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ACQUISITION OF FEMALE-ATTRACTING FRAGRANCE BY MALES OF ORIENTAL FRUIT FLY FROM A HAWAIIAN LEI FLOWER, *Fagraea berteriana*

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Abstract—Males of the Oriental fruit fly, *Bactrocera dorsalis*, are strongly attracted to and compulsively feed on a fragrant lei flower, *Fagraea berteriana*. A series of phenylpropanoid components, *trans*-3,4-dimethoxycinnamyl alcohol, its acetate, and *trans*-3,4-dimethoxycinnamaldehyde were characterized as male attractants. The alcohol stimulated the same level of feeding activity as methyl eugenol. Males that fed on flowers selectively converted the attractant components into *trans*-coniferyl alcohol and stored it in rectal glands. Males scented with the phenylpropanoids were more successful in mating than unfed males, indicating the advantage of acquiring the fragrance in mating success.

Key Words—Oriental fruit fly, *Bactrocera dorsalis*, Tephritidae, Diptera, *Fagraea berteriana*, pheromone, attractant, 3,4-dimethoxycinnamyl alcohol, coniferyl alcohol, phenylpropanoid.

INTRODUCTION

A flower's fragrance often plays an important role in mutualistic relationships: the scent signals the location of nectar rewards to insect pollinators. However, in some cases, as in euglossine bee–orchid associations, males may seek the flower fragrance itself to obtain volatile components, although the biological function of the acquired fragrance has not been clearly elucidated in these

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instances (Dressler, 1982; Whitten et al., 1989). We have shown here the utilization by a male tephritid fly of a specific flower fragrance for synthesis of a sex pheromone to entice females during courtship.

Males of the Oriental fruit fly, *Bactrocera dorsalis* (Tephritidae), are strongly attracted to the blossoms of particular plants, such as the golden shower tree, *Cassia fistula* (Fabaceae) (Kawano et al., 1968). Methyl eugenol (**1**) has been identified as the major attractant in these cases (Fletcher et al., 1975; Shah and Patel, 1976; Lewis et al., 1988), and this chemical has been successfully used in eradication efforts via the technique of male annihilation against this very destructive fruit pest (Chambers, 1977). Recently, in Hawaii we observed dense aggregations of male *B. dorsalis* feeding on (licking) the fragrant flower, *Fagraea berteriana* (Loganiaceae), which is commonly used in lei (a necklace with flowers) making and is known locally as pua-kenikeni. The males fed compulsively on the petals (Figure 1A), even though flower extracts of *F. berteriana* were found to lack even a trace amount of **1** (Figure 2). In this study, we identify the chemical attractants in the flowers and demonstrate the selective accumulation of a derivative of these chemicals in the male rectal gland. In a series of competitive mating trials conducted in the laboratory, we then show that flower-fed males had a mating advantage over control, unfed males, further suggesting a pheromonal function for the acquired flower fragrance.

METHODS AND MATERIALS

Extraction and Purification of F. berteriana *Fragrance.* About 120 flowers of *F. berteriana* were extracted with ethanol (200 ml × 3). This extract (12.73 g) was partitioned between ether (100 ml) and water (50 ml), the water phase extracted again with ether (50 ml), and the combined ether layers dried over anhyd. Na_2SO_4. Removal of the solvent by evaporation under reduced pressure gave a yellow oil (0.94 g), which was chromatographed on a silica gel column (30 g, Wako gel C-200) into eight fractions eluting in sequence with a mixture of increasing concentrations of methyl acetate (%) in hexane (v/v, ml) as follows: Fractions 1 (0%, 100), 2 (5%, 100), 3 (10%, 100), 4 (15%, 100), 5 (20%, 100), 6 (30%, 100), 7 (40%, 50), 8 (50%, 50), 9 (60%, 50), 10 (100%, 100). Active compounds (**2, 3,** and **4**) were purified by a high-performance liquid chromatography (HPLC) using YMC-Pack A-024 S5 column (8 mm ID × 300 mm, eluted with mixtures of methyl acetate and hexane, 3 ml/min).

