males for wild females.

An often-reported problem in Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann), area-wide control programs integrating the SIT, is the short period after sterile male release during which the males can be recaptured. To date, a number of very thorough field assessments of the dispersal and survival of sterile medflies have been carried out (Wong et al.1986; Baker & Chan 1991; Plant & Cunningham 1991; Baker & van der Valk 1992; Bloem et al. 1994). Most of these studies report rapid declines in numbers of released sterile flies. On average, the last recaptures of released medflies in these studies have been on d 8. This rapid decrease of flies occurred even where the recapture covered a 1 km^2 area, and fly emigration was shown to be insignificant as evidenced by the few flies that reached the outer perimeter of traps (Plant & Cunningham 1991). As a result, medfly SIT programs often have to release twice a week to ensure a sufficient presence of sterile males in a given area (Bloem et al. 1994; Dowell et al. 2000).

The rapid decrease of released sterile flies is probably the result of many abiotic and biotic factors, including predation. Various studies under natural conditions confirm the importance of vertebrate and arthropod predators in medfly biology (Hendrichs & Hendrichs 1990; Hendrichs et al. 1991; Baker & van der Valk 1992). During field-testing of dispersal and survival of a *temperature sensitive lethal* (*tsl*) genetic sexing strain, VIENNA 42 (Franz et al. 1994) in a citrus orchard, the rate of decrease was again high, and recaptured males appeared to be locating and feeding at the same natural food sites as wild males (Hendrichs et al. 1993). At the same time, there was a ten-fold difference in survival in favor of VIENNA 42 males held in control field cages in the orchard compared with those released into the same orchard. However, during the same study it was shown that in control cages with orange trees, in which the entrance was left slightly open, sterile males suffered heavy losses due to predation by a yellowjacket wasp *Vespula germanica* L. On each of the 4 occasions that the study was replicated, wasps entered the open field cage in large numbers and within 5-7 h captured all of the flies that had been released onto the field-caged host tree (Hendrichs et al. 1993). In another related field study, it could be demonstrated that foraging *V. germanica* wasps followed the odor plume to pheromone-calling medfly males aggregated in leks within dense host foliage (Hendrichs et al. 1994). Other field and field-cage studies have confirmed the sexually-biased predation suffered by mature medflies in nature (Hendrichs & Hendrichs 1998).

In view of the above findings, the objective of the present study was to develop a simple quality control test to measure predator evasion in wild and mass-reared medfly males. The development of such a test is the first step in assessing the low escape ability of mass-reared flies, with the eventual goal of improving the effectiveness of application of SIT against medfly.

Materials and Methods

Tests in Chios, Greece

All tests were conducted on orange trees, *Citrus sinensis* Ob., (approximately 2.0 m tall with 2.2 m wide crowns) within field-cages used for behavioral studies (2.2 m height × 3 m diameter). The first 2 tests (Tests 1 and 2) took place in a citrus orchard in Chios, Greece, with wild medfly males, originating from sour oranges and figs, and laboratory males of a medfly genetic sexing strain VIENNA 42 (a temperature shock in the egg stage kills the female eggs, Franz et al. 1994), mass-reared and sterilized (90 Gy) at the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf, Austria. The males were shipped from Vienna to Chios, Greece (total transport time 12 h), for field-testing (Hendrichs et al. 1993). Wild and sterile laboratory reared males were marked with a different color on the thorax and released together into the field-cage the day before testing. The methodology followed was described in Hendrichs & Hendrichs (1998).

In Test 1, the field cage entrance zipper was left open (approximately 0.15 m wide) to allow access to foraging yellowjacket wasps, *V. germanica* L. Whenever a wasp entered the cage, the zipper was closed and all attacks of the wasp on wild and sterile medfly males were followed by two observers and recorded until the wasp captured a fly and was allowed to leave the cage.

In Test 2, no wasps were allowed into the field cage by keeping the cage zipper closed. Two observers simulated predation attacks on flies by attempting to capture flies by sucking them into aspirators normally used to handle flies in laboratories. The 2 observers alternated every 20 attempts each to capture wild (10 attempts) and sterile males (10 attempts) within the orange tree foliage. The aspirators were held at the lower end of a 20-cm glass tube inserted into a flexible plastic hose, the end of which was held in the mouth of the person attempting to capture flies. The opposite end of the glass tubing, through which flies were sucked, had a 5-mm diameter opening that was bent at an angle of about 70-75 degrees to facilitate reaching for flies present on or below surfaces such as host foliage. On average there were 2 capture attempts per min. The ability of flies, involved either in pheromone-calling or other activities such as resting or feeding, to escape from aspirating humans was quantified.

