Investigation of the physiological consequences of feeding on methyl eugenol by *Bactrocera cacuminata* (Hering) (Diptera: Tephritidae)

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Males of many species of fruit flies (Tephritidae: Dacinae) respond to chemical lures of botanical origin. Such lures (e.g. cuelure and methyl eugenol (ME)) have been successfully used in fruit fly population management. The hypothesized role of these chemicals by males is exclusively ecological (pheromonal and/or allomonal) and male response has been classed as pharmacophagy. However, for a response to plant derived lures to be classified as pharmacophagous *sensu stricto*, it must be demonstrated that the role of these chemicals are ecological and not primarily metabolic (e.g. nutritional) or associated with host plant recognition. To specifically test the primary metabolic effects of feeding on ME, we investigated the physiological consequences of exposure to ME in the dacine fly, *Bactrocera cacuminata* (Hering). Our results indicate that feeding on ME does not confer any physiological advantage to *B. cacuminata*. This is reflected in the lack of any consistent difference over time between ME-fed and non ME-fed flies in terms of overall weight and lipid, carbohydrate and protein reserves. Survival was not significantly influenced by exposure to ME either. These results support the hypothesis that ME use by *B. cacuminata* could be pharmacophagous.

**KEY WORDS** pharmacophagy, pheromones, kairomones, energetic reserves, survival, nutrition

Males of many of the Tephritidae (Diptera) show strong responses to certain chemical compounds commonly referred to as male lures or parapheromones (e.g. cuelure, methyl eugenol, trimedlure). Some of these substances, such as methyl eugenol, occur naturally in plants or are analogs of substances produced by plants (Fletcher et al. 1975, Chambers 1977, Sivinski and Calkins 1986, Fletcher, 1987). The natural occurrence of others, such
as cuelure and trimedlure, is less clear (Drew 1987). It is known that some of these chemicals elicit strong chemotactic feeding responses in male flies (Meats and Hartland 1999, Meats and Osborne 2000). However, despite extensive studies of feeding and mating behavior in fruit flies (see reviews in Aluja and Norrbom 2000), the precise ecological role of these parapheromones remains an enigma.

Three principal hypotheses have been proposed to explain the role of parapheromones in the ecology of fruit flies. Metcalf et al. (1979) suggested that they serve as rendezvous stimuli used by males to locate mates. The second hypothesis explains attraction as a result of the parapheromones’ fortuitous similarity to male aggregation pheromones (Fletcher 1968). The generality of these two hypotheses with respect to cuelure and methyl eugenol responding flies is in doubt given that males of fruit fly species are least responsive to these compounds at times of peak daily sexual activity (Brieze-Stegman et al. 1978, Fitt 1981a). Furthermore, when populations are large, female flies are seldom attracted to these principally male lures (Fitt 1981a). The third hypothesis, first postulated by Fitt (1981a), suggests that these chemicals serve as pheromone precursors, vital to the synthesis of the male sex pheromone.

While the third hypothesis (Fitt 1981a) is a plausible explanation, there is considerable variability in the behavior of different species of flies towards different lures. *Ceratitis capitata* (Weidemann), the Mediterranean fruit fly, does not ingest trimedlure (Shelly and Dewire 1994; Shelly et al. 1996), while species of the subfamily Dacinae do ingest cuelure or methyl eugenol (Fitt 1981a) and appear to integrate
metabolites derived from lures into their pheromone system (Nishida et al. 1988, 1993, 1997). The strength of this hypothesis rests in the fact that females are attracted to these parapheromones when males are rare in the environment (Steiner et al. 1965, Nakagawa et al. 1970, Fletcher et al. 1975, Fitt 1981b), suggesting a mate seeking behavior based on a pheromone system. Female flies are believed to preferentially mate with males that have this phytochemically enhanced pheromone (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000).

This sequence, of attraction to and feeding on a plant derived substance, with its attendant behavioral and fitness consequences, could be described as pharmacophagous (sensu Boppré 1984). In order to examine whether this is the case it must be demonstrated that the benefits derived from these chemicals are ecological and not primarily metabolic (e.g. nutritional) or associated with host plant recognition (Boppré 1984, Nishida and Fukami 1990, Halaweish et al. 1999). In this paper, our objective was to test the primary metabolic/nutritional aspect of this hypothesis. That is, if the attraction to and feeding on methyl eugenol by the dacine fly, Bactrocera cacuminata (Hering) results in nutritional benefits to the fly. To test the hypothesis that B. cacuminata attraction to ME is physiologically beneficial, we examined whether exposure to methyl eugenol affects levels of fly nutritional reserves and survival. If the behavior is pharmacophagous, then there should be no physiological benefits in relation to feeding on parapheromones.
Materials and Methods

*Bactrocera cacuminata* is a methyl eugenol responding, non-pest, monophagous species that utilizes *Solanum mauritianum* Scopoli as its host plant. It is a member of the *dorsalis* complex of fruit flies (Drew 1989, Drew and Hancock 1994) which includes the oriental fruit fly, on which previous tests of Fitt’s (1981a) hypothesis have been done (Shelly 1994, Shelly and Dewire 1994, Tan and Nishida 1996).

