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Bioactive fractions containing methyl eugenol-derived sex pheromonal components in haemolymph of the male fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae)

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Abstract

Sex pheromonal components of the tephritid fruit fly *Bactrocera dorsalis* (Hendel), 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol, are biosynthesized from a highly potent male attractant, methyl eugenol, then sequestered and stored in the rectal gland prior to their release during courtship at dusk. These sex pheromonal components have been detected in the haemolymph and crop organ. Hence, attempts were made to separate and identify the haemolymph fractions which contained the sex pheromonal components. Identification of these bioactive fractions in methyl eugenol-fed male flies using gel filtration column chromatography and biodetection using live male flies showed two fractions as highly attractive to conspecific males. These fractions show a significant increase in protein absorbance in the elution profile of haemolymph from methyl eugenol-fed males compared with that from methyl eugenol-deprived males. The molecular mass of these bioactive fractions as determined by using gel filtration was in the peptide range of 3.3 to 5.5 kDa. Subsequent gas chromatography–mass spectrometry analyses further confirmed the presence of the pheromonal components in the bioactive fractions. The presence of these methyl eugenol-derived sex pheromonal components in specific haemolymph fractions suggests the involvement of a sex pheromone binding complex.

Keywords: Methyl eugenol, pheromone, haemolymph, *Bactrocera dorsalis* phenylpropanoids

Introduction

The unique characteristic of methyl eugenol-sensitive tephritid fruit fly species, such as *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is the strong attraction to and compulsive feeding on methyl eugenol (1,2-dimethoxy-4-(2-propenyl)benzene), a naturally-occurring phenylpropanoid found both as a plant secondary metabolite and a component of essential oils in over 200 species of plants from 32 families (Tan, 2000). While this attraction was first observed in 1915 by Howlett, recent investigations have greatly improved understanding of this phenomenon

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(Tan, 1993, 2000; Tan & Nishida, 1998, 2000; Nishida *et al*., 2004). Work on methyl eugenol-sensitive fruit fly species such as *B. dorsalis* has shown that this phenylpropanoid plays a central role in their interrelationships with plants and predators (Tan, 1993, 2000). Consumption of methyl eugenol has been known to confer protection against vertebrate predators (Nishida & Fukami, 1990; Tan & Nishida, 1998). In addition, methyl eugenol and its derivatives have also been shown to be involved in the pollination of certain *Bulbophyllum* orchids by *Bactrocera* fruit flies (Tan & Nishida, 2000; Tan *et al*., 2002; Nishida *et al*., 2004).

Consumption of methyl eugenol by males of *B. dorsalis* and *B. papayae* (Drew & Hancock), not a distinct species from the former (Naeole & Haymer, 2003; Tan, 2003), has enhanced mating competitiveness (Shelly & Dewire, 1994; Tan & Nishida, 1996, 1998; Hee & Tan, 1998; Shelly & Nishida, 2004). Chemical analyses have shown that male *B. dorsalis* convert methyl eugenol to two other phenylpropanoids, 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol (3-(4-hydroxy-3-methoxyphenyl)-2(*E*)-propen-1-ol) (Nishida *et al*., 1988; Tan & Nishida, 1996; Shelly & Nishida, 2004). These methyl eugenol metabolites are then sequestered by the male rectal gland for storage and subsequently released during courtship at dusk, to function as male sex and aggregation pheromones in *B. dorsalis* (Hee & Tan, 1998; Tan & Nishida, 1998; Khoo *et al*., 2000; Khoo & Tan, 2005).

Whilst the ecological relationship between the male attractant, methyl eugenol and the fruit fly, *B. dorsalis* has been shown to involve the production of bioactive derivatives from methyl eugenol as sex pheromonal components, little is known about the transportation of these bioactive compounds to the rectal gland. Recently, the reported occurrence of these compounds in the haemolymph of methyl eugenol-fed male *B. dorsalis* (Hee & Tan, 2004) suggests that the haemolymph transports these phenylpropanoids to the rectal gland. This paper reports on the separation and identification of bioactive fractions containing methyl eugenol-derived male sex pheromonal components in the haemolymph of the fruit fly, *B. dorsalis*.

