Pheromone Production by Male *Tribolium castaneum* (Coleoptera: Tenebrionidae) Is Influenced by Diet Quality

QING-LEI MING[1] AND SARA M. LEWIS[2]

Department of Biology, Tufts University, Medford, MA 02155, USA

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ABSTRACT *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), the red flour beetle, is a common cosmopolitan pest exploiting a variety of stored products. We experimentally manipulated diet nutritional quality by using non-nutritive filler to examine how this influenced pheromone production and olfactory attractiveness of *T. castaneum* adult males. Volatiles released by individual males reared on high versus low nutrition diets were collected using solid phase microextraction, and gas chromatography coupled to mass spectrometry was used to identify and quantify the *Tribolium* aggregation pheromone 4, 8-dimethyldecanal (DMD). Males kept on high nutrition diet showed a three-fold increase in daily DMD production, which suggests the possibility that this pheromone could act as a condition-dependent mating signal. In pitfall trap assays, there was no significant difference in the mean response of virgin females to discs kept with low versus high nutrition males, although discs carrying male cues were significantly more attractive than blank discs. These results suggest that DMD production rates by *T. castaneum* males will depend on the nutritional quality of various stored products, but such differences may not alter males' ability to attract females.

KEY WORDS aggregation pheromone, 4,8-dimethyldecanal, SPME, stored products

Tribolium flour beetles (Coleoptera: Tenebrionidae) (Coleoptera: Tenebrionidae), are cosmopolitan pests of stored cereals and grains and rank among the most important insect pests inhabiting grain processing plants and storage facilities (Sokoloff 1972, Campbell et al. 2004). In several *Tribolium* species, males produce 4,8-dimethyldecanal (DMD), a highly volatile pheromone that attracts both sexes (Ryan and O'Ceallachain 1976; Suzuki 1980, 1981; Levinson and Mori 1983; Arnaud et al. 2002; Verheggen et al. 2007). Although DMD is commonly referred to as an aggregation pheromone, it also may be a sex attractant because DMD elicited greater behavioral and electroantennogram responses from females compared with males in both red flour beetle, *Tribolium castaneum* (Herbst), and *Tribolium confusum* Jacquelin du Val (Levinson and Mori 1983, Verheggen et al. 2007). Synthetic DMD is used in commercial traps to monitor *Tribolium* populations (Campbell et al. 2002, 2004; Campbell and Arbogast 2004; Toews et al. 2005). Because *Tribolium* beetles normally inhabit stored products that vary widely in their nutritional quality (Sokoloff 1974, Baker and Loschiavo 1987), it is valuable to understand how diet nutritional quality might influence DMD production by males.

Previous studies in the red flour beetle have shown that male volatile production depends on several factors, including photoperiod, beetle age, and population density (Hussain 1993, Hussain et al. 1994). Infection by the tapeworm *Hymenolepsis diminuata* did not alter DMD production (Yan and Phillips 1996). Hussain et al. (1994) also reported lower DMD production by starved *T. castaneum* males. However, to our knowledge no studies have addressed how diet quality influences pheromone production and olfactory attractiveness in this important group of insect pests. Hence, the objectives of our study were to 1) qualitatively and quantitatively analyze DMD production by single *T. castaneum* males by using headspace solid phase microextraction (HS-SPME) coupled with gas chromatography (GC); and 2) compare DMD production and olfactory attractiveness of males reared on low and high nutrition diets.

Materials and Methods

Manipulating Diet Nutritional Quality. *T. castaneum* were from laboratory cultures (derived from the Berkeley synthetic strain) maintained on King Arthur wheat (*Triticum aestivum* L.) flour in a darkened incubator at 29°C and 70% RH. Beetles used in this experiment were obtained by rearing eggs in either high nutrition diet (100% wheat flour) or low nutrition diet consisting of 20% wheat flour mixed with microcrystalline cellulose (MCC), a non-nutritive filler

[1] Current address: School of Life Sciences, Xuzhou Normal University, Xuzhou, Jiangsu 221116, China.
[2] Corresponding author, e-mail: sara.lewis@tufts.edu.

