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Effects of Methyl Eugenol Feeding on Mating Compatibility of Asian Population of *Bactrocera dorsalis* (Diptera: Tephritidae) with African Population and with *B. carambolae*

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Abstract

Males of some species included in the *Bactrocera dorsalis* complex are strongly attracted to methyl eugenol (ME) (1,2-dimethoxy-4-(2-propenyl) benzene), a natural compound occurring in a variety of plant species. ME feeding of males of the *B. dorsalis* complex is known to enhance their mating competitiveness. Within *B. dorsalis*, recent studies show that Asian and African populations of *B. dorsalis* are sexually compatible, while populations of *B. dorsalis* and *Bactrocera carambolae* are relatively incompatible. The objectives of this study were to examine whether ME feeding by males affects mating compatibility between Asian and African populations of *B. dorsalis* and ME feeding reduces male mating incompatibility between *B. dorsalis* (Asian population) and *B. carambolae*. The data confirmed that Asian and African populations of *B. dorsalis* are sexually compatible for mating and showed that ME feeding only increased the number of matings. Though ME feeding also increased the number of matings of *B. dorsalis* (Asian population) and *B. carambolae* males but the sexual incompatibility between both species was not reduced by treatment with ME. These results conform to the efforts resolving the biological species limits among *B. dorsalis* complex and have implications for fruit fly control programs in fields and horticultural trade.

Key words: *Bactrocera dorsalis, Bactrocera carambolae*, mating compatibility, *Bactrocera dorsalis* species complex, methyl eugenol

Within the family Tephritidae, or true fruit flies, the genus *Bactrocera* contains over 500 species occurring primarily in tropical and subtropical Asia, Australia, and South Pacific Islands (Drew and Hancock 2000). Nearly 100 species have been assigned to the *Bactrocera dorsalis* complex (Drew and Hancock 1994, Clarke et al. 2005, Drew and Romig 2013), and several species within this complex (notably *B. dorsalis* (Hendel) and *Bactrocera carambolae* Drew and Hancock are among the most notorious agricultural pests because of their polyphagy and high dispersal and invasion capacity. These species (along with several other nonpest taxa) constitute a group of closely related sibling species, whose identification based on morphological characters is difficult (Clarke et al. 2005, Drew et al. 2008).

Pressing economic issues coupled with doubts on the taxonomic status of *Bactrocera invadens* Drew, Tsuruta and White, *Bactrocera papayae* Drew and Hancock, and *Bactrocera philippinensis* Drew and Hancock have prompted recent studies exploring the utility of different data sets in delimiting species within the *B. dorsalis* complex. These reports have involved molecular analyses of genetic variation both within (Dai et al. 2004, Shi et al. 2005) and between (Muraji and Nakahara 2002, Naeole and Haymer 2003, Khamis et al. 2012, Schutze et al. 2012b, Krosch et al. 2013) species, karyotyping (Baimai et al. 1995, 2000), morphometrics (Iwaizumi et al. 1997, Drew et al. 2008, Khamis et al. 2012, Schutze et al. 2012a, Krosch et al. 2013), larval host use among rainforest plants (Allwood et al. 1999, Drew 2004), and chemical analyses of male

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sex pheromones (Tan et al. 2011). Several of these studies (Tan et al. 2011, Khamis et al. 2012, Schutze et al. 2012b, Krosch et al. 2013) and mating compatibility studies provide strong evidence that *B. carambolae* is a valid species and species designations for *B. invadens*, *B. papayae*, and *B. philippinensis* are not justified and that perceived morphological differences among these taxa do not represent reproductive isolation but continuous geographic variation among *B. dorsalis* populations (Schutze et al. 2013, 2015a; Bo et al. 2014).