Materials and methods

Fruit flies

Laboratory-reared *B. dorsalis* (12 generations per year) were originally collected from infested host fruits, *Averrhoa carambola* Linnaeus (Averrhoaceae), in Penang, Malaysia, 1995, and cultured using an artificial larval diet according to Hee and Tan (1998). Adult flies were kept on a diet containing a mixture of yeast, sugar, protein and water.

Males and females were segregated one to three days after emergence (DAE) and maintained under ambient conditions in an insectary with a constant light regime (12L : 12D photoperiod) and 83–90% rh at 25–29°C. Sexually mature virgin males (14–20 DAE), that responded maximally to methyl eugenol (Tan *et al*., 1987) were used for experimentation.

Consumption of methyl eugenol

Methyl eugenol (> 98%; Merck-Schuchardt, Germany) to be used for feeding male flies was prepared by dilution to 210.4 μg μl^{-1} as an aqueous emulsion containing 1% Tween® 80 (Merck-Schuchardt, Germany). A male fly was placed with its ventral side up and wings held firmly using plasticine. It was offered 0.5 μl (containing 105 μg) of methyl eugenol directly from a capillary micropipette mounted to a micromanipulator (Narishige Co. Ltd, Japan) to avoid direct contact with other bodily parts.

Preparation of haemolymph extracts

For gel filtration chromatography, haemolymph was collected from over 500 methyl eugenol-fed males 15 min after initial feeding, when quantities of the sex pheromonal components were highest (A.K.W. Hee & K.H. Tan, unpublished data). A disposable glass capillary micropipette with a drawn-out tip attached to a micromanipulator was used to collect haemolymph of the male fly, held firmly by plasticine moulds, from a small slit made along the centre of the meso- and metathoracic segments. On average, *c*. 0.5 μl of haemolymph was collected from each male and pooled using the micropipette in a 0.6 ml microcentrifuge tube on ice, containing trace amounts of phenylthiocarbamide (1-phenyl-2-thiourea) (Sigma Chemical Company, St Louis, Missouri, USA) to inhibit tyrosinase activity. The combined haemolymph (from a batch of over 500 male flies) was then centrifuged at 12,000 *g* for 15 min at 4°C. Thereafter, the supernatant was transferred to a new tube and stored at −20°C. As an experimental control, the procedure was repeated for methyl eugenol-deprived males using a new micropipette to avoid cross contamination. Total protein content in each batch of the pooled haemolymph extract was determined at 595 nm wavelength (UVmax microplate reader, Molecular Devices Corp., Palo Alto, California, USA) using the Bio-Rad Bradford Protein Assay kit (Bio-Rad, Hercules, California) according to the manufacturer's instructions. A standard curve was prepared using bovine serum albumin as reference. Haemolymph samples were collected from three batches of methyl eugenol-fed male flies and from three batches of methyl eugenol-deprived flies.

Gel filtration

Each batch of haemolymph was fractionated using a fast protein liquid chromatography (FPLC) (Pharmacia Biotech, Sweden) system. Samples were loaded onto a 25 μl-sample loop connected to a Superdex PC3.2/30 (Amersham Pharmacia, Sweden) microbore column (300 mm × 3.2 mm) prepacked with Superdex^{TM} 200 using a column holder. To ensure total sample loop volume loading and reproducibility of the haemolymph elution profiles, a 75-μl sample was applied to the column for each chromatographic run. The column was eluted with 50 mM phosphate buffer, pH 7.0 with 150 mM NaCl at a flow rate of 40 μl min^{-1}, and elution volumes (V_e) were monitored at 280 nm. Individual fractions (30 μl) were collected manually into a 0.6 ml microcentrifuge tube. One hundred fractions were obtained.

The column was calibrated with authentic protein standards of known molecular weights (Pharmacia Biotech, Sweden) (thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa; ribonuclease A, 13.7 kDa; and vitamin B_{12}, 1.36 kDa). The void volume, V_o was determined using blue dextran (mol. wt. 2×10^6). The molecular weight of each bioactive fraction was determined from a graph of

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molecular weight plotted against K_{av} ($y = -0.1326\text{Ln}(x) + 1.8593$, $R^2 = 0.9781$).

