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ORIGINAL ARTICLE

Anatomical Localization and Stereoisomeric Composition of *Tribolium castaneum* Aggregation Pheromones

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Abstract We report that the abdomen and associated tissues are the predominant sources of male-produced pheromones in the red flour beetle, *Tribolium castaneum*, and for the first time describe the stereoisomeric composition of the natural blend of isomers of the aggregation pheromone 4,8-dimethyldecanal (DMD) in this important pest species. Quantitative analyses via GC-MS showed that the average amount of DMD released daily by single feeding males of *T. castaneum* was 878±72 ng (SE). Analysis of different body parts found the abdominal epidermis as the major source of aggregation pheromone; the thorax was a minor source, while no DMD was detectable in the head. No internal organs or obvious male-specific glands were associated with pheromone deposition. Complete separation of all four stereoisomers of DMD was achieved following oxidation to the corresponding acid, derivatization with (1*R*, 2*R*)- and (1*S*, 2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanol to diastereomeric esters, and their separation on reversed phase HPLC at –54^{o}C. Analysis of the hexane eluate from Porapak-Q-collected volatiles from feeding males revealed the presence of all four isomers (4*R*,8*R*):(4*R*,8*S*):(4*S*,8*R*):(4*S*,8*S*) at a ratio of approximately 4:4:1:1. A walking orientation bioassay in a wind tunnel with various blends of the four synthetic isomers further indicated that the attractive potency of the reconstituted natural blend of 4:4:1:1 was equivalent to that of the natural pheromone, and greater than that of the 1:1 blend of (4*R*,8*R*):(4*R*,8*S*) used in commercial lures.

Keywords Chirality, 4,8-Dimethyldecanal, insect, red flour beetle, chemical ecology, stored grain

Introduction

The red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), is a major cosmopolitan pest of stored cereal grains, beans, nuts and other durable agricultural products worldwide (Weston and Rattlingourd 2000; Campbell et al. 2010). The feeding adult male secretes an aggregation pheromone, which is attractive to both sexes. This pheromone was identified as 4,8-dimethyldecanal (DMD), which has two asymmetric carbons at C-4 and C-8 (Suzuki, 1980; Suzuki et al., 1984). Suzuki (1980) synthesized a mixture of all four stereoisomers of DMD and found it to be less active than the natural pheromone (Suzuki, 1981; Suzuki et al., 1987). Based on bioassay of the four synthetic stereoisomers of DMD against *T. castaneum*, Suzuki and Mori (1983) inferred that the natural pheromone was the (4*R*,8*R*)-stereoisomer because it appeared to be as active as the natural pheromone and Levinson and Mori (1983) supported this claim after documenting activity to each of the four synthetic stereoisomers. Subsequently, a 4:1 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-DMD was found to be ten times more active than (4*R*,8*R*)-DMD alone, although (4*R*,8*S*)-DMD itself was inactive at lower doses (Suzuki et al, 1984). However, the absolute configuration of 4,8-DMD produced by male *T. castaneum* was not further investigated, and remained unknown until the present.

The chemical synthesis of the four stereoisomers of DMD at high enantiomeric purity and a derivatization and HPLC separation method that gave baseline resolution of all four stereoisomers were recently described (Akasaka et al. 2011). The primary objective of the research reported here was to determine the stereoisomeric composition of naturally produced DMD from male beetles and to compare the attraction of natural and artificial blends to that of the synthetic pheromone blend used in commercial lures [a 1:1 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-DMD)]. Although male-specific production of DMD has been demonstrated, there has been some confusion regarding the location of tissues or glands in males that might produce and/or release the pheromone. The male-specific exocrine glands beneath the setiferous patches on the ventral side of the profemura were considered the site of production by Faustini et al. (1981), but Bloch-Qazi et al. (1998) demonstrated that males

with profemura surgically removed would continue to release pheromone. Olsson et al. (2006) provided evidence from the closely related species *Tribolium confusum* that attractive compounds were associated not only with the glands on the femurs but also with multiple locations around the body. In order to assist in future work on pheromone biosynthesis in *T. castaneum*, a second objective of this work was to identify the tissues responsible for DMD biosynthesis, deposition and/or release.