(Lattice NT200, FMC Biopolymer, Philadelphia, PA). Previous work manipulating diet quality with MCC (Fedina and Lewis 2007) has shown that such diets closely mimic pupal mass reductions seen in other nutritionally poor diets (e.g., corn flour), yet MCC provides the advantage of quantitatively reducing nutritional content without qualitatively changing diet composition. Beetles were sexed at the pupal stage, after which males were maintained individually in a darkened incubator at 29°C and 70% RH in glass vials with 2 g of either low or high nutrition diet.

Collection of Male Volatiles. To sample the volatiles emitted by *Tribolium* males, we placed 1-mo-old adults individually in 10-ml glass screw cap vials with PTFE/Silicone septa (Supelco, Bellefonte, PA) containing 0.3 g of either low or high nutrition diet. After 4 d, SPME was used to collect headspace samples of volatiles secreted by each male. For males reared on low nutrition ($n = 12$), and high nutrition ($n = 13$) diets, volatiles were sampled from closed vials for 30 min at 29°C by using a polydimethylsiloxane (PDMS) fiber (100 μm; Supelco); PDMS fibers have been shown previously to be suitable for sampling DMD (Arnaud et al. 2002). Control vials containing 0.3 g of either low or high nutrition diet without males also were sampled. Fibers were initially conditioned at 250°C until a stable baseline was obtained, and were systematically reconditioned between samples.

Identification and Quantification of DMD. Samples were analyzed by coupled GC-mass spectrometry (MS) with electron impact ionization (70 eV) by using a QP 5050 GC-MS (Shimadzu, Kyoto, Japan) equipped with a Rtx-XLB capillary column (30 m by 0.25 mm by 0.25 μm; Restek, Bellefonte, PA) in splitless mode, with helium as carrier gas. Following methods of Arnaud et al. (2002), the column was programmed from 40 to 200°C at 8°C/min and then 20°C/min to 280°C. Injector temperature was 250°C and transfer line temperature was 280°C. SPME fiber was desorbed in the injection port of the GC at 250°C for 2 min, by using a narrow-bore Supelco 0.75 mm i.d. GC inlet liner. Identifications were performed by comparison with the retention time and mass spectrum of pure DMD (Trécé Inc., Adair, OK).

Quantification of DMD was carried out by comparing GC detector response (peak area) to a calibration curve developed using solutions containing known amounts of pure DMD ranging from 1 to 400 ng in hexane, and injected under analytical conditions identical to that of SPME analyses. The calibration line showed good linearity and fit in this range ($r^2 = 0.990$), and DMD quantities were calculated using the equation: DMD (nanograms) $= 3 \times 10^{-6} \times$ DMD peak area.

Male Olfactory Attractiveness to Females. Experimental 3-mo-old males were placed individually in 0.5-ml microfuge tubes containing 80 mg of either low or high nutrition diet. In each tube, we also placed a 6-mm-diameter disc of P8 filter paper (Thermo Fisher Scientific, Waltham, MA) on the surface of the diet to adsorb male secretions. Male discs remained in tubes for 3 d before being used in assays described below; blank discs were kept for 3 d in tubes with only low or high nutrition diet.

We measured olfactory attractiveness of *T. castaneum* males reared on low nutrition ($n = 14$) and high nutrition ($n = 17$) diets compared with their respective controls by using pitfall trap assays (as described in Boake and Wade 1984, Lewis and Austad 1994, Bloch Qazi et al. 1998) conducted at 25°C. For each assay, we mounted one male disc and one control disc directly below two holes in a covered opaque 8.5- by 15- by 2-cm plastic chamber, waited 30 s, and then introduced 20 virgin females into the center. We recorded the number of females that had fallen into each pit after 15 min and calculated male olfactory attractiveness as the percentage of all responding females that were attracted to the male disc. If fewer than 10 (of 20) females responded to either stimulus (i.e., male or control discs), we excluded these results from our statistical analysis.