All flies used in the experiment were from a colony maintained at Griffith University. Wild flies were released into the colony every two to three generations to negate the effects of any laboratory induced selection pressures. Adult flies were separated by sex within 2d of emergence, well before they attain sexual maturity at approximately 10-14 d. No more than 100 adult flies were maintained in 30 x 30 x 30cm screen cages with water, sugar and protein provided *ad libitum*. The flies were maintained in a rearing room at a temperature of 25-27°C and 65-70% relative humidity. The rearing room was under semi-natural light conditions, with fluorescent tubes illuminating the room between 0800 and 1600h and natural light for the remainder of the day.

For each of two experiments (see below), 400 newly emerged male flies were isolated from females within the first 3 d since adult emergence. Two wk after emergence (i.e. when flies attained sexual maturity), half the flies were exposed to 4 ml methyl eugenol (ME) on a cotton wick for a period of 24h. Casual observations indicated that a
majority of the flies exposed to ME began feeding on it (frequent contact of the wick by their proboscis) within the first few minutes of exposure. These flies are henceforth referred to as ME-fed flies. The remaining male flies were not provided with methyl eugenol and are hereafter referred to unfed flies.

A cylindrical field cage (230cm tall \( \times \) 250cm diameter) containing three potted S. mauritianum plants was set up in a garden at least 3 d prior to the start of each experiment. This allowed for the colonization of the cage by potential competitors for resources (e.g. ants and bugs) and predators (e.g. ants, spiders and lizards) (author’s pers. obs.). The potted plants had previously been grown in their natural habitat, along a rainforest edge. For each experiment, 200 ME-fed and 200 ME-unfed male flies were released into the field cage immediately after the 24h ME exposure period. Prior to release, the unfed flies were marked with a white spot on the thorax, using liquid paper. Previous observations have shown that this does not alter their mobility or behavior in any significant way.

**Experiments.** One experiment (*Experiment 1*) was run with only sugar solution (10 ml; sprayed three timers per wk on parts of the foliage) and water provided in the field cage, while in a second experiment (*Experiment 2*) sugar solution, water and a protein autolysate solution was supplied (*Experiment 2*). The protein was supplied as 5ml of solution soaked into a sponge, with fresh protein/sponge provided three times per wk. The sponge was randomly re-positioned within the field cage every time the protein was
provided. This was done to ensure that flies had to actively forage to find the protein source.

In Experiment 1, 10 flies of each status were sampled on days 0 (day of release into cage), 1, 2, 4 and 8 for biochemical analyses. In Experiment 2, 10 flies of each status were sampled on days 0 (day of release into cage), 1, 2, 4, 8 and 16 for biochemical analyses.

**Biochemical Analysis.** Each of the flies were dessicated at 30° C for 24 h and weighed on an analytical balance (± 0.01 mg). To determine the levels of protein, lipid and carbohydrates present in the flies, the biochemical techniques of Van Handel and Day (1988), as modified by Warburg and Yuval (1996, 1997) and Yuval et al. (1998) were used as described below. All data were standardized based on the dry weight of the fly to correct for variations in size.

Flies were homogenized individually in 200 μl of 2% Na₂SO₄. Carbohydrates and lipids were extracted in 1300 μl of chloroform : methanol (1:2). Individual tubes were centrifuged at 8000 rev per min and 500 μl were taken from the supernatant of each sample and dried. Samples were then dissolved in 500 μl H₂SO₄ and incubated for 10 minutes at 90°C. Samples of 30 μl were put into wells on ELISA plates together with 270 μl of vanillin reagent (600 mg vanillin dissolved in 100 ml of distilled water and 400 ml of 85% H₃PO₄). The plate was shaken at room temperature for 30 min and then the optical density was read at 530 nm on an EL311SX Bio-tek Spectrophotometer. Total
lipids per fly were calculated from standard curves using the KCJR EIA application software (Bio-tek Instruments Inc., Winooski, Vermont).

Sugar content per fly was assessed using 300 µl from the supernantant of the chloroform: methanol extract. After adding 200 µl of water the sample was reacted with 1 ml of anthrone reagent (500 mg of anthrone dissolved in 500 ml of conc. H₂SO₄) at 90°C. Samples of 300 µl were then put into wells on ELISA plates and the optical density was read at 630 nm. Similar to the lipid content analysis, total carbohydrates per fly was estimated using standard curves.