Materials and methods

Insect cultures. Tribolium castaneum from the GA-1 strain (Haliscak and Beeman 1983), which originated nearly 30 years ago from a field site in the state of Georgia, USA, was used for pheromone collection and the KS-1 strain (Romero et al. 2009), recently collected from a commercial flour mill in Kansas, USA, was used for the wind tunnel walking bioassay. Beetles were reared on a mixture of whole-wheat flour and brewer's yeast (95:5) at 27°C and 60% RH and a 16:8 (light-dark) photoperiod. To obtain males for pheromone collection or extraction, pupae were collected, segregated by sex, and maintained separately on flour until needed for experiments.

Collection of natural pheromone. Adult males were placed in 7.5 X 2.75 cm cylindrical glass aeration chambers with 2.5 g of cracked wheat kernels (*Triticum aestivum*) and volatiles were collected according to the methods of Edde and Phillips (2006). Initial collections were made with individual beetles in aeration chambers. Subsequently, it was determined that the amount of pheromone produced per chamber could be maximized by aerating groups of five males. For remaining collections, groups of five 7-14 day-old males were used, with five aeration chamber systems running concurrently. Aerations were conducted at approximately

28°C and under 24 hr of constant light, to elicit maximum pheromone production and release (Hussain 1993), provided by a 40 W incandescent light bulb. The incoming air flow rate was 200 ml/min and the air was humidified by passing though a flask of distilled water. Volatiles were collected on small glass columns packed with Porapak-Q (Alltech Assoc., Deerfield, IL); columns were changed daily for four days. Columns were each eluted with approximately 500 µl of HPLC-grade hexane and the eluate spiked with 555 ng of *n*-dodecane in 5 µl of hexane (111 ng/µl) as an internal standard. Extracts were stored in 1.5 ml glass vials with Teflon-lined septum caps at -80°C pending analysis.

Analyses of tissues containing pheromone. Male beetles used for tissue extracts were 7-14 days old. The virgin males were placed individually into glass vials with 0.5 g cracked wheat mixed with wheat flour for feeding until analyzed. Dissection protocols were modified from Olsson et al. (2006). Briefly, the beetles were sedated with CO_2 and then were mounted ventral-side-up onto double-stick cellophane tape affixed to the bottom of a glass Petri dish. Head, thorax and abdomen were separated from the body with forceps and a group of 5 of each body part was extracted in 1 ml HPLC-grade hexane for 30 min. Five such groups of each body section were separately extracted and an internal standard of 555 ng of *n*-dodecane was added directly to each extract before further processing. Extracts were concentrated to approximately 500 µl under a gentle stream of N_2 at room temperature. After we confirmed that abdomens were the primary source of DMD, the abdomens of additional males (five groups of five abdomens each) were further dissected into two parts; the cuticle with any adhering tissues, and all other tissues, which included the digestive system (including the

hindgut, posterior midgut and Malpighian tubules), reproductive system and fat body. The two abdominal tissue groups were extracted as described above for the three body regions. All extracts were stored at -20°C for no longer than 48 h before chemical analyses.

Chemical analyses. Samples from all aeration and tissue extraction experiments were subjected to quantitative analysis by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (EI, 70eV) using a Shimadzu GC-MS QP5050A (Kyoto, Japan) equipped with a J&W Scientific DB-1 capillary column (30 m × 0.25 mm × 0.25 µm) in splitless mode, with helium as carrier gas. The injector oven was set at 250°C and the heated transfer line to the MS was set at 250°C. Oven temperature was programmed at 40°C for 0.5 min, then increased 10°C/min to 200°C and was held for 1 min, then increased to 240°C at 20°C/min, and held at 240°C for 1 min. Initial studies were conducted with the MS in the full scan mode, recording mass fragments from 35 to 350 amu. A 1:1 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-DMD was analyzed for retention time and mass spectrum, which matched the spectrum published by Suzuki (1981). In order to maximize detection sensitivity for DMD in the experiments described above, the MS was subsequently operated in the multiple ion detection mode (MID) in which only the characteristic fragment ions *m/z* = 41 and *m/z* = 57 were detected. These fragment ions are common to both the internal standard dodecane, and DMD. The quantity of DMD in each sample was determined by comparison of the peak area of the internal standard, representing 555 ng in the initial solution, and that of DMD from the MID chromatogram. Accuracy of our internal standard quantification was determined by analyzing a series of solutions with known amounts of