TLC Plate Bioassay. Small portions of the flower extract and fractions were subjected to thin-layer chromatography (TLC) on a precoated plate (HPTLC, silica gel 60 F_{254}, nano TLC, Merck) and developed with a mixture of benzene and ethyl acetate (2:1). The TLC plate, slightly moistened by misting with distilled water, was introduced into a small cage containing a number of male

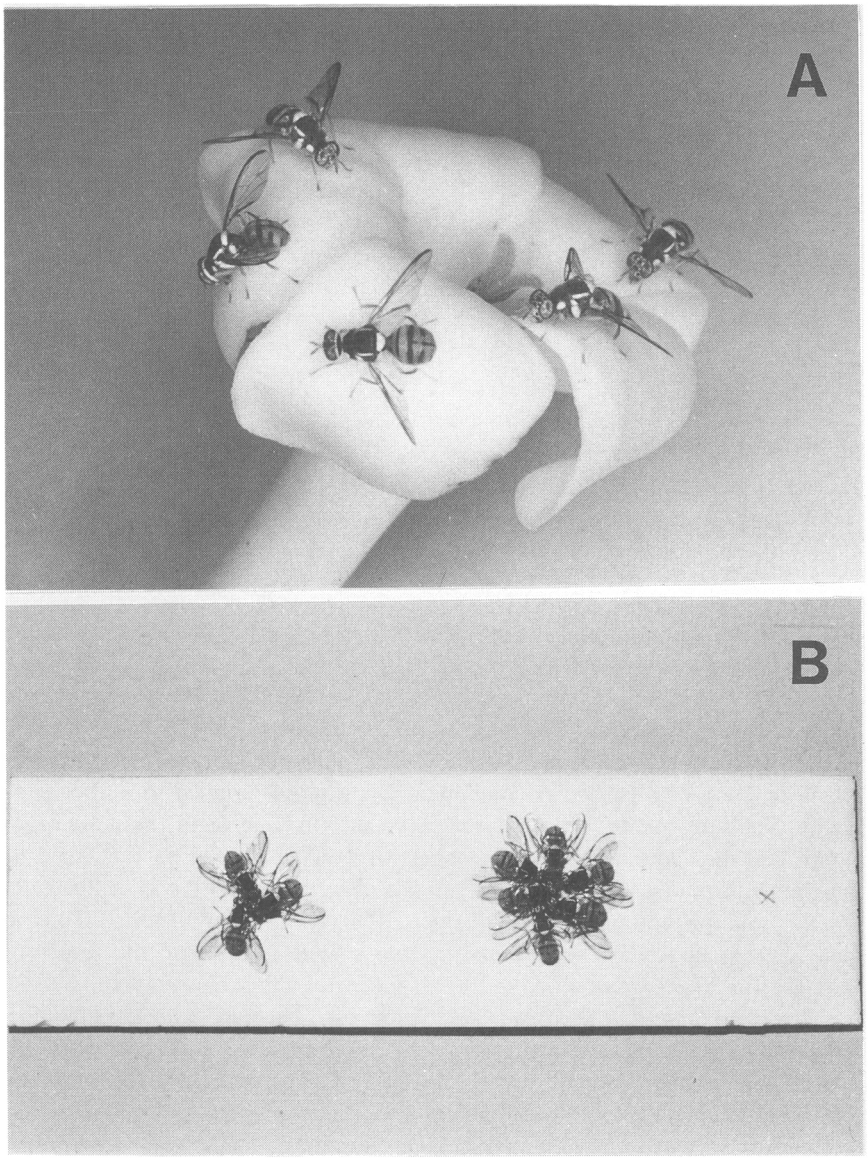

FIG. 1. A: Males of the Oriental fruit fly, *Bactrocera dorsalis*, feeding on the flower of *Fagraea berteriana*. B: Males of *B. dorsalis* feeding on attractive spots (R_f = 0.69 and 0.39) in the thin-layer plate bioassay after chromatography of a *Fagraea* flower extract (HPTLC silica gel 60 F_{254}, nano TLC Merck, developed with benzene–ethyl acetate 2:1, x-mark: original spot, left: solvent front).