Statistical Analyses. We compared daily DMD production of *T. castaneum* males reared on low versus high nutrition diets using two-tailed separate variances *t*-tests. We examined the effects of males' diet quality on their olfactory attractiveness to females with two approaches. First, for males in each diet treatment we used one sample *t*-tests of the null hypothesis that male and control discs were equally attractive to females (i.e., $\mu_0 = 50\%$). We also used a two-tailed separate variance *t*-test to determine whether mean attractiveness differed between low and high nutrition males. Descriptive statistics are reported as means ± SE.

Results

Typical chromatograms (total ion current) from the headspace SPME sampling of volatiles released by individual *T. castaneum* males (Fig. 1) showed peak at 14.33 min (retention time, t_R) that corresponded to DMD. *T. castaneum* males reared on high nutrition diet showed three-fold higher rates of DMD production compared with same-age males reared on low nutrition diet (Fig. 2: separate variance $t = 2.7$, df = 22.3, $P = 0.013$).

In pitfall trap assays testing the attractiveness of male secretions, males reared on both diets attracted significantly more females compared with control discs (Table 1: for low quality males, one-sample $t = 10.6$, df = 16, $P < 0.0005$; for high quality males, one-sample sample $t = 6.5$, df = 13, $P < 0.0005$). However, there was no significant difference in the mean response of females to discs kept with low versus high nutrition males (separate variance $t = 0.4$, df = 23.1, $P = 0.706$).

Discussion

In the past few years, headspace SPME coupled with GC-MS has been used successfully to examine pheromones in numerous Coleoptera, e.g., rhinoceros (Rochat et al. 2000) and cerambycid beetles (Lacey et al. 2004, Ginzel and Hanks 2005). This technique also

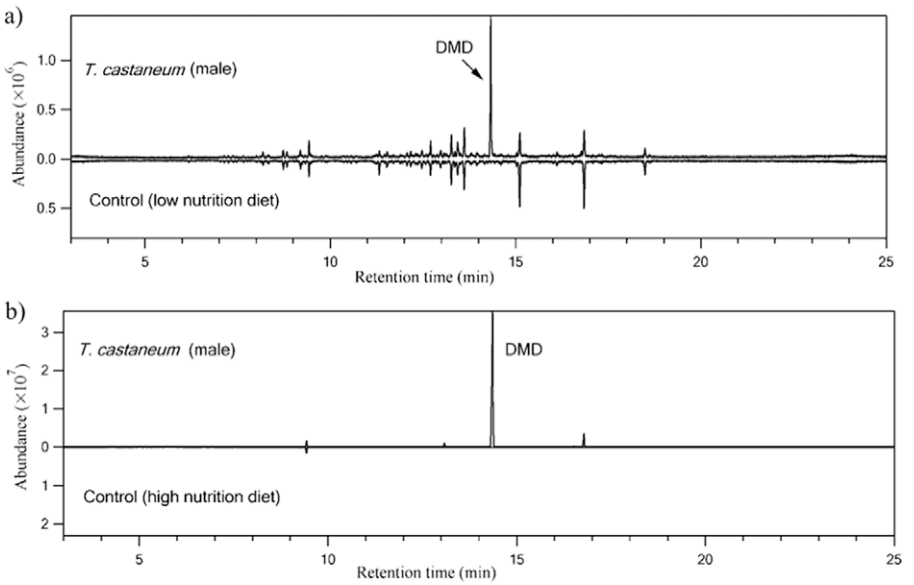

Fig. 1. Typical chromatograms from SPME-GC profiles showing volatiles released by male *T. castaneum* fed low nutrition diet (20% wheat flour mixed with non-nutritive microcrystalline cellulose) compared with diet-only control (a); male *T. castaneum* fed high nutrition diet (100% wheat flour) compared with diet-only control (b). Male-produced pheromone (t_R = 14.33 min) was identified as DMD.