147 synthetic DMD and dodecane from low to high concentrations, and the average percent
148 estimation was used to adjust the final quantities of the DMD in the samples. The total
149 amount of pheromone produced by each beetle over the six-day collection period was
150 calculated and differences among treatments were determined with ANOVA using SAS
151 software. GC and HPLC analyses of stereoisomeric composition, either with or without
152 derivatization of the naturally collected pheromones, were conducted according to the
153 methods of Akasaka et al. (2011).

154 Sample preparation procedure for analytical HPLC was as follows. To a pheromone
155 sample solution in hexane (ca. 0.4 ml containing ca. 4 μg of DMD) about 1 ml of acetone and
156 0.5 mg of $KMnO_4$ were added. The mixture was stirred at room temperature for 1 h. After
157 oxidation, 0.5 ml of 10% $NaHSO_3$ solution was added to the mixture and stirred for several
158 min. The resulting clear and colorless solution was acidified with 2 ml of 15% citric acid
159 solution (pH<3.5). The acid fraction was extracted with 2 ml of hexane 3 times. After drying
160 the hexane extract over Na_2SO_4, the solvent was removed under reduced pressure. The
161 residue was dissolved in 0.6 ml of toluene/acetonitrile (1:1, v/v). The solution was divided
162 into two portions. One was used for derivatization with
163 (1*R*,2*R*)-2-(2,3-anthracenedicarboximido)cyclohexanol and the other was for derivatization
164 with the (1*S*,2*S*)-reagent (see Akasaka 2011 for details).
165
166 *Walking orientation bioassay.* We used a wind tunnel built from the general design of Miller
167 and Roelofs (1978) for behavioral bioassays of adult *T. castaneum*. The chamber consisted of
168 an acrylic box, 100 cm in length and 40 cm in width and height with screening at each end.

Air flow was generated by a centrifugal fan, with the air passing through an activated charcoal filter to cleanse it and a porous metal plate to generate a laminar flow of 0.35–0.4 m/s. Wind tunnel bioassays were conducted at 25°C, and 60% RH. Mixed sex adults were individually isolated in 5.0 ml opened glass shell vials and starved for 24 hr prior to bioassay. The walking behavior of individual beetles to volatiles in moving air was observed on a sheet of 22 × 28 cm white photocopy paper placed on the floor of the wind tunnel at the upwind end, with paper changed between each replicate beetle. Test samples in a hexane solution were applied on 2.0 cm diameter pieces of circular filter paper (Whatman No. 1) placed on a circular metal disk, 2.0 cm diameter and 2.0 mm thick above the floor. Twenty µl of hexane for controls or hexane containing the test sample was applied to the filter paper circle and allowed to dry for 5 min in a fume hood before placing the metal disk with filter paper on the paper arena 1.0 cm from the middle of the upwind edge of the sheet of paper in the wind tunnel. Test beetles were released ~10 cm directly downwind of the source filter paper by inverting the glass vial over the paper and lifting it off once the beetle was observed to be upright. Observations of beetles terminated when one of three events occurred: the beetle walked upwind and touched the metal disk (i.e., located the odor source); the beetle walked to the edge of sheet of the paper (i.e., did not respond to the source, and attempted to leave the observation area); or the beetle remained on the paper for 2 min without one of the other two events occurring.

Twenty mixed-sex adult beetles comprised a replicate and were tested individually in direct succession, with the odor source filter paper changed after every five beetles. Five replicates (i.e., five groups of 20 individual beetle observations) were utilized for each

experimental treatment, with one replicate for each treatment and control blocked within a day. The hexane-only control bioassays were performed first in a given day and the treatment order was randomized in successive groups in a given day.