Tests in Seibersdorf, Austria

Tests 3 and 4, carried out at Seibersdorf, Austria, were based on the use of aspirators to cap-

ture flies on potted orange trees in field cages (same dimensions as above). Unmarked flies of different treatments were placed into separate but adjacent field cages. In Test 3, with laboratory flies of the pupal color dimorphism medfly genetic sexing strain SEIB 30-C (Robinson et al. 1999), we compared non-irradiated and irradiated flies, which were exposed to gamma radiation (90 Gy) as mature pupae two days before adult emergence. Flies were compared, while involved in different activities on the host tree, in their capacity to evade capture with aspirators. This comparison was carried at 2-d intervals, starting with adult emergence, to assess changes in evasive ability during the fly maturation period.

In Test 4, we carried out inter-strain comparisons to measure the capacity to evade capture in 3 different laboratory strains: genetic sexing strain VIENNA 42, pupal color genetic sexing strain SEIB 1-61, and a strain originating from coffee in Guatemala and held in the laboratory for 22 generations. Flies of all strains were mature (6-8 d old) when tested. One-half of all capture attempts in Test 4 were made by an observer with no previous experience with aspirators, the other by an experienced user of aspirators. In addition, both observers repeated the comparison of the 3 strains under 2 environmental regimes: sunny with approximately 23-25°C, or overcast and only 20-21°C.

In all 4 tests, conducted in Chios as well as Seibersdorf, care was taken that flies captured did not exceed 40% of flies released or originally emerged in a cage, in order to prevent the fly population in the cage from being constituted predominantly of individuals who had succeeded in escaping capture. Furthermore, although not recorded as part of the tests, every effort was made to remove flies that had escaped in the first capture attempt to eliminate them from the population to be sampled. However, this was not possible in the first test where wasps were often seen attacking the same fly several times in succession before capture or definite escape of the fly. Data of all tests were analyzed by Kruskal-Wallis nonparametric one-way analysis of variance. In Tests 1 and 2 each replicate consisted of the percent capture in 10 capture attempts; in Tests 3 and 4 each replicate consisted of 5 capture attempts.

Results

Tests in Chios, Greece

Results of Tests 1 and 2 conducted in Chios, comparing wasp attacks and simulated attacks with aspirators, are shown in Table 1. Three to 4 times more sterile VIENNA 42 males than wild males were captured by wasps. This highly significant difference was found for both pheromone-calling males and for males engaged in other activities (Table 1A).

In Test 2 (Table 1B), the simulated predator attacks with aspirators resulted also in significantly higher captures of sterile VIENNA 42 males, which were 2 to 3 times more likely to be captured than wild males. Overall, the percent capture of medfly males, irrespective of fly type or fly activity, was 18.2 ± 13.6% (SD) for wasps and 55.7 ± 25.6% (SD) for aspirators. In spite of this difference between wasps (Test 1) and observers with aspirators (Test 2) in capturing flies ($P < 0.0001$), the capture ratios between fly types and fly activities in general were similar for both tests. In addition (Test 2), there were no differences in percentage captures between the two observers experienced with the aspirators used to handle flies ($P = 0.4576$).

Tests in Seibersdorf, Austria

Results of Test 3, again measuring evasion of capture with aspirators, are shown in Tables 2 and 3. Overall, capacity to avoid capture increased directly with days since adult emergence in the field

Table 1. Success of capture attempts (percentage ± SD) of wild or laboratory reared medfly males by yellowjacket wasps (A) or by experimenters with aspirators (B) on a field-caged orange tree in Chios, Greece. Numbers in parenthesis represent fly capture attempts.