Males of *B. carambolae* and economically important taxa (*B. philippinensis, B. Papayae, and B. invadens*) included in the *B. dorsalis* complex are strongly attracted to methyl eugenol (ME; 1,2-dimethoxy-4-(2-propenyl)benzene (Iwahashi et al.1996, Shelly 2010, Tan et al. 2011); a phenylpropanoid compound found in > 450 plant species (Tan and Nishida 2012). This response underlies the use of ME in 1) surveys with ME-baited traps to detect the presence of incipient *Bactrocera* populations and 2) the male annihilation technique (MAT), whereby devices containing ME mixed with an insecticide are distributed on an area-wide basis in the target area to suppress/eradicate the male (and subsequently the entire) population (Vargas et al. 2010). Understanding to the biological basis of male attraction to ME will further strengthen the use of this chemical in area-wide control programs. Briefly, males convert ingested ME to several metabolites that are stored in the rectal gland and incorporated into and emitted as components of the male sex pheromone (Nishida et al. 1988a, Wee and Tan 2007). ME metabolites are known to be attractive to conspecific females (Hee and Tan 1998, Wee et al. 2007) and pathway of ME metabolism and ME metabolites is different in *B. carambolae* than other species in the *B. dorsalis* complex. *B. dorsalis* males convert the ingested ME into two main components, 2-allyl-4,5-dimethoxyphenol (DMP) and *trans*-coniferyl alcohol (CF) (Nishida et al. 1988a,b; Tan and Nishida 1996) and a later study by Tan et al. (2011) showed that similar to Asian *B. dorsalis* males, African *B. dorsalis* males also convert the ingested ME to similar metabolites and in the same ratio. *B. carambolae* males however convert ME to only CF (Wee et al. 2007). CF is a common metabolite of ME and is part of the pheromone blend of *B. dorsalis* and *B. carambolae* males and females of both species are attracted to CF.

Although female attraction and mating are not dependent on the presence of ME-derived compounds in the male pheromone (Kobayashi et al. 1978) but several studies have shown that their incorporation increases female attractiveness to the pheromone and enhances male mating success (Shelly and Dewire 1994, Wee et al. 2007). Therefore, given the prominent role of ME metabolites in the mating behavior of males in the *B. dorsalis* complex, the objectives of this study were to examine whether ME feeding by males affects mating compatibility between Asian and African populations of *B. dorsalis* and ME feeding reduces male mating incompatibility between *B. dorsalis* (Asian population) and *B. carambolae*.

Materials and Methods

Study Insects

Colonies of Asian and African populations of *B. dorsalis* and *B. carambolae* were established at the Insect Pest Control Laboratory of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria. The larvae were derived from field-collected fruits and reared in situ with subsequent pupae shipped to the Seibersdorf laboratory. The Asian *B. dorsalis* colony derived from infested mangos collected in the Saraburi Province of Thailand, the African *B. dorsalis* colony derived from pupae from Kenya, and *B. carambolae* derived from pupae from Suriname. At the start of this study, the Asian *B. dorsalis* colony had been reared in the Seibersdorf laboratory for ~8 generations, the African *B. dorsalis* colony for ~27 generations, and the *B. carambolae* colony for ~9 generations. Although younger colonies would have been more desirable, coordinating shipment of field-derived insects is logistically difficult and thus limited the experiment to these particular colonies. Colonies were maintained in screen cages (60 by 60 by 120 cm) held at 25 ± 1°C and 65% relative humidity (RH). Adults were provided a mixture of sugar and hydrolyzed yeast (3:1 ratio by weight) and water; eggs were collected in perforated bottles, and larvae were reared on a carrot powder-based diet modified from bulk diet (Hooper 1987) by replacing wheat bran with carrot powder. All ingredients in both diets were similar with little variation of preservatives and acidifying agents used to standardize the pH of diet. The flies used in this study were separated by sex within 3 d of emergence (well before sexual maturity at 10 d for African *B. dorsalis* Ihsan, unpublished data) and 16 d for the Asian *B. dorsalis* and *B. carambolae* (Schutze et al. 2013) held in cylindrical plexiglass cages (30 cm long, 45 cm diameter) with the ends covered with screen mesh with food and water as above. Males and females of the three species were 17- to 19-d old when tested.

Mating Trials

In a set of experiments using Asian *B. dorsalis* and African *B. dorsalis*, four different mating tests were carried out: experiment 1) ME was withheld from males of both Asian and African populations, experiment 2) ME was provided to males of Asian *B. dorsalis* only, experiment 3) ME was provided to males of African *B. dorsalis* only, and experiment 4) ME was provided to males of both populations.