Biodetection of bioactive haemolymph fractions

As *B. dorsalis* males are attracted strongly to methyl eugenol-derived sex pheromonal components found in the haemolymph (Hee & Tan, 2004), conspecific males were used to detect for bioactivity in the fractions obtained by the above chromatographic separation of haemolymph. An aliquot of each fraction (10 µl) was spotted onto a separate filter paper (2 cm^2; Whatman No. 1, UK) and placed on a plastic Petri dish (3 cm diameter). The filter papers impregnated with 10 successive fractions were then introduced into a cage (43 cm^3) containing 200 male flies. Attraction and behaviour of flies were observed for a 10-min observation period. Following that, the filter papers were removed and filter papers impregnated with the next 10 fractions were presented. This was repeated successively until all the 100 eluted fractions from each batch of haemolymph were tested.

Gas chromatography–mass spectrometry (GC–MS)

Bioactive fractions, as detected using live male flies in the preceding section, were further subjected to GC–MS and GC analyses. Identification of sex pheromonal components in the haemolymph bioactive fractions was based on methods used by Nishida *et al*. (1988). GC–MS analysis was performed on a HP 5989B mass spectrometer (Hewlett Packard, Palo Alto, California) (electron impact, at 70 eV) connected to a non-polar GC column (30 m × 0.25 mm fused silica column coated with cross-linked 5% phenyl-methylpolysiloxane HP-5MS, 0.33 µm film thickness) (J&W Scientific, Folsom, California) programmed from 80°C (1 min hold) to 240°C at a rate of 10°C min^{-1}. Chemical identification was performed by comparison with the retention time and mass spectra fragmentation pattern of authentic standards.

Gas chromatography quantifications of the sex pheromonal components were done on a Shimadzu GC-14A gas chromatograph (Shimadzu, Japan) using a HP Ultra-1 capillary column (25 m × 0.2 mm fused silica column coated with cross-linked methyl siloxane, 0.33 µm film thickness) (J&W Scientific, Folsom, California) and under the same programme conditions as above by comparing the flame ionization intensities (FID) with those of the authentic standard sample of known concentrations by using a C-R6A integrator (Shimadzu, Japan). For each batch of haemolymph, the bioactive fractions were combined and aliquots (10 µl) were added to ethanol (10 µl) in a small 'V' tube and sealed using parafilm. The mixture was centrifuged at 5000 × *g* for 10 min at 4°C to obtain a clear supernatant. Aliquots (1 µl) of the supernatant were used for gas chromatography–mass spectrometry and gas chromatography injections.

Results

Gel filtration chromatography and biodetection

The haemolymph elution profiles showed a significant increase in protein absorbance for fractions 65 to 75 for methyl eugenol-fed male *B. dorsalis* when compared with methyl eugenol-deprived male haemolymph (fig. 1). This result was also substantiated by significantly higher protein content in methyl eugenol-fed males (0.401 ± 0.011 mg ml^{-1}) when compared with methyl eugenol-deprived males (0.366 ± 0.004 mg ml^{-1}) (mean ± SE, $n = 3$, $P < 0.05$, Student's *t* test). In biodetection of the bioactive haemolymph fractions using live male flies, fractions 69 and 70 from methyl eugenol-fed males were identified as bioactive fractions. Attraction of *B. dorsalis* males to these bioactive fractions was observed as early as 5 min as male flies (between 16–20 males) would exhibit rapid instances of short zig-zag flying before landing and feeding on those particular spots (fig. 2). No males were observed to be feeding on other fractions. The bioactive fractions were determined, from the elution profile, to be located at the peak absorbance of the methyl eugenol-fed male haemolymph proteins (fig. 1). The molecular mass of these fractions was estimated to be in the range of 3.3 to 5.5 kDa.