All four stereoisomers of 4,8-DMD were synthesized as previously described (Akasaka et al. 2011); chemical purities: (4*R*,8*R*)-isomer at 92.2%, (4*R*,8*S*)-isomer at 95.3%, (4*S*,8*R*)-isomer at 91.7% and (4*S*,8*S*)-isomer at 94.4%; stereoisomeric purities of all four stereoisomers were 97% ee at C-4 and over 99% ee at C-8. We compared the behavioral responses of beetles to the following treatments in bioassays: 0.1 ng, a very small yet biologically relevant amount determined to give an acceptable positive response from among a arrange of concentrations (unpublished data), of a 1:1 blend of synthetic stereoisomers (4*R*,8*R*)- and (4*R*,8*S*)-DMD, which mimics the blend used in commercial pheromone lures; a 1:1:1:1 blend of all four synthetic stereoisomers (4*R*,8*R*)-, (4*R*,8*S*)-, (4*S*,8*R*)-, and (4*S*,8*S*)-DMD; a 4:4:1:1 blend of synthetic stereoisomers (4*R*,8*R*)-, (4*R*,8*S*)-, (4*S*,8*R*)-, and (4*S*,8*S*)-DMD, which mimics the natural blend of stereoisomers produced by male beetles (reported below); naturally collected DMD, which included other potential semiochemicals in the complete eluate collected on Porapak-Q during aeration of feeding males; and a solvent control. The percentage of beetles in a given test group contacting the odor source within two minutes (i.e., percent of positive response out of 20 beetles) was subjected to arcsine square root transformation to normalize distribution and then analyzed using ANOVA. Differences among means were determined using the Student-Newman-Keuls (SNK) Test with SAS software (SAS Institute, 2001).

Results

Quantitative analysis of volatiles collected on Porapak-Q from aerations showed that the average amount of DMD released by a single feeding male was 878±72 (SE) ng per 24 h period (n=275 beetle-day-equivalents). There was no 4,8-DMD found in extracts of the head or from internal organs and tissues. The amount of DMD in extracts of whole abdomens averaged 285 ± 51 ng, which was significantly greater than the 62 ± 14 ng extracted from thoraces or the 159 ± 25 ng extracted from the abdominal epidermis lacking internal organs (ANOVA: $F_{2,25}$=10.39, p<0.01, n=25) (ANOVA , SAS software). Results clearly indicated that the abdomen of male *T. castaneum* was the predominant source of aggregation pheromone, and that approximately half of this amount derived from the abdominal cuticle and epidermis. The thorax contained a much smaller amount. No DMD was found in extracts of internal abdominal organs.

Enantioselective GC analysis on a chiral stationary phase column (details in Akasaka et al. 2011) enabled good separation of (4*R*,8*R*)-DMD and (4*R*,8*S*)-DMD, but did not resolve the (4*S*,8*R*)-DMD from the (4*S*,8*S*)-DMD stereoisomers, which co-eluted as one peak (Fig. 1). Complete separation of the four stereoisomers of 4,8-DMD was achieved following oxidation with $KMnO_4$ to the corresponding acid, its derivatization with (1*R*,2*R*)- and (1*S*,2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanol to diastereomeric esters, their analysis on the reversed-phase HPLC column immersed in a cooling bath at -54°C (Fig. 2), and the absolute configuration of naturally produced DMD was revealed. The natural ratio of the four stereoisomers of 4,8-DMD was approximately 4:4:1:1 [(4*R*,8*R*):(4*R*,8*S*):(4*S*,8*R*):(4*S*,8*S*)] , measured by calculating the area under each stereoisomer

peak in the HPLC chromatogram, and this represents the average of 15 analyses of samples collected from June 2, 2010 to July 2, 2010.