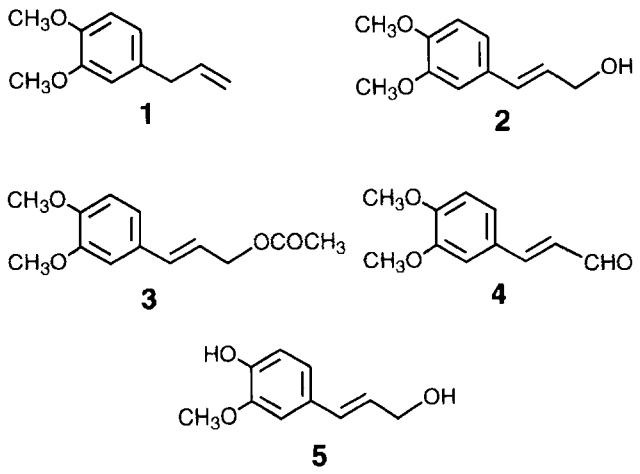

FIG. 2. Phenylpropanoids associated with the oriental fruit fly: methyl eugenol (**1**), *trans*-3,4-dimethoxycinnamyl alcohol (**2**), *trans*-3,4-dimethoxycinnamyl acetate (**3**), *trans*-3,4-dimethoxycinnamaldehyde (**4**), and *trans*-coniferyl alcohol (**5**).

flies (10–30 days old), and licking behavior was observed for about 10 min to determine the active zones (see Figure 1B).

Outdoor Cage Bioassay. B. dorsalis males (150–200 flies, 10–30 days old) were released in outdoor screen cages (2.2 m height × 3.0 m diameter) containing sticky traps suspended from the roof. Traps contained either a solvent blank or 1 mg of a particular compound (**2, 3,** and **4**) applied to a filter paper pellet. Each treatment was replicated in a cage (making eight traps per cage). Traps and flies were placed in the cages for 6 hr during the daytime, and the number of captured flies was recorded. The attractant activity was evaluated by Dunn's multiple comparison test (Daniel, 1990) (N = 2 for all tests). To compare the attractancy between **1** and **2,** males were exposed to the respective samples for 20 min period (N = 2 tests) following the same protocol.

Feeding Stimulant Bioassay. A strip of test filter paper, the tip (10 mm^2) of which was impregnated with each sample (compounds **1–4**) and moistened with distilled water immediately before the test, was brought to within 3 mm of the mouthparts of individual males (N = 50/compound) for 3 sec. The presence or absence of continuous licking on the test sample was scored.

Rectal Volatile Analysis. Males (20 days old) were exposed to *F. berteriana* flowers (3 flowers/male) and allowed to feed freely for one day. Rectal glands were dissected on the second day after feeding, and the gland and the remaining body tissues were extracted separately with ethanol for gas chromatographic quantification (cf. Nishida et al., 1988a, 1993). To test if synthetic **2** and **3** were incorporated into rectal glands, the males were exposed to each compound sup-

plied as a thin film (30 μg/male), allowed to feed for 1 hr, and then processed as above.

Flower Feeding and Mating Success Test. Males (20–25 days old) were allowed to feed on *F. berteriana* flowers for 6 hr and then held 2, 7, and 21 days before testing. Fed and unfed males (21–28 days old) were marked by placing enamel paint on the thorax. Although fed males held 21 days before testing were older than unfed males, Shelly and Dewire (1994) found age to have no effect on the mating success of mature males of *B. dorsalis*. In mating trials, three fed males, three unfed males, and three females (21–31 days old) were placed in glass cages (30-cm cubes) several hours before dusk (the period of sexual activity). Cages were checked 3–4 hr after dusk, and the identities of copulating males were scored (mating occurs at dusk, and pairs remain coupled until dawn). Males fed the synthetic mixture of **2** and **3** (1 : 1) were tested three or seven days after feeding. During exposure, 50 males were given access to the mixture (approximately 30 μg/male) for 6 hr. All flies were virgins and were used only once.

