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Assessment of non-methyl eugenol-responding lines of *Bactrocera dorsalis* (Diptera: Tephritidae) males on lure response and mating

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Abstract

Bactrocera dorsalis (Hendel) (Diptera: Tephritidae), is a major global pest of fruits. Currently, the sequential male annihilation technique, followed by the sterile insect technique has been used to significantly reduce the population of feral males in this species. However, issues with sterile males being killed by going to male annihilation traps have reduced the efficacy of this approach. The availability of males that are non-methyl eugenol-responding would minimize this issue and increase the efficacy of both approaches. For this, we recently established two separate lines of non-methyl eugenol-responding males. These lines were reared for 10 generations and in this paper, we report on the assessment of males from these lines in terms of methyl eugenol response and mating ability. We saw a gradual decrease in non-responders from *ca.* 35 to 10% after the 7th generation. Despite that, there were still significant differences until the 10th generation in numbers of non-responders over controls using laboratory strain males. We did not attain pure isolines of non-methyl eugenol-responding males, so we used non-responders from the 10th generation of those lines as sires to initiate two reduced-responder lines. Using these reduced responder flies, we found that there was no significant difference in mating competitiveness when compared with control males. Overall, we suggest that it may be possible to establish lines of low or reduced responder males to be used for sterile release programs, that could be applied until the 10th generation of rearing. Our information will contribute to the further development of an increasingly successful management technique incorporating the use of SIT alongside MAT to contain wild populations of *B. dorsalis*.

Introduction

Male lures are widely used in tephritid fruit fly management programmes for detection and monitoring of pest population (Dominiak *et al.*, 2003; Vargas *et al.*, 2010; Manrakhan *et al.*, 2017). They are also used in control programmes such as male annihilation technique (MAT) (Vargas *et al.*, 2014). In that approach, a potent male lure such as methyl eugenol (ME) is widely used against the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Vargas *et al.*, 2010). ME is mixed with a toxicant and placed in bait traps of high density in particular areas to reduce the male fly population (Steiner *et al.*, 1970). The MAT has been successfully applied to eradicate fruit fly populations in several instances (Steiner *et al.*, 1970; Hancock *et al.*, 2000; Seewooruthun *et al.*, 2000; Manrakhan *et al.*, 2011) and MAT was the most frequently used method to manage the *B. dorsalis* population worldwide (Suckling *et al.*, 2014). MAT was highly successful in many cases (Hancock *et al.*, 2000; Allwood *et al.*, 2002; Lloyd *et al.*, 2010). However, the presence of non-ME-responsive *B. dorsalis* males was thought to be one reason why MAT did not work well in the Ogasawara Islands of Japan (Ito and Iwahashi, 1974; Habu *et al.*, 1984). Thus, some studies were conducted to identify the reasons for reduced response to the male lures by male fruit flies. These reasons included possible factors due to genetics (Ito and Iwahashi, 1974; Shelly, 1997; Liu *et al.*, 2017), prior ME exposure for adults (Shelly, 1994; Shelly, 2000; Khan *et al.*, 2017) and larval lure feeding (Shelly and Nishida, 2004; Manoukis *et al.*, 2018).

The presence of non-ME-responsive wild males is an impediment to any MAT programmes against *B. dorsalis*. This also includes application of MAT together with sterile insect technique (SIT). In SIT, sterile males that are mass-reared are released into the field to mate with feral females resulting in no progenies produced (Lance and McInnis, 2021). Interestingly, the development of non-ME-responsiveness in sterile males may prove to be beneficial in combined application of MAT and SIT. Modelling studies have suggested that simultaneous application of MAT and SIT is synergistic (Barclay *et al.*, 2014). However, the MAT and SIT approach was not developed because of the fear that those field-released sterile males

may be attracted and killed by existing ME traps that are used to kill feral males. These sterile males would die even before mating with feral females, but these deaths can be prevented with release of sterile males that are not attracted to ME (Pereira *et al*., 2022).

There was low incidence of repeat feeding on ME by males of *B. dorsalis* (Shelly, 1994), but ME consumption actually increases the mating competitiveness of sterile males (Shelly *et al*., 2010). Similar results were also demonstrated in subsequent combined MAT-SIT trials where there were reduction of post-release attraction of sterile male flies to ME-baited traps, and enhancement in post-release mating competitiveness (Shelly, 2020). Additionally, releases of *B. tryoni* sterile males fed with raspberry ketone have resulted in lower captures in MAT traps (Khan *et al*., 2017).