	Successful capture attempts (%)		
Fly activity	Fertile wild males	Sterile lab. males	Significance[1]
A. Wasps			
Sexual activities	8.2 ± 8.7 (100)	36.7 ± 13.4 (90)	$P = 0.0003$**
Other activities[2]	13.7 ± 6.8 (540)	40.6 ± 12.1 (80)	$P < 0.0001$**
B. Aspirators			
Sexual activities	27.6 ± 6.9 (29)	80.7 ± 8.0 (150)	$P = 0.0058$**
Other activities[2]	28.4 ± 11.7 (101)	62.3 ± 19.9 (220)	$P < 0.0001$**

[1]Kruskal-Wallis One-Way Nonparametric ANOVA.
[2]Includes feeding and resting males, both on the cage wall and foliage.

cage (Table 2). Although this trend was observable in non-irradiated flies, it was not statistically significant. For irradiated flies the difference was highly significant, with recently emerged flies having the lowest capacity for evading predators. The comparison of non-irradiated and irradiated flies involved in different activities on an orange tree is shown in Table 3. Overall, irradiated flies were significantly more susceptible to being captured than non-irradiated flies ($P = 0.0019$). This was particularly so for activities or locations for which alertness or wariness of flies is normally higher, such as male presence on leaf tops or female approaching male leks (females on the same or neighboring leaves within a radius of 10-15 cm of pheromone calling males). On the other hand, there was no significant difference for activities in which the attention of males is diverted in sexual activities, for example pheromone-calling, male-male aggressive encounters, courting or mating.

Results of Test 4 are presented in Tables 4 and 5. As shown in Table 4, no differences were found in capture avoidance between the 3 laboratory strains under comparison ($P = 0.2544$) and capture rates were only slightly, but not significantly, higher at the cooler temperatures (Table 4). While differences in capture rates between the respective fly activities (Table 5) were similar to the preceding aspirator tests, observer experience in with aspirators played a role for those fly activities in which alertness is normally higher, such as male presence on leaf tops or females on fruit. For these activities significant differences in fly capture were obtained between the experienced and the inexperienced observer, whereas for those activities in which flies are less wary because of involvement in sexual activities or feeding no such differences were found (Table 5).

Discussion

Our results, comparing wasp attacks and simulated attacks with aspirators under semi-natural conditions, show that sterile laboratory medflies are less likely to evade capture than wild flies. This confirms the relevance of measuring predator evasion in strains mass-produced for many generations under standard medfly mass-rearing conditions.

Considering the significantly higher susceptibility of laboratory reared flies to capture by foraging predators, resulting in significant losses for fruit fly control programs that integrate the SIT, it appears important to develop and apply a simple quality control test for tephritid fruit flies that addresses sterile fly capacity to avoid capture. Presently, the FAO/IAEA/USDA (2003) manual, which is used as a standard for quality control procedures in fruit fly programs incorporating the SIT, does not make reference to this aspect of sterile fly behavior, or fly agility or irritability, in general. Even the "startle" test, recommended as part of the RAPID quality control system (Boller et al. 1981), and listed in a quality control manual used in Latin America (Orozco et al. 1983), is not included in the above international fruit fly quality control manual.

Open field mating tests consistently require much higher sterile to wild male overflooding ratios than are needed within relatively protected field cages or large field enclosures to achieve the same levels of egg sterility (Rendón et al. 2004; Shelly et al. 2005). As the successful food foraging ability of sterile males has been repeatedly confirmed (Maor et al. 2004), this discrepancy between open field and field enclosure results must derive from the very high mortality that sterile males incur in the field before maturing sexually and encountering potential mates. Thus, ability to avoid capture is a fundamental aspect of sterile male quality that needs to be considered in operational programs.

The startle test measures a complex of reactions involved in fly response to light. It was originally developed by Schroeder et al. (1973), later modified by Boller et al. (1981), and further improved by C. O. Calkins (personal communication), including a compact mobile startle test

Table 2. Success of capture attempts (percentage ± SD) of irradiated and non-irradiated laboratory reared male and female medflies by experimenters with aspirators on a field-caged orange tree over successive periods after fly emergence. Numbers in parenthesis represent fly capture attempts.

	Fly age (days after emergence)			
	1-2	3-4	5-6	
Treatment	Successful capture attempts (%)			Significance[1]
Non-irradiated flies[2]	85.0 ± 15.5 (80)	78.0 ± 17.4 (200)	71.3 ± 25.3 (160)	$P = 0.2113^{N.S.}$
Irradiated flies[2]	93.8 ± 12.0 (80)	83.5 ± 14.2 (200)	73.9 ± 23.0 (160)	$P = 0.0022^{**}$

[1]Kruskal-Wallis One-Way Nonparametric ANOVA.
[2]Mass reared flies of pupal color sexing strain SEIB 30-C.