Behavioral Bioassay of B. dorsalis *Females Toward Chemically Treated Males and Dummies.* Males were individually fed on a mixture of **2** and **3** (30 μg each/male) at age 20 days and tested three days later. Males were anesthetized in a freezer and then slowly dragged (using a forceps) 3–4 mm in front of a virgin female (30–35 days old) for approximately 20 mm. A positive response was scored if the females chased and contacted the males and/or extruded their ovipositors toward the males within 10 sec ($N = 32$). Likewise, a male-sized piece of filter paper was impregnated with compound **5** (50 μg/dummy) and tested in the same manner as intact males ($N = 32$). The bioassay, both with the anesthetized fly and with the dummy, was carried out under dim light at 19:00–20:30 hr.

Instruments. Gas chromatography (GC)-mass spectra (MS) for the volatile components were obtained with a Hitachi M-80 mass spectrometer (electron impact, at 70 eV) connected to a GC column (24-m × 0.2-mm fused silica column coated with cross-linked methyl silicone HP-1, 0.33-μm film thickness, helium as carrier gas) programed from 80°C (approximately 2 min holding) to 240°C at a rate of 10°C/min. The ^{1}H NMR spectra were recorded with a Bruker AC300 spectrometer (300 MHz) with TMS as an internal standard (letters s, d, t representing singlet, doublet and triplet, J values in Hz).

RESULTS

Identification of Male Attractants in F. berteriana *Flower*

Males of *B. dorsalis* were exposed to a thin-layer plate which had been chromatographed with a crude extract of *F. berteriana* flowers. The flies were

attracted to and licked two restricted areas centered at the R_f values of 0.39 and 0.69 (Figure 1B). The pure active compounds responsible for stimulating feeding were isolated from fresh flowers of *F. berteriana*. Silica gel column chromatography separated two major active compounds (**2** and **3**) in fractions 8 and 5, which corresponded to the spots at the R_f values of 0.39 and 0.69, respectively. Fraction 8 was subjected to preparative HPLC, eluting with 60% methyl acetate in hexane. Active compound **2** was isolated at a retention time (R_t) of 15.0 min. Fraction 5 was separated by HPLC, eluting with 32% methyl acetate in hexane, and compound **3** was isolated at R_t = 13.0 min. A minor active component **4** was also recovered from R_t = 18.7 min from fraction 5. The quantities of compounds **2**, **3**, and **4** (mean content ± SD, N = 4) were 33.8 ± 15.1, 12.1 ± 5.6, 0.4 ± 0.1 μg/flower, respectively.

Compounds **2** and **3** were identified as *trans*-3,4-dimethoxycinnamyl alcohol and its acetate, respectively, from the following spectral data and synthesis: **2** was prepared from *trans*-3,4-dimethoxycinnamic acid methyl ester by reduction with $LiAlH_4$ (Nishida et al., 1988b); acetylation of **2** with acetic anhydride in pyridine yielded **3.**

Compound **2.** MS: *m/z*(%) 194 (98), 165 (22), 151 (100), 138 (67), 91 (41), 77 (33). ^{1}H NMR ($CDCl_3$): δ 3.88 (3H, s), 3.90 (3H, s), 4.31 (2H, double d, J = 1.4, 5.9), 6.23 (1H, double t, J = 5.9, 15.8), 6.56 (1H, broad d, J = 15.8), 6.83 (1H, d, J = 8.1), 6.93 (1H, double d, J = 1.8, 8.1), 6.95 (1H, broad s).