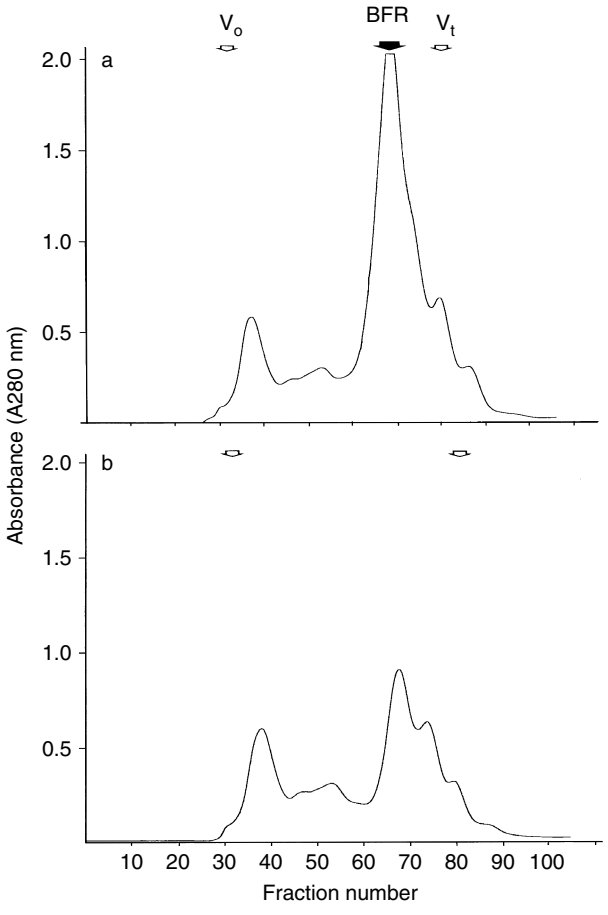

Fig. 1. Elution patterns after fast protein liquid chromatography (FPLC) gel filtration. (a) Elution pattern of methyl eugenol-fed *Bactrocera dorsalis* male haemolymph. The bioactive fractions (BFR) were detected using live male flies (biodetection). (b) Elution pattern of methyl eugenol-deprived *B. dorsalis* male haemolymph (V_o = void volume, V_t = total column volume.).

Gas chromatographic analyses of the bioactive fractions

Capillary gas chromatograms showed the presence of 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol in the

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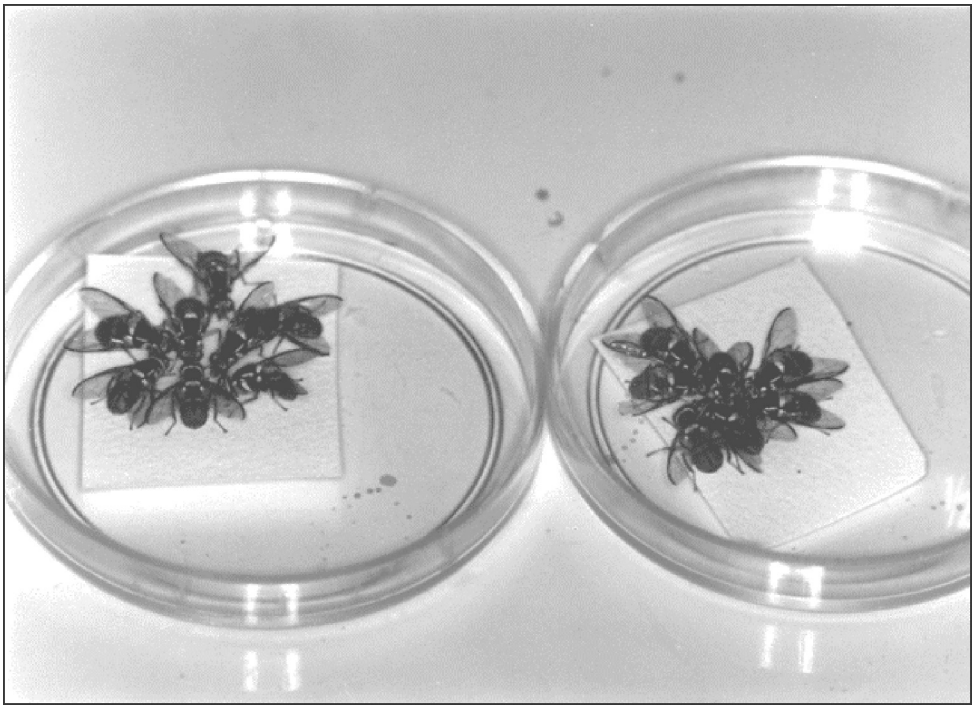

Fig. 2. Sexually mature males of *Bactrocera dorsalis* feeding on bioactive fractions 69 and 70 from fast protein liquid chromatography gel filtration of haemolymph from methyl eugenol-fed males.

combined bioactive fractions (fig. 3). The compounds were identified and confirmed from their mass-spectra fragmentation patterns. Quantities of 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol detected in the bioactive fractions were calculated to be 149.04 ± 15.84 ng per male fly and 52.08 ± 29.16 ng per male fly respectively (mean $\pm$ SE; $n = 3$).