The behavioral responses of *T. castaneum* to the four synthetic stereoisomers of 4,8-DMD at the naturally produced ratio ([(4*R*,8*R*)-:(4*R*,8*S*)- : (4*S*,8*R*)-DMD = 4 : 4 : 1 : 1]) and the natural blend of pheromones and other semiochemicals collected from virgin feeding males were similar and significantly greater than the response to the (4*R*,8*R*)-DMD : (4*R*,8*S*)-DMD = 1 : 1 (as used in commercial pheromone lures) or the (4*R*,8*R*)- : (4*R*,8*S*)-: (4*S*,8*R*)- : (4*S*,8*S*)-DMD =1:1:1:1 ratio treatment (Fig. 3). All treatments with stereoisomers of DMD elicited significantly higher responses than the solvent control in the walking bioassay (Fig. 3). The results confirmed that a synthetic blend of DMD stereoisomers in the same relative ratio as the natural aggregation pheromones is more attractive to *T. castaneum* males and females than the ratio of stereoisomers currently used in commercial pheromone lures.

Discussion

The present study demonstrates that feeding male *T. castaneum* release DMD at rates similar to the 635 ng·24 h^{-1} reported by Hussain (1993), and 7-fold higher than rates reported by Bloch-Qazi et al (1998). These differences are most likely due to the fact that our aerations and those of Hussain (1993) were conducted under 24 hr light conditions, and those of Bloch-Qazi et al. (1998) were conducted with dark periods separating photoperiods, which would have reduced pheromone collection relative to results from the current study.

The pheromone production site in *T. castaneum* was originally believed to be associated with setiferous glands on the ventral side of the prothoracic femurs (Faustini et al., 1981).

However, the study by Bloch-Qazi et al. (1998) clearly demonstrated that these patches and their associated glands were not the predominant sources of DMD biosynthesis or deposition in *T. castaneum*. Our findings show more conclusively that male pheromones are produced and/or deposited predominantly in the abdominal epidermis, and possibly to a lesser extent in the thorax. The results are consistent with the report by Olsson et al. (2006) for *T. confusum*, who found that females were attracted not only to extracts of male legs but also to whole body extracts and extracts of male bodies without legs.

Chiral specificity in pheromones or allelochemicals as single isomers or in precise ratios is essential for chemical communication in numerous insect species (Silverstein 1979, Mori 2007). The two asymmetric carbons of DMD potentially provide for four different stereoisomers, and it has been hypothesized that the isomeric composition may differ among species of *Tribolium* (Suzuki et al., 1984, 1987; Arnaud et al., 2002, Verheggen et al., 2007). Enantiomers typically are identical in various physical properties except the direction of optical rotation, such that they can not be separated by conventional and achiral chromatographic methods. However, the method of Akasaka et al. (2011) provided a means of separating all four stereoisomers of 4,8-DMD.

This is the first report of the natural composition of stereoisomers of DMD produced by male *T. castaneum*. Insects that use chiral pheromones typically produce and respond to either a single stereoisomer or to a species-specific blend of only some of the possible stereoisomers. Seldom does an insect produce and employ all possible stereoisomers of a pheromone molecule, particularly for compounds with more than a single chiral carbon. In such cases, alternative stereoisomers are more often either not produced or are inactive (Mori

2007). Here we found that feeding *T. castaneum* males produce all four stereoisomers of DMD at a specific ratio of approximately 4:4:1:1, and that this blend has greater activity as an attractant than either racemic DMD or the commercial blend. Although (4*R*,8*R*)- + (4*R*,8*S*)-blends of stereoisomers used for commercial pheromone lures in traps have high biological activity, as does the pure (4*R*,8*R*)-stereoisomer alone (unpublished data), the natural blend of four stereoisomers reported here proved to be more active than any of the other blends tested, which is a relatively rare situation in insects (reviewed in Mori 1997 and Mori 2007). Despite the natural production of all four stereoisomers by male beetles in our study it is possible that all four stereoisomers are not absolutely required for response, which future work will need to address. We note also that the total amount of DMD eliciting the positive responses in our walking bioassays was very small, at 0.1 ng applied to filter paper, or approximately 1/10,000th the amount produced by a single male in one day, which points to the extremely high level of biological activity DMD has for orientation of these beetles. Further, since the summed amount of all DMD isomers tested in any bioassay replicate was 0.1 ng applied to the paper, the amount of the most active (4*R*,8*R*)-isomer differed by a considerable 4-fold amount among the blends tested. Thus the relative effects of dose versus blend ratio could not be unambiguously separated in this experiment. Additional details of the effects of individual stereoisomers of DMD, and more concentrations and combinations of them, will be reported separately.