Compound **3.** MS: *m/z*(%) 236 (100), 193 (25), 177 (67), 165 (38), 146 (42), 91 (38), 77 (16), 43 (50). ^{1}H NMR ($CDCl_3$): δ2.09 (3H, s), 3.88 (3H, s), 3.90 (3H, s), 4.01 (2H, d, J = 6.6), 6.17 (1H, double t, J = 6.5, 16.1), 6.59 (1H, d, J = 16.1), 6.82 (1H, d, J = 7.9), 6.92 (1H, d, J = 7.9), 6.94 (1H, s).

The additional stimulant component (**4**) was identified as *trans*-3,4-dimethoxycinnamaldehyde from its diagnostic mass spectrum [*m/z*(%) 192 (100), 177 (22), 161 (71), 121 (24), 91 (31), 77 (49), 51 (43)]. Compound **4** was synthesized from **2** by oxidation with pyridinium chlorochromate in dichloromethane [^{1}H NMR ($CDCl_3$): δ 6.62(1H, double d, J = 7.7, 15.8), 6.91 (1H, d, J = 8.3), 7.08(1H, d, J = 2.0), 7.17(1H, double d, J = 2.0, 8.3), 7.41 (1H, d, J = 15.8), 9.67(1H, d, J = 7.7)].

Behavioral Effects of Phenylpropanoids **2**, **3**, *and* **4** *on Males*

Male response to *F. berteriana* volatiles consisted of two distinct behavioral steps: long-distance attraction followed by feeding. Attractant bioassays were conducted by releasing males into outdoor screen cages containing sticky traps with test chemicals. Compounds **2** and **3** were highly active, while the aldehyde **4** was not significantly different from the solvent blank (average number of male

flies captured ± SD/trap, $N = 2$ for all tests; compound **2:** 51.0 ± 9.7, **3:** 20.3 ± 8.6, **4:** 2.0 ± 2.4, blank: 1.5 ± 2.6; Dunn's multiple comparison test: **2** = **3** > **4** = blank; $\alpha = 0.15$). In tests comparing only **1** and **2**, **1** was found to be more attractive than **2** (**1:** 50.5 ± 16.8, **2:** 8.3 ± 6.7, blank: 0 ± 0 males/trap; $P < 0.05$, Mann-Whitney test).

Compounds **2**, **3**, and **4** evoked compulsive feeding responses from males when the chemicals were brought near the flies. Figure 3 shows the dose-response of phenylpropanoids (**1–4**), revealing that at low doses (both 1.0 and 3.0 ng) compound **2** stimulated a higher level of feeding than did **1** ($P < 0.001$; G test (2 × 2 contingency) with Yates correction). Compounds **3** and **4** also stimulated feeding activity but only at higher doses.

Accumulation of Compound 5 in Rectal Glands

B. dorsalis males used in the preceding experiment selectively converted both compounds **2** and **3** into *trans*-coniferyl alcohol (**5**), which was identified by GC–MS [*m/z*(%) 180 (69), 137 (100), 124 (56), 91 (34), 77 (20)] (cf. Nishida et al., 1988a).

When males were fed flowers of *F. berteriana*, they accumulated substantial quantities of **5** in the body tissues (2.7 ± 1.3 μg/male, $N = 8$), a large portion of which was concentrated in the rectal glands (1.4 ± 0.7 μg/gland, $N = 8$). Likewise, when males fed on a mixture of synthetic **2** and **3**, **5** accumulated in the rectal glands within a day after ingestion (rectal contents of **5** after ingestion of **2:** 17.4 ± 4.6; **3:** 15.1 ± 5.1 μg; unfed control: 0 μg/gland, $N = 5$).