has been used to detect the aggregation pheromone and other volatile compounds of the red flour beetle (Arnaud et al. 2002, Villaverde et al. 2007). Our study confirms the usefulness of HS-SPME for comparing DMD production by individual *T. castaneum* males, and we measured 24-h production rates of 54.8 ± 9.2 ng for males reared on wheat flour (a high nutrition diet). Using Super-Q columns, Hussain et al. (1994) and Bloch Qazi et al. (1998) reported that DMD production by single *T. castaneum* males averaged 637 ng/24 h and 81.5 ng/24 h, respectively. Although different sampling methods were used, our DMD production rates were similar to those reported by Bloch Qazi et al. (1998) by using males from the same laboratory strain. However, using similar methods (SPME with PDMS fiber) Arnaud et al. (2002) measured three *T. castaneum* males and reported an average of 0.71 ± 0.12 ng DMD per beetle: this low rate might represent differences among laboratory strains. Consistent with previous studies (Hussain 1993, Hussain et al. 1994, Bloch Qazi et al. 1998), we also found considerable variation among males in their DMD production: in this study, coefficients of variation were 60 and 208% for males on high and low nutrition diets, respectively. Because these were same-aged males from the same strain, our results suggest that *T. castaneum* males vary in their ability to produce pheromone under nutritionally stressful conditions.

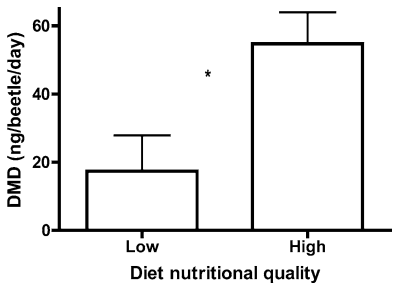

Fig. 2. Effects of diet nutritional quality on production of aggregation pheromone DMD (nanograms per beetle per day; means ± SE) by *T. castaneum* males reared in either low nutrition diet (20% wheat flour mixed with non-nutritive microcrystalline cellulose; n = 12) or high nutrition diet (100% wheat flour; n = 13). The asterisk (*) indicates $P <$ 0.05 (t-tests).

Our results demonstrate that DMD production by *T. castaneum* males is strongly affected by their diet quality (Fig. 2). Previous research has shown that starvation lowers DMD production by *T. castaneum* males (Hussain et al. 1994), but the current study is the first indication that diet quality also influences DMD production in this species. These results suggest the potential for *T. castaneum* females to use DMD as a condition-dependent indicator of male quality, similar

Table 1. Behavioral response of *T. castaneum* females to olfactory cues from males reared on low and high nutrition diets (mean ± SE percentage of responding females attracted to male versus control discs)

Diet nutritional quality	No. behavioral tests	Response (%)	P^a
Low	14	80.9 ± 2.9	<0.0005
High	17	78.9 ± 4.5	<0.0005

[a] P values from one-sample t-tests of null hypothesis that females respond equally to male versus control cues (mean response = 50%).

to numerous other condition-dependent traits used in mate choice (Andersson 1994). Food quality has been shown to affect the output of male aggregation pheromone in other grain beetles (Edde et al. 2007). They studied the lesser grain borer, *Rhyzopertha dominica* (F.) and found significant differences in male production of aggregation pheromones when adults were kept on different food sources.

Based on this higher DMD production rate, we expected that well-fed males would be more attractive to females. Many previous studies with *T. castaneum* have used similar pitfall trap assays to measure the degree to which females are attracted to olfactory cues from individual males collected onto filter paper discs, which reflect a time-integrated composite of male secretions (Boake and Wade 1984, Boake 1986, Lewis and Austad 1994, Bloch Qazi et al. 1998, Pai and Yan 2003). Our pitfall assays showed that for males from both diet types, virgin *T. castanuem* females were significantly more attracted to male discs than to diet-only control discs, but we found no significant difference between males reared on high versus low nutrition diets in their ability to attract virgin females (Table 1). One possible explanation for this result could be that females do not respond to this magnitude of difference in DMD. Another possibility that has been suggested previously (Verheggen et al. 2007, Fedina and Lewis 2008) is that long-range mate attraction in *Tribolium* may involve several compounds that work synergistically or in opposition to DMD.

In conclusion, this study demonstrates that *T. castanuem* males reduce endogenous production of DMD when they are reared on low nutrition diet. For both sexes of *T. castaneum*, Fedina and Lewis (2007) found higher response to commercial traps baited with DMD when beetles were reared on low nutrition diet. Together, these studies indicate that more accurate assessment of pest population densities using trap-based insect monitoring programs can be achieved by taking into account the nutritional quality of the stored products.

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