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Source: Journal of Economic Entomology, 111(4) : 1644-1649

Published By: Entomological Society of America

URL: https://doi.org/10.1093/jee/toy095

Journal of Economic Entomology, 111(4), 2018, 1644–1649
doi: 10.1093/jee/toy095
Advance Access Publication Date: 12 April 2018
Research Article

Terminalia Larval Host Fruit Reduces the Response of *Bactrocera dorsalis* (Diptera: Tephritidae) Adults to the Male Lure Methyl Eugenol

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Subject Editor: Charles Burks

Received 12 December 2017; Editorial decision 21 March 2018

Abstract

Methyl eugenol (ME) is a powerful semiochemical attractant to males of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), and is the keystone of detection, control, and eradication programs against this polyphagous and highly invasive tephritid pest. Despite its status as a model lure against *B. dorsalis*, variation among individuals in their attraction is known, independent of the generally increasing attraction with age and decreases with previous exposure. Here we report that adult male *B. dorsalis* that fed on *Terminalia catappa* L. (Myrtales: Combretaceae) (tropical almond) fruit as larvae have a significantly lower behavioral response to ME compared with wild males from *Psidium guajava* L. (Myrtales: Myrtaceae) or colony-reared males raised on artificial larval diet. F1 males from the tropical almond stock reared on artificial larval diet did not show reduced attraction to ME, suggesting that the lowered response of parental males (sires) results from the host fruit itself, perhaps its relatively high amount of ME. Experiments with ME added to artificial diet lend some support to this interpretation. In addition to the results above, we report on quantities of ME in three different host fruits (*T. catappa, P. guajava,* and *Carica papaya* L. (Brassicales: Caricaceae)) of *B. dorsalis*. This study indicates the need for further research on the effect of host fruit on adult response to lures in economically important tephritids.

Key words: trapping, semiochemical, invasive, detection, eradication

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is one of the world's most damaging insect pests of fruits and vegetables, owing to its high vagility (Froerer et al. 2010), polyphagy (>120 larval host plants, Clarke et al. 2005), and fecundity (Huang and Chi 2014). Endemic to Southeast Asia, the species is highly invasive and now occurs throughout tropical Asia and Africa as well as the Pacific Islands, including Taiwan and Hawaii (Clarke et al. 2005). Individuals of *B. dorsalis* are regularly detected in California, where the species is considered an imminent and serious threat to the state's economically important agribusiness (Barr et al. 2014).

Detection and control of *B. dorsalis* rely largely on the male-specific lure methyl eugenol (ME, 4-allyl-1, 2-dimethoxybenzene), which is voraciously consumed by *B. dorsalis* males and thus used to bait traps in target areas (Tan et al. 2014). Used in conjunction with an insecticide, ME is used both as a survey tool to detect the presence of incipient infestations and as a control measure in the so-called male annihilation technique (Vargas et al. 2014), where a high density of ME + toxicant traps are established to eliminate males and ultimately the entire population.

Other male lures, most notably trimedlure (*tert*-butyl 4- and 5-chloro-*cis* and *trans*-2-methylcyclohexane-1-carboxylate) and cue-lure (4-(*p*-acetoxyphenyl)-2-butanone), are similarly used against other tephritid species, but ME is generally considered the most powerful of the tephritid male attractants. Mark-release-recapture studies support this notion, as *B. dorsalis* males were captured in higher proportions in ME-baited traps than were males of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) in trimedlure-baited traps or males of *Zeugodacus* (formerly *Bactrocera*, Virgilio et al. 2015) *cucurbitae* (Coquillett) (Diptera: Tephritidae) in cue-lure-baited traps (Shelly et al. 2014). Although ME is a powerful attractant, males of *B. dorsalis* nonetheless vary in their response, and even in small laboratory cages not all males are attracted to an ME source (Shelly 1994). The complete set of factors underlying this variability is not known with certainty, but several parameters have been identified, including:

i. Genetics: The use of non-ME-responding males as sires resulted in increased levels of lure nonresponsiveness in several generations in laboratory lines of *B. dorsalis* (Itô and Iwahashi 1974, Shelly 1997), indicating a genetic component to ME attraction. This result supports the claim by Japanese workers (Itô

and Iwahashi 1974, Habu et al. 1984) that prolonged use of ME-baited kill stations at low levels on the Ogasawara Islands selected for nonresponding males of *B. dorsalis*, which thwarted eradication efforts on that island.