Non-responsiveness to ME in feral adults of *B. dorsalis* was detected in very low numbers (<6%) (Shelly, 1994, 1997; Chen *et al*., 2018), and earlier studies have shown that lines of non-ME-responsiveness in males can be selected and established (Ito and Iwahashi, 1974; Shelly, 1997). Also, our laboratory studies have indicated the possibility that non-ME-responsiveness (NR) in male flies can be raised from a single generation through successive exposures to ME. Therefore, it would be ideal if successive exposures of males to ME from a single generation can be used to select for NR flies with little or no mortality during the selection process. It is essential to assess NR line progenies across the generations to understand how those non-ME-responsiveness traits can be inherited. This study will provide information on the viability of those NR lines and how strongly expressed non-responsiveness to ME in development of NR lines from *B. dorsalis*. Therefore, in this paper, we evaluated the exposure of *B. dorsalis* NR lines from F2 through F10 generations to ME and their subsequent mating performance.

Materials and method

Insects

Colonies of *B. dorsalis* were provided with adequate sugar: yeast hydrolysate enzymatic (USB Corporation, USA) (3:1) food and water *ad libitum*. Flies were maintained at 25–29°C with 83–90% relative humidity under a 12: 12 (L:D) photoperiod. Rearing was done using cages of 40 cm × 40 cm × 40 cm and density of flies in those cages maintained at 400–500 flies to prevent overcrowding. Males and females were segregated within 3 days after emergence (DAE) and placed in separate cages and provided adequate food and water until used to the experiments.

Establishment of NR lines of B. dorsalis *colonies*

Two separate lines were initiated from flies that originated from 2 different collection sites in Serdang, Selangor, Malaysia, hereafter known as line 1 and line 2 respectively. Both lines were established from larvae of infested fruits of wild *Syzygium* sp.

In the fruit, we found the presence of sympatric species of *B. carambolae,* and flies have intermediate characteristics of *B. dorsalis* and *B. carambolae* (Wee and Tan, 2005) in the field. Therefore, adult emergence was subjected to identification of *B. dorsalis* (Drew and Romig, 2016) for each generation during colony domestication that ran for 5 generations to ensure laboratory adaptation (Schutze *et al*., 2015). Thereafter, sexually virgin males of 14–15 DAE were initially exposed to ME until at least two consecutive exposures demonstrated that those males had not responded to ME at all, and those nonresponsive males were used as sires to initiate those NR lines (Mandanayake and Hee, unpublished data). Those lines were being bred at 8 generations a year. We used about 200 pairs to initiate each generation. Correspondingly our laboratory strain (reared since 2009 with nine generations per year with irregular introgression) was reared as controls for all experimentations.

1. **Exposure of male NR lines to ME**. We conducted the cage assay to evaluate the presence of non-ME-responsiveness across the generation for two NR line progenies i.e., lines 1 and 2 respectively which were initiated using the non-ME-responsive sires obtained from successive exposures to ME from earlier studies. Initially, we did not use the NR F1 generation to screen for non-ME-responsiveness due to the lower number of flies available. Instead, sexually mature 200 virgin males (14–15 DAE) from F2 generation of each NR line and laboratory strain were kept separately in a screen cage (40 cm × 40 cm × 40 cm) for exposure to ME. Food and water were removed 0.5 h before the assays commenced. Five microlitres of ME (99.8% purity, Merck®, Germany) was applied by using a 10 microlitre syringe (Hamilton®, U.S.A.) on a roll of cotton wick (15 mm × 5 mm) that was placed on a Petri dish. Then, we covered the wick with a semi-spherical metal mesh (11.5 cm diam. × 4.5 cm height), before the entire unit was introduced into the cage.