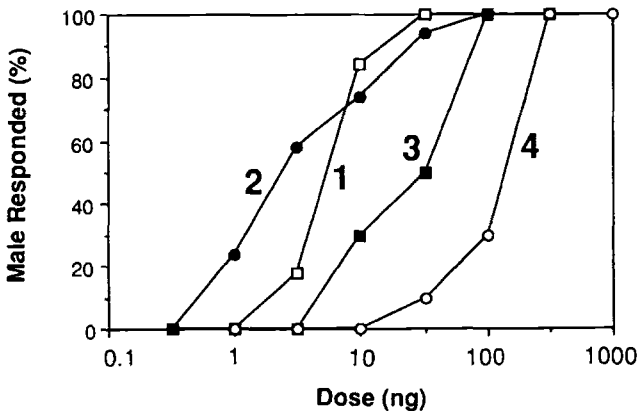

FIG. 3. Feeding stimulant activity of compounds **2–4** isolated from *Fagraea berteriana* flowers in the male feeding assay compared with methyl eugenol (**1**) ($N = 50$).

Effect of Flower and Phenylpropanoid Feeding on Mating Success

After feeding on pua-kenikeni flowers, males mated more successfully than unfed males. Males given access to flowers accounted for 67% (56/83), 80% (53/66), and 77% (78/101) of the total matings in tests conducted 2, 7, and 21 days after flower feeding ($P < 0.05$ all tests; binomial test) (Figure 4). Similarly, males fed a synthetic mixture of **2** and **3** had a mating advantage over unfed males both three days (67% = 51/76) and seven days (65% = 74/113) after feeding ($P < 0.05$ in both cases; binomial test). In these latter tests, many of the unmated females were observed chasing males that had been fed alcohol **2,** sometimes extruding their ovipositors directly toward the males. Such behavior was rarely observed towards the unfed males. Copulation often occurred immediately after ovipositor extrusion, suggesting this action to be a possible mate acceptance response of the female.

Behavioral Effect of Phenylpropanoids 2 and 3 on Females

To associate more rigorously female attraction with male feeding status, we monitored female response to anesthetized (and hence behaviorally equivalent) males fed or unfed the synthetic mixture of compounds **2** and **3.** Males treated with the chemicals attracted females more strongly than untreated males (15/32 vs. 5/32, respectively; $P < 0.05$; G test with Yates' correction). Similar results were obtained using the paper dummies treated with **5** (17/32 vs. 2/32, respectively; $P < 0.001$; G test with Yates' correction). In addition, among

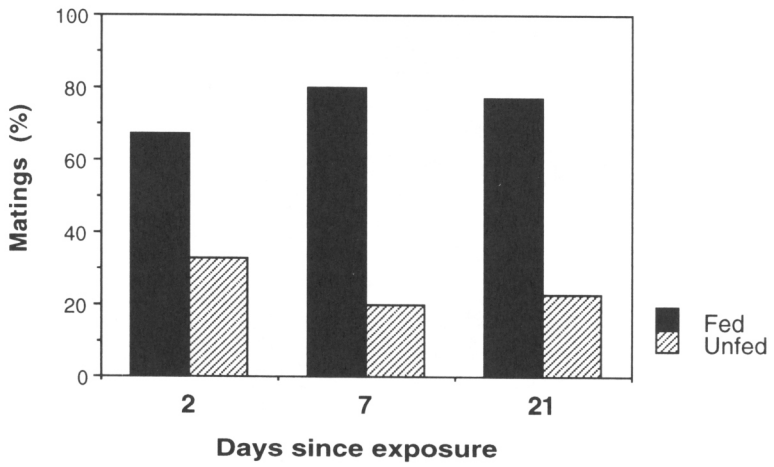

FIG. 4. Percent mating obtained by fed and unfed (control) males in trials conducted 2, 7, and 21 days after a 6-hr exposure of males to *Fagraea berteriana* flowers.

attracted females ovipositor extrusion was occasionally observed toward the fed males (2/15) or treated dummies (5/17) but was never observed toward unfed males or untreated dummies. Metabolite **5** thus appears to act as a short-range attractant and arrestant pheromone to elicit female acceptance of male courtship.