ii. Age: Although young males may display low attraction, sexually mature males show the strongest response to ME (Wong et al. 1989, Shelly et al. 2008). This temporal relationship presumably reflects the incorporation of ME into the male sex pheromone, which is released only upon attaining sexual maturity, and immature males that do feed on ME are apparently incapable of sequestering the compound for later use in pheromone synthesis (Shelly et al. 2008).

iii. Prior ME exposure: In mark-release-recapture studies, mature males of *B. dorsalis* that were fed ME applied to cotton wicks were captured in lower numbers than were control males not provided ME prior to release (Shelly 1994). This effect may depend on the amount of ME consumed, as a similar study that used a natural ME source, flowers of *Cassia fistula* L. (Fabales: Fabaceae) and *Fagraea berteriana* A. Gray (Gentianales: Gentianaceae) with a much lower concentration of ME, failed to detect any reduction in male attraction following floral exposure (Shelly 2000).

The objective of this study was to examine another potential cause of inter-male variability in responsiveness to ME, namely the influence of larval host fruit in attenuating ME response in adults. This test arises from anecdotal observations indicating low response to ME by sexually mature adult male *B. dorsalis* emerging from fruit of *Terminalia catappa*, a preferred host of *B. dorsalis* with high ME content (Siderhurst and Jang 2006). Thus, we further hypothesized that the cause of lowered response in these males was the high amount of ME in this particular fruit. To our knowledge, only one prior study has indirectly investigated this possibility (Shelly and Nishida 2004). In that work, the addition of ME to artificial larval diet did not result in either the sequestration of ME metabolites in the adult male rectal gland (site of pheromone synthesis) or increased mating success relative to males reared on the same larval diet but lacking ME. However, the effect of larval ME ingestion on adult male response to ME has not been directly tested, and the relationship between male individuals' attraction to ME and mating success is not clear.

Here, we report the effect of larval consumption of *T. catappa* fruit and ME on *B. dorsalis* male response to ME. Specifically, we 1) evaluated male response to ME using wild and colony male flies originated from two field-collected host fruits (*T. catappa and Psidium guajava* L., the latter suspected to have low ME content) and artificial larval diet, respectively, 2) tested whether the effect of larval consumption of *T. catappa* on ME response was genetic and thus transferred to next generation, 3) determined the effect of ME consumption on male response to ME by manipulating ME content in artificial larval diet, and 4) compared ME content in three different host fruits (*T. catappa, P. guajava,* and *Carica papaya*) of *B. dorsalis*.

Materials and Methods

Insects

Field-collected insects were obtained from *T. catappa* fruit ('tropical almond', also known as 'false kamani' in Hawaii) from Keaukaha, Hawaii Island (19°43′55.42″N, 155°1′11.66″W) and also from *P. guajava* fruit ('guava') from Keaau, Hawaii Island (19°36′26.04″N, 155°4′15.52″W). Fruit were held following the method described in Gayle et al. (2013), by placing infested fruit into containers with dry wheat mill feed and artificial larval diet. Pupae plus eclosing adults were maintained at 24°C (±4°C) and 70% RH (±10%) in 25-cm cubical cages. Approximately 150–600 pupae resulted from each collection.

Colony-reared *B. dorsalis* for the experiments came from the research colony at the Daniel K. Inouye–U.S. Pacific Basin Agricultural Research Center (DKI-PBARC) in Hilo, HI. This colony was derived from wild flies collected in Puna, Hawaii Island, in 1984, and has been maintained in the laboratory on artificial larval diet (Tanaka et al. 1969) in large cages (0.6 × 1.18 × 1.32 m [w by h by d]) at a density of approximately 50,000 per cage. The colony has been periodically refreshed with wild flies from Hawaii Island to maintain genetic diversity.