   Shelly (1994) reported reduced incidence of repeated male flies feeding on ME, but it has not been established if non-ME-responsiveness is due to olfactory and/or gustatory factors. Identification of those factors was important in designing appropriate methods of inducing non-ME-responsiveness in competing sterile males for feral females. Thus, initially in our study, the male flies were only exposed to ME without feeding. The unit was placed inside the cage for 0.5 h to attract the flies. Then unit was carefully covered with an inverted plastic container (11 cm diam. × 21 cm height) and taken out of the cage to remove the flies attracted to the ME source. We considered the flies remaining in the cage as non-ME responsive flies (NR flies) and their number was recorded. The experiment was conducted from 2nd to 10th each generation, but F3 and F4 generations were not evaluated as there was no access to laboratory due to mandatory closure caused by the coronavirus disease 2019 (Covid-19) pandemic. NR lines were only bred, and density of flies increased for those generations. We used laboratory strain males as controls. The experiment was conducted during 07:45–09:00 h. The experiment was repeated for six times (using different cohorts) for each generation, with an interval of every two days.
2. **Effects of subsequent exposure of ME on male mating success.** In this experiment, male flies' exposure to ME after 1 and 3 days respectively, was evaluated for effects on mating. It was important to ascertain if exposure to ME would confer mating advantage over males that have not been exposed to ME. Sexually mature virgin males (14–15 DAE) from two NR lines were separately placed in screen cages, and flies were exposed to ME using similar methods as described previously. We collected male flies that were attracted to ME and kept in a separate cage with food and water before being subjected to mating trials as follows. One hundred males that were earlier attracted to ME source were paired with same number of females. At the same time, one hundred males from each NR lines, which was not exposed to ME (non-ME-exposed), were paired with females in separate cages. All the flies used

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in the experiment were of similar age. The experiment was repeated using laboratory strain flies as controls. Mating was considered successful when the pair of male and female was *in copula* for over 0.5 h. The mating pairs were collected at dusk, using glass vials and numbers were recorded. The experiment was replicated for 6 times.

3. **Mating ability of NR line progenies.** We conducted a cage assay to determine for the mating ability of NR flies compared to the normal laboratory strain males. Sexually matured virgin males (14–15 DAE) from two NR line progenies were exposed to ME as described previously and only those not attracted to ME was used for this experimentation. Laboratory strains of sexually mature males (15–16 DAE) were used as controls. Flies were kept in a rearing screen cage (40 cm × 40 cm × 40 cm) with adequate food and water. Females flies from laboratory strain of similar age were used for the mating study. Females were introduced to the cages in the morning hours of the same morning when the experiment was initiated. The observations were made between 18:30 and 19:30 since *B. dorsalis* mating occurred during dusk. Mated pairs were collected using a glass vial and the number was recorded. Experiment was repeated for 6 times using different cohorts of flies.

Statistical analysis

We analysed data obtained from males exposed to ME from F2 to F10 generation in each NR line 1 and line 2 separately. Data were subjected to Shapiro–Wilk normality test ($P = 0.05$) followed by analyses of variance. Percentage of non-ME-responsive males from each F2 to F10 of NR line 1 and line 2 were compared separately with the laboratory strain. Data were subjected to Shapiro–Wilk normality test ($P = 0.05$) followed by analyses of variance. Means were compared using Tukey's tests *at* $P = 0.05$.

We subjected mating pairs recorded for ME-exposed and non-ME-exposed males from NR line 1 and line 2 to the Student's *t*-test at $P = 0.05$. The number of mated pairs recorded on NR lines and laboratory were subjected to Shapiro–Wilk normality test ($P = 0.05$) followed by analyses of variance. Means were compared using Tukey's tests at $P = 0.05$. All analysis was done using Statistical Package for the Social Sciences software (IBM SPSS Statistics for Windows, Version 25.0. IBM Corp., Armonk, NY).