In another set of experiments between Asian *B. dorsalis* and *B. carambolae*, four different mating tests were performed: experiment 5) ME was withheld from males of both species, experiment 6) ME was provided to males of Asian *B. dorsalis* only, experiment 7) ME was provided to males of *B. carambolae* only, and experiment 8) ME was provided to males of both species. Hereafter, we refer to males provided with ME as treated and ME-deprived males as control. When provided, 0.5 ml of ME was applied to a strip of filter paper (5 by 20 cm), which was then placed in a cylindrical plexiglass cage containing 100 males (16 ± 1 d old). ME exposure commenced at 10.30 h and lasted 1 h and was conducted 2 d prior to the mating test.

The mating trials were conducted in four field cages housed in an outdoor greenhouse at the Seibersdorf laboratory. The cages had a nylon mesh screening with a base of 4.0 m^2 and a height of 1.8 m and each contained a single potted orange tree (*Citrus sinensis* (L.) Osbeck) with a canopy that occupied much of the cage interior. On a given test day, 20 males of each species were released 90 min before sunset (the period of peak mating activity in these species, (Arakaki et al. 1984), and 40 females of each species were released 15 min later. The 2:1 female:male ratio was used to ensure that females of both species were available to males throughout the entire trial. In studies of mating competitiveness, the sex ratio is typically reversed but here the focus was on compatibility between the populations and not competition. All flies had been marked 1 d earlier by holding nonanaesthetized individuals motionless in nylon netting and applying water-based paint to the prothorax directly through the netting. Males and females of each population were marked with the same color, and different colors distinguished the treatments. In the case of treated males, marking was performed

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Table 1. Summary of matings recorded in the four mating experiments involving Asian and African *B. dorsalis*

Experiment matings	ME treatment (males)	DD	DV	VD	VV	Total
1	Neither population	67 (24.2)	73 (26.3)	62 (22.4)	75 (27.1)	277
2	Asian *B. dorsalis* only	76 (26.9)	68 (24.0)	82 (29.0)	57 (20.1)	283
3	Afr. *B. dorsalis* only	69 (26.4)	53 (20.3)	62 (23.8)	77 (29.5)	261
4	Both populations	82 (27.8)	66 (22.4)	84 (28.5)	63 (21.3)	295

In the four possible pairings, D represents Asian *B. dorsalis*, V represents African *B. dorsalis*, the first letter represents the male, and the second letter represents the female. For each mating combination, values represent the total number of matings recorded over all replicates for a given experiment and in parentheses the percentage of the total matings for the experiment that this value constitutes. The total number of matings observed per experiment is given in the far right column.

before the period of ME exposure. The cages were monitored continuously beginning with the release of the females until 1 h after sunset. Mating pairs were collected by gently coaxing them in vials (one pair per vial), and the time of collection was recorded. The vials were placed on the floor of the cage and checked every 15–20 min throughout the trial to note any decoupling [in dusk-mating *Bactrocera* pairs typically remain in copula throughout the night until sunrise (Arakaki et al. 1984)]. For each of the four experiments, trials were conducted in the four cages on each of two nights for a total of eight replicates per experiment. Air temperatures during the mating trials were 25 ± 2°C.

Random mating for each of four experiments was tested with a χ^2 test separately. In addition, we computed the isolation index (ISI) (Cayol et al. 1999), which provides a measure of mating compatibility or its converse mating isolation:

$$\mathrm{ISI} = \frac{(\mathrm{DD} + \mathrm{VV}) - (\mathrm{DV} + \mathrm{VD})}{(\mathrm{DD} + \mathrm{VV} + \mathrm{DV} + \mathrm{VD})}.$$

where DD is the number of couples between Asian *B. dorsalis* males and females, VV is the number of couples between African *B. dorsalis* males and females, DV is the number of couples between Asian *B. dorsalis* males and African *B. dorsalis* females, and VD is the number of couples between African *B. dorsalis* males and Asian *B. dorsalis* females. The index ranges from −1 (negative assortative mating) to +1 (positive assortative mating or total sexual isolation). A value of 0 represents random mating (equal proportions of the four possible mating combinations), i.e., complete mating compatibility. Conversely if index range didn't include value of 0 it represents nonrandom mating which is deviation from random mating (toward assortative mating) having higher proportion of homotypic couples. For each experiment, values of ISI were computed for the individual replicates, these values were used to compute an overall mean ISI for the entire experiment, and mating compatibility was identified if the 95% confidence interval of the overall mean included 0 (Schutze et al. 2013). ISI values for the Asian *B. dorsalis*–*B. carambolae* tests were computed and analyzed in identical fashion.