TABLE 3. SUCCESS OF CAPTURE ATTEMPTS (PERCENTAGE ± SD) OF IRRADIATED AND NON-IRRADIATED LABORATORY REARED MALE AND FEMALE MEDFLIES IN RELATION TO FLY ACTIVITIES BY EXPERIMENTERS WITH ASPIRATORS ON A FIELD-CAGED ORANGE TREE. NUMBERS IN PARENTHESIS REPRESENT FLY CAPTURE ATTEMPTS.

	Successful capture attempts (%)		
Fly activity	Non-irradiated flies[1]	Irradiated flies[1]	Significance[2]
Females approaching leks	62.5 ± 21.8 (80)	81.3 ± 20.0 (80)	$P = 0.0157$*
Males present on leaf tops	65.8 ± 16.1 (120)	80.0 ± 19.6 (120)	$P = 0.0086$**
Males resting on leaf bottoms	75.8 ± 22.1 (120)	82.5 ± 18.9 (120)	$P = 0.2979^{N.S.}$
Males involved in sexual activities	81.3 ± 18.6 (80)	80.0 ± 14.6 (80)	$P = 0.6872^{N.S.}$
Mating pairs	86.7 ± 17.3 (40)	95.0 ± 8.9 (40)	$P = 0.2139^{N.S.}$

[1]Mass reared flies of pupal color sexing strain SEIB 30-C.
[2]Kruskal-Wallis One-Way Nonparametric ANOVA.

machine with 10 test units. It is the only test currently available that, indirectly, may give an indication of fly agility or predator evasion capacity in mass-reared flies. However, the startle test is actually a type of flight propensity test that distinguishes between flies that will fly when stimulated from those that do so at a much slower rate. This flight is not in response to a sudden approach by a dark object (e.g., a predator) or a sudden falling object (Schroeder & Chambers 1977), but to a sudden onset of light experienced by flies held previously in total darkness for 30 min.

In terms of measuring capacity to avoid capture, there are additional disadvantages with the startle test. Not only is the test carried out under unnatural conditions, but previous placement of flies into the test units requires considerable fly manipulation, often involving CO_2. There is also the possibility of pheromone contamination of the test units. However, it is not possible to distinguish between fly activities nor to detect some of these problems because flies are concealed within black holding containers. Finally, the startle test lasts for a period of 3 min, allowing flies during this rather long period to fly in the direction of the light source, whereas responses to a predator attack occur within fractions of a second to, at most, several seconds.

Fly response to real predation attacks is complex and its measurement even more so. It requires extensive observation not readily amenable to standardization, as well as the availability of predators (Hendrichs & Hendrichs 1998). Predators are not available at all locations and during all seasons. To overcome these complications, we have described here a simple quality control method for measuring male medfly capacity to avoid capture. Unlike the startle test, the test requires no special equipment and does not measure flight response to onset of light and other stimuli but rather reflects more directly fly escape ability under more realistic conditions. Although we found absolute losses to simulated predation with aspirators higher than to real predation by foraging yellowjacket wasps, the relative ability to avoid capture is similar in mass-reared and wild males.

Our quality control method for measuring capacity to avoid capture in fruit fly males would not require routine testing as part of the RAPID laboratory quality control tests (Boller et al. 1981). Rather, it could be conducted at longer intervals as part of the more valuable confirmation quality control tests carried out under semi-natural conditions (Chambers et al. 1983). Only such tests, which must be carried out in greenhouses or on

TABLE 4. SUCCESS OF CAPTURE ATTEMPTS (PERCENTAGE ± SD) OF LABORATORY REARED MEDFLIES WITH ASPIRATORS ON A FIELD-CAGED ORANGE TREE UNDER DIFFERENT ENVIRONMENTAL CONDITIONS. NUMBERS IN PARENTHESIS REPRESENT FLY CAPTURE ATTEMPTS.

	Successful capture attempts (%)		
Laboratory strain[1]	Sunny (23-25°C)	Overcast (20-21°C)	Significance[2]
Pupal color sexing strain SEIB 1-61	88.9 ± 14.1 (90)	96.0 ± 8.2 (100)	$P = 0.0845^{N.S.}$
tsl sexing strain VIENNA 42	92.0 ± 10.1 (200)	95.0 ± 8.9 (200)	$P = 0.3173^{N.S.}$
Bisexual strain from Guatemala	95.0 ± 8.9 (100)	97.9 ± 6.3 (95)	$P = 0.2452^{N.S.}$

[1]Only those fly activities are included in which there were no differences in capture rates between experimenters (see Table 5). No difference between the 3 strains in percent capture ($P = 0.2544^{N.S.}$).
[2]Kruskal-Wallis One-Way Nonparametric ANOVA.