Results and discussion

The occurrence of NR flies from F2 to F10 generations of two NR line progenies (except information for F3 and F4 generations that were absent due to mandatory Covid-19 laboratory closure) was evaluated and reported as follows. As shown in table 1, when male flies were exposed to ME, in NR line 1, amongst the generations tested, we found significantly higher NR numbers were obtained at F2 generation but those numbers were significantly reduced at the F5 generation ($F = 35.78$; $df = 6, 39$; Tukey's test, $P = 0.001$). We found that the F10 generation contained the lowest number of NR males obtained among all the tested generations. Also, we found a similar trend in line 2 with relatively higher numbers of NR flies in F2 generation among all the tested generations, with lowest number in the F10 generation ($F = 19.85$; $df = 6, 35$; Tukey's test, $P = 0.001$) (table 1). In both lines, there appeared *ca.* 4-fold reduction in NR males from F2 to F10 generation. Moreover, from F6 to F10 generations, the presence of NR flies in both lines were significantly higher than the laboratory strain (as controls), which only showed 5–6% of naturally occurring NR files throughout the study. The number of NR flies recorded in F2 generation for lines 1 and 2 were significantly higher than the laboratory strain ($F = 16.98$; $df = 2, 14$; Tukey's test, $P = 0.001$) with *ca.* 6-fold increment over that of the laboratory strain (table 1). This trend of higher NR flies over that of controls continued through the F10 generation although from F9 onwards, there was a marked decreased in the number of NR males obtained at below 10% but still significantly higher than that of controls ($F = 8.54$; $df = 2, 14$; Tukey's test, $P = 0.004$). There was no difference between the presence of NR flies reported between both NR lines ($P = 0.938$). Based on these results, we suggest the possibility that 10 successive generations from a single line of reduced responder flies be exposed to ME to obtain NR males although yields of NR males were higher in earlier generations. Further, there may be a weak phenotypic expression of non-ME-responsiveness in the generations tested although after the F7 generation, the trend of downward non-ME-responsiveness appeared to have stabilized.

Table 1. Non-attraction of B. dorsalis males to methyl eugenol following males' exposure to the lure

Generation	NR Line 1	NR Line 2	Laboratory strain
F2	$35.4 \pm 2.2^{a, A}$	$34.3 \pm 4.2^{a, A}$	$5.7 \pm 0.8^{b, A}$
F5	$19.2 \pm 2.6^{a, B}$	$20.4 \pm 3.0^{a, B}$	$6.7 \pm 1.3^{b, A}$
F6	$16.8 \pm 1.7^{a, BC}$	$16.9 \pm 0.7^{a, BC}$	$6.7 \pm 1.3^{b, A}$
F7	$13.8 \pm 1.1^{a, BC}$	$15.8 \pm 1.6^{a, BC}$	$6.4 \pm 1.3^{b, A}$
F8	$10.1 \pm 0.7^{a, BCD}$	$10.5 \pm 0.7^{a, CD}$	$6.1 \pm 1.2^{b, A}$
F9	$8.3 \pm 0.9^{a, CD}$	$9.3 \pm 0.5^{a, CD}$	$5.2 \pm 0.7^{b, A}$
F10	$8.0 \pm 0.4^{a, D}$	$8.3 \pm 0.4^{a, D}$	$5.3 \pm 0.7^{b, A}$

Percentage of means (bars = ± SE) with different lowercase letters for each row; and different uppercase letters for each column are significantly different at $P < 0.05$ using Tukey's Test, $n = 250$ males, 5–6 replicates of different cohorts tested. Absence of data for F3 and F4 generation due to mandatory Covid-19 laboratory closure.

Cunningham (1989) reported that though some proportion males appear not to respond to lures in any particular population, but development of a complete non-respondent strain for a specific attractant has never been proven. Efforts to artificially select those non-lure-responding lines have not succeeded in obtaining lines that demonstrated complete disappearance of lure attraction. Instead, we suggest that reduced response had occurred across generations in response to a particular male lure (Shelly, 1994; Liu *et al.*, 2017; Yazdani, 2022). Our results are consistent with those findings as non-responsiveness to ME could not entirely disappear even when males obtained from initial non-ME-responsive parent lines used as sires, and those males were subjected to successive exposures to ME until persistent non-attraction to lure was demonstrated in only extremely low numbers of males.

We attained a higher percentage of non-ME-responsiveness in the 2nd generation compared to other studies that demonstrated lower non-ME-responsiveness (Shelly, 1997; Liu *et al.*, 2017; Chen *et al.*, 2018). Repeating selection of NR sires to initiate lines, and assessment across generations could be done for the domesticated colonies originating from different collection sites to optimize the current result. However, yields of NR males may vary due to differences originating from those lines. We found that the percentage of