Experiments were conducted on sexually mature male *B. dorsalis*, taking into account the longer prematuration interval of wild flies compared with colony-reared individuals (Vargas et al. 2000). For Experiment 1, the physiological age of wild males at the time of the assays averaged 575 degree-days Celsius [DDC] (SD = 24 DDC), which was equivalent to approximately 41 calendar days under our insectary conditions. For colony males, the average age at experiment time was 301 DDC (SD = 11 DDC), equivalent to about 22 calendar days. F1 males were tested at an average of 601 DDC (SD = 23 DDC), about 43 calendar days of age. We dissected 2–3 each of males and females to check for sperm and insemination, respectively, and confirm sexual maturation before experiments. As age is known to have an important role on ME response (Wong et al. 1989), we examined its effect via a separate experiment, identified here as Experiment 2. For Experiment 2, age ranged between approximately 200 and 450 DDC (further details in the "Experiments" section).

Measuring ME Response

ME-response bioassays were conducted using Y-tube glass olfactometers (5.5 cm diameter and 25 cm arm and base length). Air flow in each arm was set at 175 ml/min through a carbon filter, with one arm containing the odor from 5-µl ME applied to a cotton dental wick placed in the chamber attached to the end of that arm and the other arm with no odor (empty chamber). Room temperature and RH were maintained at 24.5°C and 50%, respectively. After a 5-min equilibration period, 15 male *B. dorsalis* were introduced to the bottom of the base tube, and the number of individuals in each arm was recorded after 15 min. For a single trial (N = 1), we repeated this procedure with an additional 15 males with the lure on the other arm to avoid positional effects; therefore, a single trial result is the average of right- and left-arm lure placement assaying a total of 30 males.

We calculated the responsiveness of males in the olfactometer to ME following Bertschy et al. (1997). Briefly, the proportion activated ('responsive', R) was the number in both arms/number introduced; the proportion selective (S) was the number in the lure arm/number in both arms. Finally, the proportion responders was SR, equivalent to number in the lure arm/number introduced.

Experiments

We conducted two experiments to investigate the effect of larval consumption of tropical almond on adult ME responsiveness. In Experiment 1, we compared the response of wild males emerged from field-collected tropical almond fruits or guava fruits with colony-derived males reared on artificial diet (control). We also tested an F1 generation produced from wild flies that emerged from field-collected tropical almond (tropical almond F1/control). To create F1 generation, females were provided papayas (*C. papaya*), thought to

be low in ME, for oviposition, which were then supplemented with artificial larval diet to complete development as previously described. Comparison of ME response was conducted via Wilcoxon Rank Sum Tests in R version 3.3.2 with $\alpha = 0.05$ (R Core Team 2016).

In Experiment 2, we tested our hypothesis on the role of ME in larval diet on reduced adult male response to ME. Based on the ME level tested in Shelly and Nishida (2004), we compared the response of colony-derived males raised in plain artificial diet with those from artificial diet with 0.5% ME added. For each treatment, we followed cohorts of flies set up sequentially between August 2016 and March 2017 (control: 4 cohorts, $N = 25$ trials; ME-fed: 4 cohorts, $N = 21$ trials), and sampled individuals for response assays between the physiological ages of 225 and 420 DDC. For this experiment, overall responses were compared via two-tailed unpaired *t*-test on variance-stabilized [arcsin] transformed data. Linear regression was used to test the effect of physiological age while controlling for cohort. Both analyses were conducted in R.

Measuring ME in Host Fruit

ME content of tropical almond ($N = 17$), guava ($N = 7$), and papaya ($N = 7$) was quantified from the flesh portions of the fruits, where *B. dorsalis* larvae would typically feed (i.e., excluding the seeds of the papaya and guava or the hardened core of the tropical almond). To determine ME content in fruit, we used the extraction method described in Siderhurst and Jang (2006). In brief, 1 g of homogenized tissue from each fruit was extracted with 500 µl of dichloromethane (DCM). The samples were pulse vortexed for 2 h and then centrifuged at 0°C and 10,000 rpm for 30 min to separate the DCM from the fruit pulp; the DCM analyte was then removed from the centrifuge tube for analysis.