Discussion

The two phenylpropanoids, 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol, are known to be highly attractive to male flies and elicit a searching behaviour similar to that of male response to methyl eugenol (Nishida *et al*., 1988; Hee & Tan, 1998; Khoo *et al*., 2000; Tan, 2000). These methyl eugenol metabolites have also been shown to occur in the haemolymph and crop of methyl eugenol-fed male flies (Hee & Tan, 2004). In the present study, gel filtration column chromatography was used to fractionate the haemolymph and the biological activity of all eluted fractions subjected to biodetection using live male flies. The use of *B. dorsalis* males to successfully detect the bioactive fractions demonstrates the feasibility of such a technique, which does not rely on the use of expensive radiolabelled methyl eugenol or methyl eugenol-derived sex pheromonal components as a probe. Radiolabelled hydrocarbon sex pheromone components have previously been used in detection of haemolymph fractions containing such bioactive compounds (Schal *et al*., 1998). However, in this paper, the method of biodetection and subsequent confirmation using GC–MS analysis of the compounds involved has been shown to be sensitive, reliable and economical. This technique enables preliminary screening for the presence of pheromonal components that are also male attractants, prior to chemical analyses that are time-consuming to perform and sometimes less sensitive. Further, the response of *B. dorsalis* males to methyl eugenol has been analysed (Wee *et al*., 2002) and initial results of male response to 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol showed probit values within the range of methyl eugenol (A.K.W. Hee & K.H. Tan, unpublished data).

Although the sex pheromonal components are attractive to female flies (Nishida *et al*., 1988; Tan & Nishida, 1996, 1998; Khoo *et al*., 2000) it was not possible to use female attraction to determine the exact location of components on a thin layer chromatography plate. When a female fly is attracted to the pheromonal components, the fly is constantly moving for < 30 s, at times rapidly, near the attractant spot before leaving; and at very low quantity (in nanograms), no female has been observed to extrude her ovipositor (Tan & Nishida, 1996, 1998). It would thus be very difficult or impossible to pin-point precisely the exact spot to which female flies are attracted. In contrast, the use of male flies facilitates the detection of the bioactive haemolymph sample as the males feed on the spot containing any one of the pheromonal components and leave a distinct salivary mark. Further, the use of male flies in biodetection allows for a longer testing period than that of females. Male flies are most sensitive to the pheromonal components between 0800 and 1100 h (Tan, 1985; Nishida *et al*., 1988; Tan & Nishida, 1996, 1998; Khoo *et al*., 2000; Wee *et al*., 2002), whilst the corresponding period for females is only 30 min between 1845 and 1915 h (Hee & Tan, 1998; Khoo *et al*., 2000).

Recent investigations into the nature of pheromone transport in male *B. dorsalis* following methyl eugenol consumption revealed the presence of methyl eugenol-derived sex pheromonal components in the crop and haemolymph (Hee & Tan, 2004). The latter was suggested