Two additional indices, which indicate the overall level of mating activity of males and females, respectively, were computed to complement ISI values (Cayol et al. 1999). The male relative performance index (MRPI) was computed as:

$$\mathrm{MRPI} = \frac{(\mathrm{VV} + \mathrm{VD}) - (\mathrm{DV} + \mathrm{DD})}{(\mathrm{DD} + \mathrm{VV} + \mathrm{DV} + \mathrm{VD})}.$$

The female relative performance index (FRPI) was computed as:

$$\mathrm{FRPI} = \frac{(\mathrm{VV} + \mathrm{DV}) - (\mathrm{DD} + \mathrm{VD})}{(\mathrm{DD} + \mathrm{VV} + \mathrm{DV} + \mathrm{VD})}.$$

Both of these indices range from −1 (all matings achieved by Asian *B. dorsalis* males or females, respectively) to +1 (all matings achieved by African *B. dorsalis* males or females, respectively). Same interpretation applies for Asian *B. dorsalis* and *B. carambolae*. A value of 0 represents equal mating performance (activity) between males or females, respectively, of the two species. The same computation and interpretation was applied to the Asian *B. dorsalis* and *B. carambolae* crosses. Analysis of the MRPI and FRPI indices was the same as that described above for ISI.

Comparisons among crosses subject to parametric assumptions were made with one-way ANOVA (analysis of variance). If significant variation was detected, a multiple comparisons procedure was performed to identify differences ($\alpha = 0.05$) in pairwise comparisons (Holm-Sidak). Analyses were performed using SigmaPlot 11 statistical software.

Results

Mating Patterns

Asian versus African B. Dorsalis. Random mating was tested with a χ^2 test using data pooled over all replicates as the data were homogenous across the individual replicates for all experiments ($P > 0.05$ in all cases). For each experiment, mating was random among individuals of Asian *B. dorsalis* and African *B. dorsalis* as approximately equal proportion of couples were observed for all four of the possible mating combinations and ME feeding by either of males didn't affect the status of random mating (Table 1; χ^2 values and associated P values were: experiment 1: $\chi^2 = 1.51$, $P = 0.68$; experiment 2: $\chi^2 = 4.73$, $P = 0.19$; experiment 3: $\chi^2 = 4.96$, $P = 0.18$; experiment 4: $\chi^2 = 4.79$, $P = 0.19$).

The ISI values likewise indicated random mating between Asian *B. dorsalis* and African *B. dorsalis* as the 95% confidence interval included zero in each experiment (Table 2). Likewise, the indices of male (MRPI) and female (FRPI) mating propensity revealed similar levels of mating activity between the species for all experiments, with the exception of experiment 2 in which Asian *B. dorsalis* females showed higher mating participation than African *B. dorsalis* females (56 vs. 44% participation in the total matings, Tables 1 and 2). Thus, in general, the ISI values obtained were not influenced by uneven levels of mating readiness between the species.

Asian B. dorsalis versus B. Carambolae. Similar to the Asian versus African *B. dorsalis* data, random mating between Asian *B. dorsalis* and *B. carambolae* was tested with a χ^2 test using data pooled over all replicates as the data were homogenous across the individual replicates for all experiments ($P > 0.05$ in all cases). For each experiment, nonrandom mating (higher proportion of homotypic couples) among individuals of Asian *B. dorsalis* and *B. carambolae* were observed for all four of the possible mating combinations and ME feeding by either of males didn't affect the status of nonrandom mating (Table 3; χ^2 values and associated P values were: experiment

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Table 2. ISI, MRPI, and FRPI as obtained during the field cage tests with Asian and African *B. dorsalis*

Experiment	ME treatment(males)	ISI Mean (95% CL)	MRPI Mean (95% CL)	FRPI Mean (95% CL)
1	Neither population	0.02 (±0.12)	−0.01 (±0.05)	0.07(±0.21)
2	Asian *B. dorsalis* only	−0.05 (±0.12)	−0.02 (±0.09)	−0.11 (±0.12)
3	Afr. *B. dorsalis* only	0.12 (±0.14)	0.06 (±0.14)	0.00 (±0.26)
4	Both populations	−0.02 (±0.12)	0.00 (±0.05)	−0.12 (±0.11)

Formulae for computing indices are given in text. For each experiment, mean values and 95% confidence limits (CLs) were calculated over all replicates ($n = 8$).