A 1-µl aliquot of the analyte was injected onto a gas chromatograph mass spectrometer (Agilent 6890N GC–Hewlett Packard 5973 MSD, Santa Clara, CA), fitted with a Zebron ZB-WAX Plus column (30 m × 0.25 mm ID × 0.25 µm, Phenomenex, Torrance, CA) for analysis. The samples were run in splitless mode with 1-min sampling time, with a temperature program starting at 40°C held for 1 min, increased by 5°C/min, up to 250°C, and held for 5 min. To increase sensitivity for detecting sub-ng quantities of ME, select ion monitoring (SIM) was used to scan for the *m/z* 178 ion, the molecular and base ion of ME.

We quantified ME in DCM analyte by creating a quantification curve for ME (ME [pg/µl] = $0.0084X + 34.952$, where X = the peak area of ME in GC-MS; $R_2 = 0.9962$; Supp Fig. 1 [online only]) based on a dilution series of concentrations ranging from 5 to 2,000 pg/µl using authentic ME standards. The concentration data were heterogeneous for variances based on Bartlett's test and thus were analyzed with the multiple comparison max-*t* test (Herberich et al. 2010) in R, as this test is robust to departures from assumptions of normality and homogeneity of variances.

Results

Experiment 1

We observed significantly lower ME response of wild male *B. dorsalis* emerging from tropical almond compared with all other flies (tropical almond compared with control, $W = 219.5$, $P < 0.001$; compared with guava, $W = 240.5$, $P < 0.001$; compared with tropical almond F1, $W = 12$, $P < 0.001$; no other comparisons were significant; see Fig. 1). The wild and F1 flies generally had lower response rates compared with the colony *B. dorsalis* reared on artificial diet. Guava and F1 wild flies reared on diet had nearly identical response rates. Average activation proportions (R) between the different groups followed the same trends as seen in the overall proportion responders (control mean = 0.71, SD = 0.16; guava mean = 0.61, SD = 0.20; tropical almond mean = 0.22, SD = 0.19; Tropical almond F1 mean = 0.59, SD = 0.19).

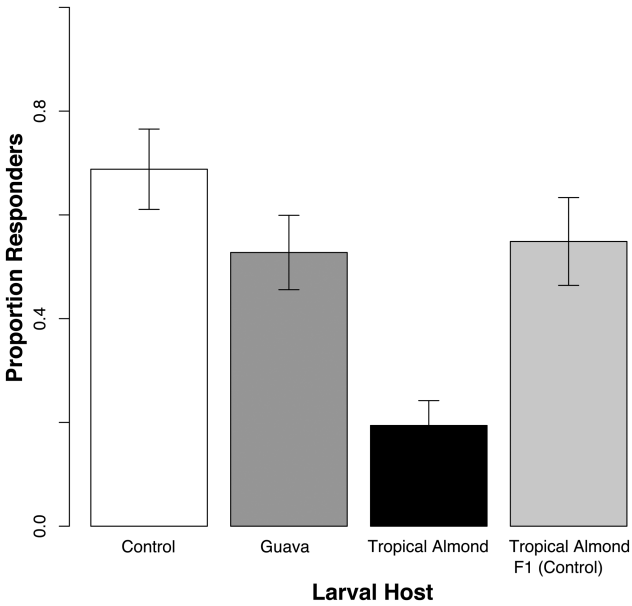

Fig. 1. Response of sexually mature adult male *Bactrocera dorsalis* to ME from colony stock reared on artificial diet (control; $N = 5$), wild collected from *Psidium* (guava; $N = 5$), *Terminalia* (tropical almond; $N = 8$), and F1 (reared on artificial diet using eggs from *Terminalia* origin wild *B. dorsalis*; $N = 4$).

Experiment 2

There was an overall lower response to ME by colony *B. dorsalis* reared with 0.5% ME in their larval diet compared with those raised without ME in their artificial diet (ME mean responders = 0.684; control responders = 0.787; $t = 2.418$, df = 35, $P = 0.021$). For the adult flies emerging from diet with 0.5% ME, we observed a significant effect of physiological age on ME responsiveness, which was not the case for adults emerging from control diet (Fig. 2, Table 1).