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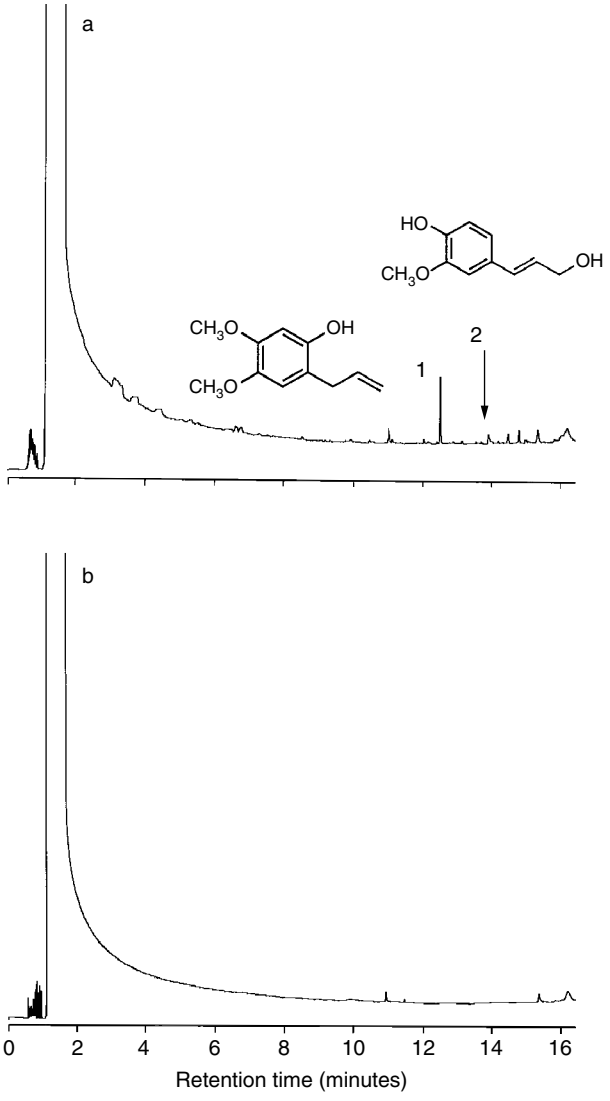

Fig. 3. Gas chromatographic analyses of the fractions obtained from column gel filtration of haemolymph from *Bactrocera dorsalis* males: (a) chromatogram showing the sex pheromonal components, 2-allyl-4,5-dimethoxyphenol (1) and (*E*)-coniferyl alcohol (2) present in the bioactive fractions of haemolymph from methyl eugenol-fed *Bactrocera dorsalis* males; (b) absence of the pheromonal components in the corresponding fractions from haemolymph from methyl eugenol-deprived conspecific males (control).

to transport the bioactive compounds to the rectal gland where sequestration and storage occurs prior to release as sex pheromone during dusk. Furthermore, thin layer chromatography (TLC) analyses using a hexane : ethyl acetate solvent system (2 : 1; v/v) showed that over 80% of those sex pheromonal components remained at the origin after chromatographic separation of the haemolymph, compared with total separation of authentic 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol applied as positive controls (Hee & Tan, 2004). This suggests that the TLC solvent system was not able to separate effectively the pheromonal components in the haemolymph and that these components are possibly bound in polar protein complexes, as confirmed here by the presence of the sex pheromonal components in the bioactive fractions in the large peak of protein absorbance of methyl eugenol-fed male haemolymph. Therefore, the significant increase in protein absorbance of methyl eugenol-fed males in the present study and detection of sex pheromonal components in the bioactive protein fractions clearly warrant further separation of these bioactive fractions to determine the involvement of male haemolymph protein(s).

Acknowledgments

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References

Hee, A.K.W. & Tan, K.H. (1998) Attraction of female and male *Bactrocera papayae* to conspecific males fed with methyl eugenol and attraction of females to male sex pheromone components. *Journal of Chemical Ecology* **24**, 753–764.

Hee, A.K.W. & Tan, K.H. (2004) Male sex pheromonal components derived from methyl eugenol in the hemolymph of the fruit fly *Bactrocera papayae*. *Journal of Chemical Ecology* **30**, 2127–2138.

Howlett, F.M. (1915) Chemical reactions of fruit flies. *Bulletin of Entomological Research* **6**, 297–305.

Khoo, C.C.H. & Tan, K.H. (2005) Rectal gland of *Bactrocera papayae*: ultrastructure, anatomy, and sequestration of autofluorescent compounds upon methyl eugenol consumption by the male fruit fly. *Microscopy Research and Technique* **67**, 219–226.

Khoo, C.C.H., Yuen, K.H. & Tan, K.H. (2000) Attraction of female *Bactrocera papayae* to sex pheromone components with two different release devices. *Journal of Chemical Ecology* **26**, 2487–2496.

Naeole, C.K.M. & Haymer, D.S. (2003) Use of oligonucleotide arrays for molecular taxonomic studies of closely related species in the oriental fruit fly (*Bactrocera dorsalis*) complex. *Molecular Ecology Notes* **3**, 662–665.