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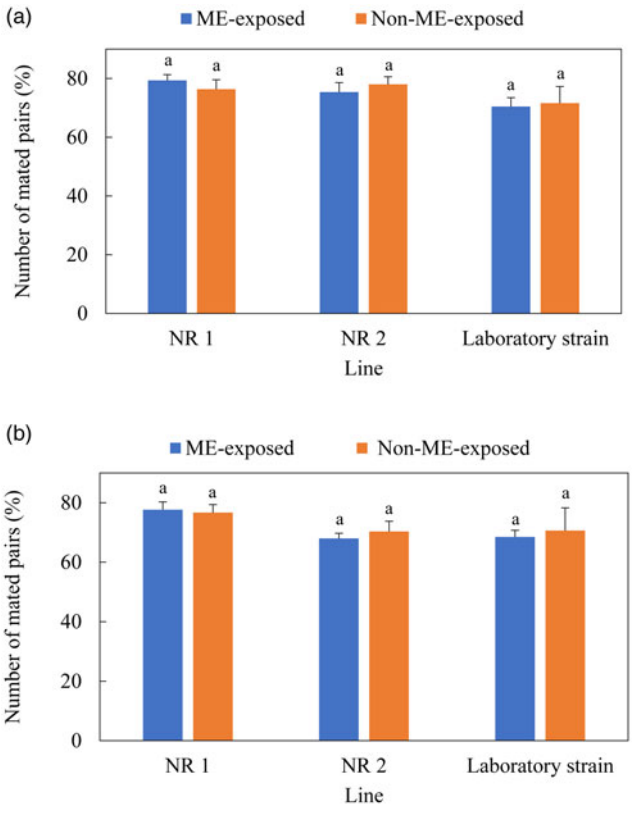

Figure 1. Copulating males from NR lines and laboratory strain following (a) 1-day, and (b) 3-days' exposure to methyl eugenol (ME). Percentage of means (bars = ± SE) with same letters are not significantly different at $P > 0.05$, $n = 100$ pairs, 6 replicates, Tukey's test.

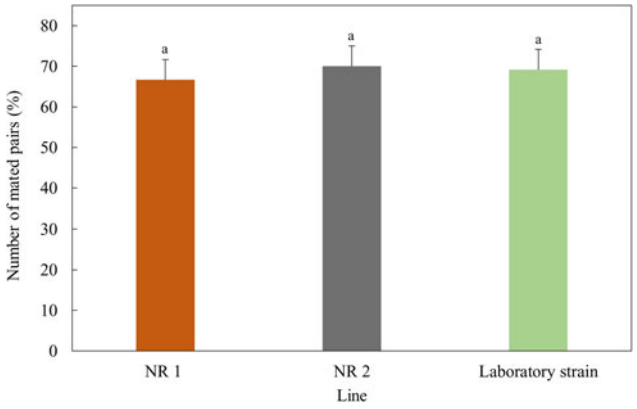

Figure 2. Non-ME-responsive copulating males from NR lines and laboratory strain following exposure to ME respectively. Percentage of means (bars = ± SE) with same letters are not significant differently at $P > 0.05$, $n = 50$ pairs, 6 replicates, Tukey's test.

NR flies was reduced across the generations, and one reason may be attributed to artificial selection not commenced across the generations. Similar results were found when *B. tryoni* was screened for cuelure-non-responsiveness (Yazdani, 2022). In that particular study, the non-responsiveness percentage was highest in F2 generation and remained constant during artificial selection. However, the percentages of unresponsive males were reduced after stopping the artificial selection for two generations and followed by repeating the screening procedure (Yazdani, 2022). However, as our study only evaluated the phenotypic variation of the NR flies, we think that further investigation on the molecular and genetic basis on non- or reduced-ME-responsiveness particularly affecting olfactory receptors and/or sensilla concerned is warranted.

We found that exposure of NR males to ME did not increase the number of those males that successfully copulated with conspecific virgin females (fig. 1). There was no significant difference in the number of mating pairs between the ME-exposed and non-ME-exposed males 1 day after exposure of NR line 1 ($t = -0.266$; $df = 10$; Student's t-test, $P = 0.796$) and NR line 2 ($t = -0.642$; $df = 10$; Student's t-test, $P = 0.536$) to ME. Additionally, we found similar results 3-days after exposure to ME as in NR line 1 ($t = -0.276$; $df = 10$; Student's t-test, $P = 0.746$) and NR line 2 ($t = 0.607$; $df = 10$; Student's t-test, $P = 0.557$) respectively. Observations involving laboratory strains of males as controls were similar. Thus, based on the result of our experiment, we suggest that exposure to ME (without feeding) did not increase the mating performance of male flies. In contrast, Cáceres *et al.* (2018) have demonstrated that ME-aromatherapy had increased mating competitiveness of male *B. dorsalis*. In those experiments, male flies were exposed to 0.5 ml of ME for 5 h.