DISCUSSION

We have shown here that males of *B. dorsalis* are attracted to and compulsively feed on flowers of *F. berteriana* stimulated by three phenylpropanoid components **2, 3,** and **4.** Males selectively converted compounds **2** and **3** to coniferyl alcohol (**5**) for storage in the rectal glands and mated more frequently than unfed males, indicating the advantage of acquiring the fragrance in obtaining matings. Wild males of *B. dorsalis* and related species in the Oriental fruit fly complex incorporate methyl eugenol (**1**) metabolites in their body after foraging on methyl eugenol-containing plants (Nishida et al., 1988a,b; Nishida and Fukami, 1990; Tan and Nishida, 1996). Males of *B. dorsalis* fed on methyl eugenol were shown to have a mating advantage over the unfed males (Shelly and Dewire, 1994). Enhancement of mating success in *B. dorsalis* has also been shown by intact feeding on methyl eugenol-bearing flowers of *C. fistula* (Shelly, unpublished). Thus, acquisition of phenylpropanoid compounds by *B. dorsalis* and related species appears to have evolved within the context of sexual selection, particularly female preference for males scented with chemicals derived from flowers. However, the basis of this preference remains unknown. Females do not appear to derive any direct benefits (in terms of increased fecundity or offspring hatch rate) by selecting males fed compound **1** (Shelly, unpublished). Female preference could reflect a runaway process. If the ability to collect phenylpropanoid compounds has a heritable component, by mating with scented males, females may be increasing the likelihood that their sons will successfully locate and collect these compounds and thereby enjoy a mating advantage. Aditionally, phenylpropanoid **5** and its analogs have been shown to be repellent to avian predators (Nishida and Fukami, 1990; Jakubas et al., 1992). Since a large proportion of the wild population of *B. dorsalis* males stores these compounds in significant quantities, the acquired phenylpropanoids seem to play a role as defense substances (Tan, 1993; Tan and Nishida, 1996).

Although the rectal phenylpropanoid appears to play an important role as a sex pheromone under natural conditions, indoor males mate at a high frequency even without access to phenylpropanoids. Regardless of whether they had prior access to phenylpropanoid sources, males of *B. dorsalis* emit a smoke of a series of compounds that have been suspected to act as a sex pheromone (Ohinata et al., 1982). The rectal chemicals include fatty acids, trisodium phosphate, spiroketals, alkylamides, and other minor volatiles; the behavioral functions of

these in the courtship sequence have not, however, been clarified (Ohinata et al., 1982; Perkins et al., 1990). Further study is needed to understand the nature of the mating system of *B. dorsalis* in association with those endogenous rectal chemicals and pharmacophagously acquired phenylpropanoids (Nishida and Fukami, 1990).

The feeding location on *F. berteriana* flower (well away from the anthers) and the absence of pollen on examined individuals suggests that this *B. dorsalis*–flower association is not mutualistic. By contrast, floral spikes of *Spathiphyllum cannaefolium* (Araceae) attract males of *B. carambolae* and *B. papayae* with compounds **1, 2,** and **3** during the time when the spadix produces the characteristic fragrance and a large amount of pollen (Nishida and Tan, 1993; Chuah et al., 1996). Wild *B. carambolae* males bearing pollen clusters on the body surface were found to store substantial, although variable, quantities of metabolite **5** in the rectal glands, suggesting a possible mutualistic interaction through pollination (Nishida and Tan, 1993; Nishida and Tan, unpublished).

Regardless of the adaptive significance of lure attraction, the knowledge that feeding on lures enhances the mating performance of male tephritids may have tremendous practical value. The sterile insect technique (SIT) is a widely used, and environmentally safe control method involving the release of irradiated (sterile), mass-reared males to achieve sterile male–wild female matings and the production of infertile eggs. As the success of SIT hinges on the mating competitiveness of sterile males, prerelease exposure of sterile males to the lure may be a logistically simple way to enhance their mating frequency with wild females and consequently increase the effectiveness of the control program (Shelly, 1995; Shelly et al., 1996).

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