Haemolymph dissolved protein was extracted in 1200 µl phosphate buffer saline (PBS). Samples of 300 µl were taken and after adding 500 µl of PBS, were reacted with 200 µl of Bradford reagent (Bradford 1976). Samples of 300 µl were then put into wells on ELISA plates and optical density was read at 595 nm. Total dissolved protein per fly was calculated from standard curves.

**Survival.** In each of the two experiments (mentioned above) the field cage was censused every alternate day for a period of 1 mo from release of the flies. For a focused period of 5 min, the entire cage was scanned for the two groups of flies and the number of individuals observed was noted. Since equal numbers of ME-fed and unfed flies were sampled from the field cage for biochemical analyses, the sampling protocol did not bias the survival estimates in favor of either state.
**Data Analyses.** Differences in the weight, lipid, protein and carbohydrate reserves between ME-fed and unfed flies were analysed using Analyses of Variance (ANOVA). The ANOVA model used status (ME-fed vs. Unfed) as a fixed factor with days since exposure to methyl eugenol as a covariate. All data were checked for assumptions of the ANOVA and appropriately transformed (if required) prior to analysis. Specific comparisons between status within day were made using t-tests. In the case of carbohydrate reserves in *Experiment 2* (sugar, protein and water were provided in the field cage) heteroscedasticity could not be eliminated by standard transformations of the data for the overall analysis. Hence the data were only analyzed by ANOVAs for each of the day intervals since exposure to methyl eugenol.

Survival data was analyzed using linear regression analyses on log transformed data. The slopes were compared to examine differences between survival rates between ME-fed and unfed flies (Zar 1999).

**Results**

**Experiment 1 (Only sugar and water provided).** There was no significant difference in weight ($F = 0.747; df = 1.97; P = 0.390; \text{Fig. 1a}$), lipid ($F = 0.100; df = 1.97; P = 0.752; \text{Fig. 1b}$), protein ($F = 0.310; df = 1.97; P = 0.579; \text{Fig. 2a}$) or carbohydrate reserves ($F = 0.882; df = 1.97; P = 0.350; \text{Fig. 2b}$), between ME-fed and unfed flies over the course of the entire experiment. There were no differences between
flies of either state in weight, lipid, protein or carbohydrate reserves within day (Figs. 1 and 2).

The regression models fitted to the data explained 91.9% and 95.4% of the variation in the survival of ME-fed and unfed flies respectively. Comparisons of the rate of survival of flies of the two states indicated no significant differences between them ($t = 1.3059; \text{df} = 28; P = 0.2022$; Figure 3a).

**Experiment 2 (Sugar, water and protein provided).** There was no significant difference in weight ($F = 3.094; \text{df} = 1,117; P = 0.081$; Fig. 4a) or lipid ($F = 1.668; \text{df} = 1,117; P = 0.199$; Fig. 4b) reserves. Protein reserves did vary with status ($F = 7.533; \text{df} = 1,117; P = 0.007$; Fig. 5a). Carbohydrate reserves varied significantly on days 0, 2, 8 and 16, but not consistently (Fig. 5b; Day 0 – $F = 25.878; \text{df} = 1,18; P < 0.001$; Day 1 – $F = 1.818; \text{df} = 1,18; P=0.194$; Day 2 – $F = 6.396; \text{df} = 1,18; P = 0.021$; Day 4 – $F = 1.870; \text{df} = 1,18; P = 0.188$; Day 8 – $F = 47.297; \text{df} = 1,18; P < 0.001$ (log transformed); Day 16 – $F = 27.052, \text{df} = 1,18; P < 0.001$) between ME-fed and unfed flies over the course of the entire experiment. The only other difference between flies of either state within day was in protein reserves on day 4 and day 16 (Fig. 5a) with unfed flies having higher protein reserves than ME-fed flies on both occasions.

The regression models fitted to the data explained 94.1% and 96.3% of the variation in the survival of ME-fed and unfed flies respectively. Comparisons of the rate
of survival of flies of the two states indicated no significant differences between them \((t = 0.3387; \text{df} = 28; P = 0.7374; \text{Fig. 3b})\).

**Discussion**

The functional basis of attraction of dacine fruit flies to botanically derived lures is central to our understanding of their ecology and evolution (Metcalf 1990). Male response to these lures has been inferred (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000), and occasionally stated to be pharmacophagy (Khoo and Tan 1998). In addition, this putative functional role of these parapheromones has been generalized to the Dacinae in general (Shelly and Dewire 1994, Tan and Nishida 1996, Shelly 2000). However, as pointed out by Boppré (1984), for a behavior to be defined as pharmacophagy, it is vital to demonstrate that the consequences of feeding on the plant-derived chemical are primarily ecological and not physiological. Hence we investigated the physiological consequences of feeding on methyl eugenol by *B. cacuminata*.