In our study, only 5 μl of ME was exposed for 0.5 h to the male flies. Cáceres *et al.* (2018) believed that exposure to higher amounts of ME at longer periods of time may have resulted in those exposed male flies acquiring volatiles of ME as evident from the action of proboscis pumping that is a function of feeding (Vijaysegaran *et al.*, 1997). However, more investigations are needed to ascertain if the male rectal gland of ME-exposed males contain ME-derived male sex pheromone components such as 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol. These compounds were sequestered into the rectal gland by haemolymph transport as a result of ME consumption (Hee and Tan, 2004, 2006).

We found that there was no significant difference in the percentage of mating pairs of NR males compared with laboratory strain males ($F = 0.179$; $df = 2, 15$; Tukey's test, $P = 0.838$). We speculate that NR flies could behave in a similar way to laboratory reared *B. dorsalis* males in terms of successful copulation. The implications from our observations are that those NR males have not lost their mating ability and therefore, can be expected to be as competitive as laboratory reared males. As such, assuming that sufficient NR or ME-reduced responders were obtained for field releases, operationally, in simultaneous MAT-SIT programme, we predict that there will be less one factor of sterile males being lured to MAT traps containing ME. Therefore, the removal of this factor would optimize the overall efficiency of the control programme (fig. 2).

It has been reported that feeding at the fly larval stage on ME may decrease the responsiveness to lure at the adult stage. Manoukis *et al.* (2018) raised *B. dorsalis* larvae on *Terminalia catappa* host fruit that contained relatively higher ME concentrations (3.88 $\mu g\, g^{-1}$ fruit) to test the reduced ME responsiveness of adults. Larvae of male flies fed on guava, *Psidium guajava,* had higher non-ME-responsiveness compared to flies reared on artificial food without ME (Chen *et al.*, 2018) though guava contained lower amounts of ME (0.023 $\mu g\, g^{-1}$ fruit) (Manoukis *et al.*, 2018). In contrast, Shelly and Nishida (2004) found that inclusion of ME to the *B. dorsalis* artificial larval diet did not result in either the sequestration of ME metabolites in the adult male rectal gland nor increased mating success relative to males reared on the same larval diet without ME.

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Nevertheless, although larval feeding on ME was not tested in our study, we recommend that this approach should be integrated in subsequent studies involving selected single line of non- or reduced ME-responsive colony that is successively exposed to ME in order to increase the numbers of NR males. It must be noted that while inclusion of ME in larval diet has been shown not to improve mating success of those adult flies, this is not surprising based on the current results as long as there is no significant difference between those flies and controls. At the very least, there were the practical implications from our findings. We suspect that NR males can compete as well as those laboratory-reared males. This finding warrants further investigation especially if there is possible synergistic action with inclusion ME in the larval diet of NR lines in producing NR males that demonstrate persistent non-attraction to ME. Further at this stage, it is not known if chemoreceptor or gustatory receptor(s) at the olfactory organs leading to neuronal action are responsible for the non-responsiveness of males to ME. Reduction in male flies' attraction to ME was achieved by gene silencing (Liu *et al.*, 2017; Chen *et al.*, 2021). However, the molecular basis of non-ME-responsiveness is still not understood as higher expressions of those genes were recorded instead in those non-ME-responsive males (Fan *et al.*, 2022).

Thus, in this proof-of-concept, we demonstrated that first, it was possible that NR or reduced responder males can be obtained from a single generation of colony through successive exposures of males to ME. Secondly, the non-ME-responsiveness trait decreased to less than 10% in the 7th generation, but the subsequent 3 generations continued to show significant numbers of non-ME-responsiveness compared to controls. Finally, based on similar mating competitiveness between NR males and controls, we suggest that no loss of mating ability in the former. Additionally, direct ingestion of ME offered a mating advantage over non-ME-fed males instead of only exposure without feeding of ME when attracting conspecific females. Furthermore, when sterile release programs are combined with MATs, the use of these males may significantly improve the effectiveness of such programs by reducing the loss of males to lure entrapments.

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