We anecdotally observed increased mortality at higher ME doses from separate experiments (up to 100% mortality from diet with 1.0% ME added). At the levels of ME used here, we did not observe significant and systematic survivorship differences.

ME Amounts in Host Fruit

The mean ME content in fruit was significantly greater in tropical almond (mean = 3.88 ± 0.84 µg/g fruit) than that in guava (0.023 ± 0.001 µg/g fruit) or papaya (0.019 ± 0.0004 µg/g fruit; both $P < 0.001$). The concentration of ME in guava was significantly higher than that of papaya ($P = 0.028$).

Discussion

Wild *B. dorsalis* adults emerging from tropical almond fruit were less responsive to ME compared with those from guava or colony-reared males raised on artificial larval diet. F1 progeny of the wild flies from tropical almond, reared on artificial diet, had a response rate equivalent to that of males from guava and the colony flies reared on artificial diet, which suggests that the tropical almond larval host is causing reduced response rather than any genetic difference between

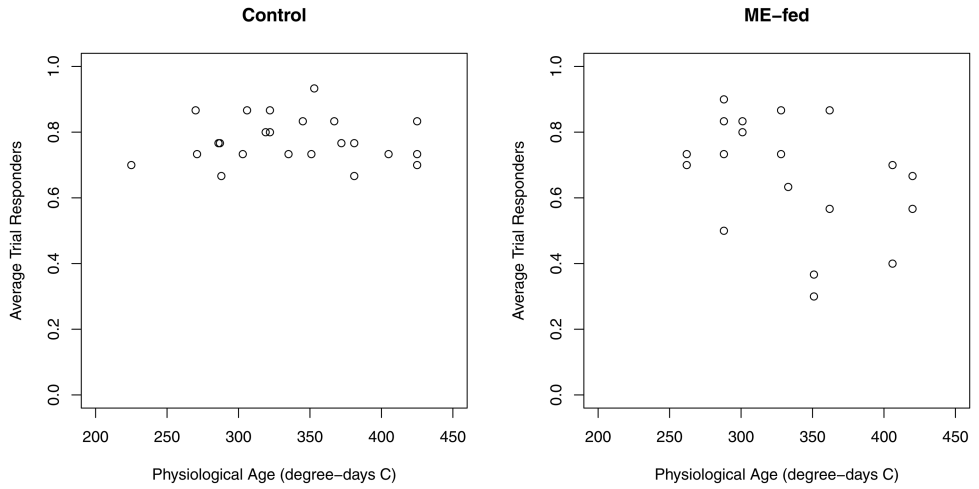

Fig. 2. Relationship between physiological age (DDC since pupal eclosion) and ME responsiveness for adult *Bactrocera dorsalis* emerged from larvae reared with normal artificial diet ('Control') and artificial larval diet with 0.5% ME ('ME-fed').

Table 1. Linear regression of the effect of physiological age on ME response by adults from ME-fed and control colony-derived larvae

	Control larvae: $R^2 = 0.17$; $F = 2.30$ on 2 and 22 df			
	Estimate	SE	*t*	*P*
Intercept	0.8172	0.0840	9.73	<0.001*
Cohort	0.0295	0.0139	2.12	0.046*
Physiological age	–0.0004	0.0003	1.31	0.203
	ME-fed larvae: $R^2 = 0.48$; $F = 8.32$ on 2 and 18 df			
Intercept	1.2763	0.1967	6.49	<0.001*
Cohort	0.1135	0.0364	3.12	0.006*
Physiological age	–0.0027	0.0007	3.90	0.001*

Cohort identity was significant in both models and so is included to control for its effects. * indicates statistical significance at $\alpha = 0.05$.

wild flies collected from the coastal area where tropical almond trees are found and those from Keaau where guava fruits were collected. Reduced response to ME by adults fed on ME is known (Shelly 1994), but this study represents the first demonstration of reduced response to ME by adults reared on a larval diet rich in ME.