Consistent with the expectations of pharmacophagous behavior, our data show that there are no physiological benefits of feeding on methyl eugenol by *B. cacuminata*. Though the transformation of methyl eugenol directly into energetic reserves is unlikely by biochemical pathways (Fletcher and Kitching 1995), it is not impossible (M. Fletcher and B. Kitching, Uni of Qld pers comm.), and thus the lack of specific ecological and/or physiological tests has meant that a possible physiological benefit of ME feeding could not be ignored in the ongoing scientific development of this field. Therefore, we pursued
this question to assess if feeding on methyl eugenol influenced foraging for nutrients and thereby conveyed physiological benefits to males feeding on it. This does not appear to be the case for either measures of primary metabolism (Figs. 1, 2, 4 and 5) or for survival (Fig. 3).

Some differences were evident in relation to feeding on ME. When flies were not provided with a supplement of protein, no significant difference in nutrient reserves was detected on any of the days sampled (Figs. 1b, 2a and 2b). However, when we added a source of protein to the field cage, significant differences in reserves of carbohydrate and protein (but not lipid) were evident on some of the sampling days. Particularly striking was the trend seen in carbohydrate reserves, where after the second day in the field cage, through to day 16 (with the exception of day 4), ME fed males had significantly higher levels of carbohydrates than males who had no exposure to ME. Conversely, on day 4 and day 16 unfed males had higher protein levels than ME fed males (Figs. 5a and 5b). This pattern may indicate that, when protein was available in abundance, ME fed males engaged in a different pattern of behavior than unfed flies, and (or) utilized their resources in a different manner.

Benefits to defense as a result of feeding on ME have been hypothesized in male dacine fruit flies. The Asian house gecko (*Hemidactylus frenatus* Dumeril and Bibron) is deterred from feeding on ME-fed *Bactrocera papayae* Drew and Hancock and cuelure-fed *Bactrocera cucurbitate* (Coquillett) males (Tan 2000, Tan and Nishida, 1998). In the present study, our large field cage housed numerous predators, including ants, spiders,
lizards and reduviids. Though we did not quantify predation, the survival data (Fig. 3) suggest that feeding on methyl eugenol did not enhance survival in the presence of potential predators in the field cage. Whether these differences in physiology and survival may be interpreted as a reflection of an advantage enjoyed by the ME fed flies is moot.

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Figure captions

**Fig. 1.** Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) weight (mg) and (b) lipid reserves (µg/ mg of fly) when flies had access to sugar and water in the field cage. Bars within day that are marked with the same letter are not significantly different.

**Fig. 2.** Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) protein reserves (µg/ mg of fly) and (b) carbohydrate reserves (µg/ mg of fly) when flies had access to sugar and water in the field cage. Bars within day that are marked with the same letter are not significantly different.

**Fig. 3.** Difference in survival between ME-fed (filled circles) and Unfed *Bactrocera cacuminata* (open circles) (a) when provided with sugar and water and (b) when provided with sugar, protein and water.

**Fig. 4.** Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) weight (mg) and (b) lipid reserves (µg/ mg of fly) when flies had access to sugar, protein and water in the field cage. Bars within day that are marked with the same letter are not significantly different.

**Fig. 5.** Differences between ME-fed and Unfed *Bactrocera cacuminata* in (a) protein reserves (µg/ mg of fly) and (b) carbohydrate reserves (µg/ mg of fly) when flies had access to sugar, protein and water in the field cage. Bars within day that are marked with the same letter are not significantly different.
Fig. 1(a) and (b)

(a)

Days since exposure to methyl eugenol

(b)

Days since exposure to methyl eugenol
Fig. 2(a) and (b)

(a) Protein - µg/mg of fly (mean ± st.dev.)
- Protein (methyl eugenol fed flies)
- Protein (unfed flies)

(b) Carbohydrates - µg/mg of fly (mean ± st.dev.)
- Carbohydrates (methyl eugenol fed flies)
- Carbohydrates (unfed flies)
Fig. 3(a) and (b)
Fig. 4(a) and (b)
Fig. 5(a) and (b)

(a) Protein content in flies exposed to methyl eugenol over time. The graph shows a significant increase in protein content between days 0 and 16, with differences between methyl eugenol fed flies and unfed flies indicated by different letters.

(b) Carbohydrate content in flies exposed to methyl eugenol over time. The graph shows a decrease in carbohydrate content over time, with differences between methyl eugenol fed flies and unfed flies indicated by different letters.