TABLE 5. SUCCESS OF CAPTURE ATTEMPTS (PERCENTAGE ± SD) OF LABORATORY REARED MEDFLIES WHEN COMPARING EXPERIMENTERS WITH DIFFERENT EXPERIENCE IN USING ASPIRATORS ON A FIELD-CAGED ORANGE TREE. NUMBERS IN PARENTHESIS REPRESENT FLY CAPTURE ATTEMPTS.

	Successful capture attempts (%)		
Fly activity[1]	Inexperienced[2] person	Experienced person	Significance[3]
Females present on fruit	72.0 ± 11.0 (25)	96.0 ± 8.9 (25)	$P = 0.0139$*
Males present on leaf tops	72.0 ± 29.1 (75)	94.7 ± 9.2 (75)	$P = 0.0057$**
Males resting on leaf bottoms	88.0 ± 14.7 (75)	93.3 ± 9.8 (75)	$P = 0.3373^{N.S.}$
Males feeding	96.0 ± 8.3 (75)	98.7 ± 5.2 (75)	$P = 0.2909^{N.S.}$
Males pheromone-calling	90.7 ± 10.3 (75)	92.0 ± 10.1 (75)	$P = 0.7172^{N.S.}$
Mating pairs	98.5 ± 5.5 (65)	97.8 ± 7.3 (70)	$P = 0.5930^{N.S.}$

[1]Combined data of comparison of 3 mass reared laboratory strains.
[2]No previous experience.
[3]Kruskal-Wallis One-Way Nonparametric ANOVA.

field-caged host trees, and preferably including wild females as a quality baseline, will provide a more definite verification of sterile fly quality.

Results, both with wasps and aspirators, confirm that mass-rearing conditions favor the production of flies with a decreased irritability or "nervousness". Highly irritable flies appear to die sooner in crowded adult colony cages of mass-rearing facilities than do more sedentary flies (Calkins, personal communication), and less irritable flies appear to have a higher mating success under these conditions (Briceño & Eberhard 2002).

To counter this inadvertent selection, the described method, besides measuring capacity to avoid capture, could be useful for the systematic selection of strains with a lower threshold for startle activity (Ewing 1963; Schroeder & Chambers 1977). The "filter rearing system" (Fisher & Cáceres 2000; Cáceres et al. 2004), involves managing a mother colony that is filtered to maintain the purity of genetic sexing strains, and from which large colonies are regularly derived for sterile male mass-production. This same approach could be extended to establish small "pre-filter" mother colonies under relaxed low density conditions, preferably in greenhouses or field-enclosures with trees and include the selection for males that achieve matings with wild females, and that maintain a certain irritability to reduce easy capture.

As in the case of the startle test, a number of variables have to be controlled to standardize the aspirator method to make it more reproducible. These include temperature and light conditions, fly location, fly sex, fly age, fly numbers (in relation to foliage surface), and nutritional history of the flies. For example, temperatures during testing should not fall outside the 24-28°C range, and flies located on the cage wall should not be included in the measurements, in view that flies away from the host tree are less able to evade capture attempt than those present within the host foliage.

A major disadvantage, as shown by our tests, is individual variation in skill in the use of aspirators. However, preliminary tests have shown that better uniformity can be achieved by with battery-powered mechanical aspirators or by not applying suction until just before the tip of the aspirator reaches the fly. In addition, as shown in Table 5, the observer effect can largely be reduced by restricting measurements to males involved in sexual activities, as they are the main targets of foraging predators (Hendrichs et al. 1994; Hendrichs & Hendrichs 1998), presumably because sexually active males are, in general, less wary of predator attacks (Nagamine & Itô 1980; Burk 1982; M.A.H., unpublished data).

In summary, we present a method that allows measuring the capacity of medflies to evade capture by predators, essential for sterile males to reach sexual maturation under open field conditions. It is recognized, that further standardization of the test is required to allow more accurate inter-strain comparisons to be made. However, it can be concluded that standard medfly mass-rearing conditions result in the production of sterile males that are much less able to evade predation than are wild males. Recognition of this problem and development of a corresponding quality control test could significantly improve the reliability and economics of sterile insect technology for fruit flies.

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