Table 3. Summary of matings recorded in the four mating experiments involving *B. dorsalis* and *B. carambolae*

Experiment matings	ME treatment (males)	DD	DC	CC	CD	Total
5	Neither species	91 (39.7)	41 (17.9)	65 (28.3)	32 (13.9)	229
6	*B. dorsalis* only	86 (32.7)	47 (17.9)	81 (30.8)	49 (18.7)	263
7	*B. carambolae* only	92 (33.6)	53 (19.3)	89 (32.4)	40 (14.6)	274
8	Both species	109 (37.7)	32 (11.0)	83 (28.7)	65 (22.5)	289

In the four possible pairings, D represents *B. dorsalis*, C represents *B. carambolae*, the first letter represents the male, and the second letter represents the female. For each mating combination, values represent the total number of matings recorded over all replicates for a given experiment, and in parentheses the percentage of the total matings for the experiment that this value constitutes. The total number of matings observed per experiment is given in the far right column.

Table 4. ISI, MRPI, and FRPI as obtained during field cage tests with *B. dorsalis* and *B. carambolae*

Experiment	ME Treatment (males)	ISI Mean (95% CL)	MRPI Mean (95% CL)	FRPI Mean (95% CL)
5	Neither species	0.36 (−0.16 ± 0.57)	−0.14 (±0.01)	−0.07 (±0.12)
6	*B. dorsalis* only	0.3 (−0.11 ± 0.42)	0.02 (±0.06)	0.01 (±0.10)
7	*B. carambolae* only	0.32 (−0.14 ± 0.5)	−0.06 (±0.16)	0.03 (±0.05)
8	Both species	0.33 (−0.16 ± 0.51)	0.03 (±0.05)	−0. 2 (±0.11)

Formulae for computing indices are given in text. For each experiment, mean values and 95% CL were calculated over all replicates ($n = 8$).

5: $\chi^2 = 36.69$, $P < 0.05$; experiment 6: $\chi^2 = 24.25$, $P < 0.05$; experiment 7: $\chi^2 = 17.79$, $P < 0.05$; experiment 8: $\chi^2 = 43.44$, $P < 0.05$.

The ISI values indicated mating isolation between Asian *B. dorsalis* and *B. carambolae* as the 95% confidence interval didn't include zero in each experiment (Table 4). The indices of male (MRPI) and female (FRPI) mating propensity revealed similar levels of mating activity between the species for all experiments (Tables 3 and 4).

In cross-mating studies, between Asian *B. dorsalis* and African *B. dorsalis* treating only Asian *B. dorsalis* males with ME or having males of both populations treated with ME increased the total number of matings but statistically nonsignificant in comparison to untreated control males. However, in the case where only African *B. dorsalis* males were treated with ME, there were fewer matings (statistically nonsignificant) as compared withthe control or when both species had been treated with ME. Although in cross-mating studies between Asian *B. dorsalis* and *B. carambolae* treating either of males with ME or having males of both populations treated with ME increased the total number of matings but statistically nonsignificant in comparison to untreated control males.

Discussion

We examined the effect of ME treatment on the incidence of pairing Asian with African *B. dorsalis* and Asian *B. dorsalis* with *B. carambolae*, by treating males of either one species only or males of both species. The data indicate that ME treatment didn't influence the sexual ISI in favor of one or the other taxa. Tan et al. (2011) reported that similar to Asian *B. dorsalis* males, African *B. dorsalis* males also convert the ingested ME to similar metabolites and in the same ratio. Thus, no change in sexual isolation index in favor of one or the other taxa due to ME feeding is understandable. The observed mating compatibility between Asian *B. dorsalis* and African *B. dorsalis* in this study is supportive of another study by Bo et al. (2014) where *B. dorsalis* populations from Pakistan and China were evaluated against *B. dorsalis* (*B. invadens*) from Kenya and both populations had mating compatibility.