Experimental addition of ME to artificial larval diet resulted in lower response to ME by emerging adult males compared with males reared on the same diet without ME added. However, this effect seems to be modulated by age: older males fed on ME had a lower response, but this was not seen in controls. This was unexpected, as ME response is thought to increase with male age, tied as it is to sexual maturation (Wong et al. 1989). In addition, we know from measurements presented here that the amount of ME we added to the diet was much higher than the amount found naturally in tropical almond fruit. Comparing the concentration of ME in the tropical almond with the artificial diet, the concentration of the artificial diet would be 5 mg/g, four orders of magnitude greater than the mean concentration of ME in tropical almond, at around 0.004 mg/g. Despite the high amount, the effect of larval ME feeding only modestly reduced response in older adult males.

Since adding large amounts of ME to diet seemed to only modestly reduce response, it seems likely that there are additional compounds in tropical almond, or some other characteristic besides the presence of ME, that contribute to the strongly reduced response by adults reared from this host fruit. Alternatively, the high amount of ME introduced to the diet compared with tropical almond fruit might have somehow reversed the nonresponsive behavior of the adults back to something more like the response rates seen in unexposed males or other from fruit other than tropical almond, but we consider this possibility remote.

Another characteristic of tropical almond fruit is that there is very little pulp, especially compared with fruit like guava. It is possible that nutritional deficiency is driving lower ME response by adult males from tropical almond. However, this hypothesis is not supported by anecdotal observation of accelerated development of larvae from tropical almond through the pupal stage compared with those from guava during this experiment (approx. 1–2 d earlier emergence compared with artificial diet), which may not suggest a nutritional deficiency.

Reduced response to ME has been observed before. Itô and Iwahashi (1974) found reduced response by males from an island near Okinawa but attributed that to a genetically distinct population in that area given its isolation. However, that population was not studied further, so the possibility that host fruit played a role remains. In our own experiments, we have observed large fluctuations in response rates (Manoukis et al. 2015), but often it is difficult to discern the precise cause, especially since the males tested were from the same colony and were reared on the same artificial larval diet. This observation, coupled with the present findings, suggest that the strength of response of *B. dorsalis* males to ME may have both genetic and environmental components, though we know of no studies assessing the role of a genetic component on ME response.

Any increase in our understanding of factors affecting adult *B. dorsalis* response to ME will be significant to program managers and others dealing with this invasive species because of the importance of this lure for detection and control of this species. From the findings of this study, we suggest that further research on host fruit effects on adult *B. dorsalis* response rates to ME should be conducted. This information on host effects could be used to alert program managers to the possibility of reduced response when particular host trees are in an outbreak area.

ME consumed by adults has been shown to increase mating competitiveness for males in at least three species of *Bactrocera* (McInnis et al. 2011, Orankanok et al. 2013, Haq et al. 2014). If ME, additional factors, or both consumed at the larval stage could be identified as both reducing responsiveness and increasing mating competitiveness, this could be useful for producing mass-reared flies for SIT programs, following the example from *C. capitata* and ginger root oil (Shelly and McInnis 2001). Though evidence to date does not show increased mating competitiveness of adult male *B. dorsalis* via larval ME feeding alone (Shelly and Nishida 2004), we are not aware of mating competitiveness experiments with larvae reared from ME-containing fruit such as that of *Terminalia*. Reduced response rates to ME would also mean treated males would be less susceptible to ME-based control measures against wild males such as male annihilation technique, allowing use of this control measure in combination with sterile insect technique (Akter et al. 2017, Khan et al. 2017).

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

This study would not have been possible without the many hours of hard work rearing and assaying the response of many flies by many talented individuals. Among those who contributed to collecting these data were Lori Carvalho, Thomas Mangine, Caley Saragosa, Stephanie Gayle, and Shannon Wilson. We also thank the rearing crew at DKI-PBARC, including Keith Shigetani, Mike McKenney, and Masayuki Osako. This work was funded by USDA-ARS. Opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the USDA. USDA is an equal opportunity provider and employer.

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