Nishida, R. & Fukami, H. (1990) Sequestration of distasteful compounds by some pharmacophagous insects. *Journal of Chemical Ecology* **16**, 151–164.

Nishida, R., Tan, K.H., Wee, S.L., Hee, A.K.W. & Toong, Y.C. (2004) Phenylpropanoids in the fragrance of the fruit fly orchid, *Bulbophyllum cheiri*, and their relationship to the pollinator, *Bactrocera papayae*. *Biochemical Systematics and Ecology* **32**, 245–252.

Nishida, R., Tan, K.H., Serit, M., Lajis, N.H., Sukari, A.M., Takahashi, S. & Fukami, H. (1988) Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Experientia* **44**, 534–536.

Schal, C., Sevala, V.L. & Cardé, R.T. (1998) Novel and highly specific transport of a volatile sex pheromone by hemolymph lipophorin in moths. *Naturwissenchaften* **85**, 339–342.

Shelly, T.E. & Dewire, A.L.M. (1994) Chemically mediated mating success in male oriental fruit flies

https://doi.org/10.1079/BER2005392 Published online by Cambridge University Press

(Diptera: Tephritidae). *Annals of the Entomological Society of America* **87**, 375–382.

Shelly, T.E. & Nishida, R. (2004) Larval and adult feeding on methyl eugenol and the mating success of male oriental fruit flies, *Bactrocera dorsalis*. *Entomologia Experimentalis et Applicata* **112**, 155–158.

Tan, K.H. (1985) Estimation of native populations of male *Dacus* spp. by using Jolly's stochastic method using a new designed attractant trap in a village ecosystem. *Journal of Plant Protection in the Tropics* **2**, 87–95.

Tan, K.H. (1993) Ecohormones for the management of fruit fly pests – understanding plant–fruit fly–predator interrelationships. pp. 495–503 in *Proceedings of an International Symposium on Management of Insect Pests: Nuclear and Related Molecular and Genetic Techniques*. Austria, IAEA.

Tan, K.H. (2000) Behaviour and chemical ecology of *Bactrocera* flies. pp. 647–656 *in* Tan, K.H (*Ed.*) *Area-wide control of fruit flies and other insect pests*. Penang, Penerbit Universiti Sains Malaysia.

Tan, K.H. (2003) Interbreeding and DNA analysis of sibling species within the *Bactrocera dorsalis* complex. pp. 113–12. in *Recent trends on sterile insect technique and area-wide integrated pest management – economic feasibility, control projects, farmer organization and* Bactrocera dorsalis *complex control study*. Research Institute for Subtropics, Japan.

Tan, K.H. & Nishida, R. (1996) Sex pheromone and mating competition after methyl eugenol consumption in the *Bactrocera dorsalis* complex. pp. 147–153 *in* McPheron, B.A. & Steck, G.J (*Eds*) *Fruit fly pests*. Florida, St Lucie Press.

Tan, K.H. & Nishida, R. (1998) Ecological significance of male attractant in the defence and mating strategies of the fruit fly pest, *Bactrocera papayae*. *Entomologia Experimentalis et Applicata* **89**, 155–158.

Tan, K.H. & Nishida, R. (2000) Mutual reproductive benefits between a wild orchid, *Bulbophyllum patens* and *Bactrocera* fruit flies via a floral synonome. *Journal of Chemical Ecology* **26**, 533–546.

Tan, K.H., Kirton, L.G. & Serit, M. (1987) Age response of *Dacus dorsalis* (Hendel) to methyl eugenol in (A) a wind tunnel and (B) traps set in a village, and its implication in population estimation. pp. 425–432 *in* Economopoulos, A.P (*Ed.*) *Fruit flies*. Amsterdam, Elsevier.

Tan, K.H., Nishida, R. & Toong, Y.C. (2002) Floral synomone of a wild orchid, *Bulbophyllum cheiri*, lures *Bactrocera* fruit flies for pollination. *Journal of Chemical Ecology* **28**, 1161–1172.

Wee, S.L., Hee, A.K.W. & Tan, K.H. (2002) Comparative sensitivity to and consumption of methyl eugenol in three *Bactrocera dorsalis* (Diptera: Tephritidae) complex sibling species. *Chemoecology* **12**, 193–197.

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