A certain proportion of Asian *B. dorsalis* and *B. carambolae* individuals mated with each other but there were low levels of compatibility between these species. ME treatment is reported to enhance mating success of *B.dorsalis* and *B. carambolae* males (Shelly and Dewire 1994, Tan and Nishida 1996, Wee et al. 2007). Nonsignificant increase in number of matings in this study should neither be considered contradictory to previous studies nor misleading that ME feeding didn't enhance mating success because the experimental design was not fulfilling the basic requirement of the standard mating competitiveness test where two or more than two (treated vs. nontreated) males are competing for single female (FAO/IAEA/USDA 2003). These experiments were designed to test

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whether ME treatment influence mating compatibility between both species and 2:1 female:male ratio was used to ensure that females of both species were available to males throughout the entire experiment. These results showed that increased number of matings due to ME feeding was more in favor of homotypic couples but feeding didn't reduce mating isolation. The low frequency of interspecific matings between these two species is supportive of previous studies reporting interbreeding of *B. dorsalis* (*B. dorsalis* s.s.) and *B. carambolae* (McInnis et al. 1999) and *B. dorsalis* (*B. papayae*) and *B. carambolae* (Wee and Tan 2000) during field cage experiments. Furthermore, hybridization between *B. dorsalis* (*B. papayae*) and *B. carambolae* in Malaysia has also been reported (Wee and Tan 2000). Fewer *B. dorsalis* (*B. papayae*) versus *B. carambolae* hybrid males were captured in ME-baited traps compared with parental males indicating low interbreeding between *B. dorsalis* (*B. papayae*) and *B. carambolae* (Wee and Tan 2000). This study results showed cross-mating frequency between a Thai population of *B. dorsalis* and *B. carambolae* ranging between 30 and 36% of the total observed matings, which is in accordance to the results reported by Wee and Tan (2000). ME treatment didn't shift the assortative mating trend to random mating between both species. Therefore, the hypothesis that ME feeding may result in increased attractiveness of the males and reduced mating isolation by reducing discrimination of the females for mate choice in hetero-specific matings was not supported by these results. Furthermore, despite of different pathway of ME metabolism in *B. dorsalis* and *B. carambolae* where *B. dorsalis* males are known to convert the ingested ME into DMP and CF (Nishida et al. 1988a,b; Tan and Nishida 1996) and *B. carambolae* males convert ME to only CF (Wee et al. 2007) and females of both species are attracted to CF, but, there was no evidence of reduced discrimination of females for hetero-specific males.

In conclusion, our findings are supportive of previous studies (Schutze et al. 2015a,b) that integrated mating compatibility, post-zygotic compatibility, chemotaxonomy, cytogenetics, and genetic analysis to resolve the species delimitation of *B. dorsalis s.l.* and *B. carambolae*, and which strongly indicate that *B. dorsalis* (*B. dorsalis* s.s.) and *B. dorsalis* (*B. invadens*) are the same biological species, while *B. carambolae* is a different entity. ME feeding by males increased the overall number of matings but did not reduce the sexual incompatibility between *B. dorsalis* and *B. carambolae*.

Additionally, these findings have implications for area-wide control of fruit flies. One of the most commonly used technique for ME responding species is the male annihilation technique; MAT (Steiner 1952) which can be applied to suppress fruit flies more successfully in those areas having single species. However, the geographical regions having overlapping populations of *B. dorsalis* and *B. carambolae*, fruit fly control strategies if relying on MAT should be devised for each of the species seperately. For example, laboratory bioassay showed that *B. carambolae* had low sensitivity to ME than *B. dorsalis* and *B. papaya* (Wee et al. 2002) and field data from Surinam also revealed that four to five times as many ME fiber blocks per hectare were needed to suppress populations of *B. carambolae* compared with the number typically used to reduce populations of *B. dorsalis* (Van Sauers Müller 2008). Similarly for fruit flies control strategies incorporating the sterile insect technique; SIT (Knipling 1955), a great body of SIT knowledge exists for *B. dorsalis* but much less so for the other members of the *B. dorsalis* complex. These studies validating the previous studies for species status in the *B. dorsalis* complex will allow *B. dorsalis* SIT to be applied for other members of the complex.

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