The commonly used lure for commercially available traps to monitor pest populations of *Tribolium* beetles is a synthetic mixture of the (4*R*,8*R*)- and (4*R*,8S)-isomers at a 1:1 ratio, but the current work indicates that, at least in the case of the red flour beetle, the optimal ratio

for use in traps may be that which mimics the naturally produced ratio for *T. castaneum.* The racemic mixture of all four stereoisomers at 1:1:1:1, presumably the lowest cost to produce, was found to be similar in activity to the 1:1 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-isomers and may be adequate for commercial use. Efficacy of the chiral derivatization and HPLC separation developed by Akasaka et al. (2011) will now allow for resolution of DMD stereoisomer ratios in additional populations of *T. castaneum* and in other species of *Tribolium* beetles. Future research on synthesis of DMD stereoisomers may lead to cost-effective production of improved pheromone lures for several species of pest *Tribolium*. Information from pheromone traps is increasingly critical for decision-making in integrated pest management of stored durable food products, as consumers and government regulatory agencies call for reduced use of chemical insecticides, adoption of biologically based pest management methods, and provision of safe, high-quality foods (Phillips and Throne 2010).

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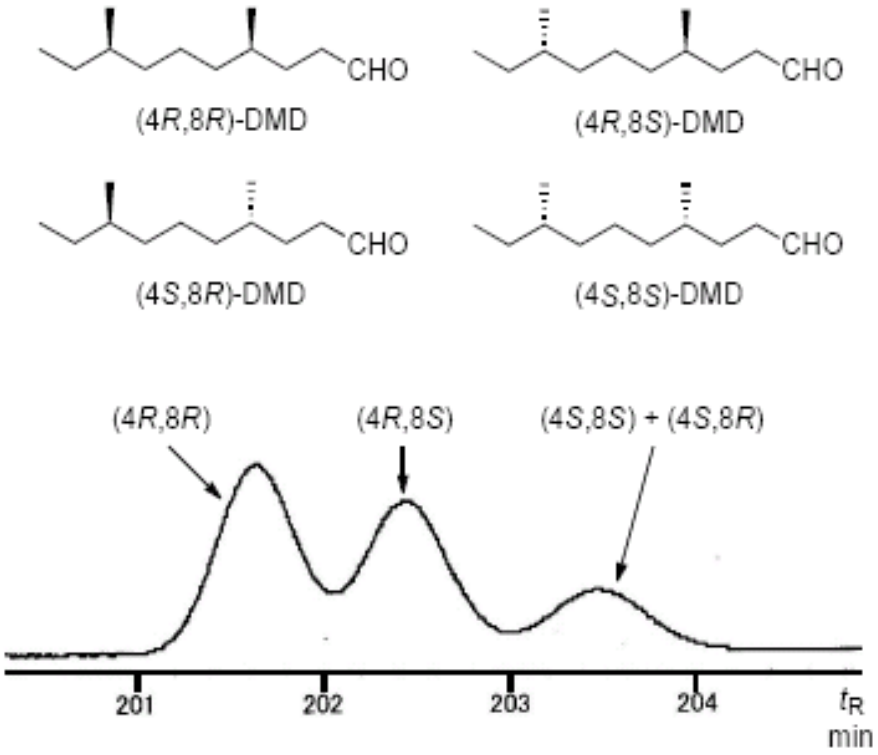

Fig.1 Separation of naturally collected aggregation pheromone stereoisomers on a cyclodextrin-based chiral stationary phase column (50% MOMTBDMSGCD) via GC-flame ionization detection of volatiles from Porapak-Q aerations of feeding males. The *RR* and *RS* stereoisomers resolve but the *SS* and *SR* stereoisomers co-elute as one broad peak.

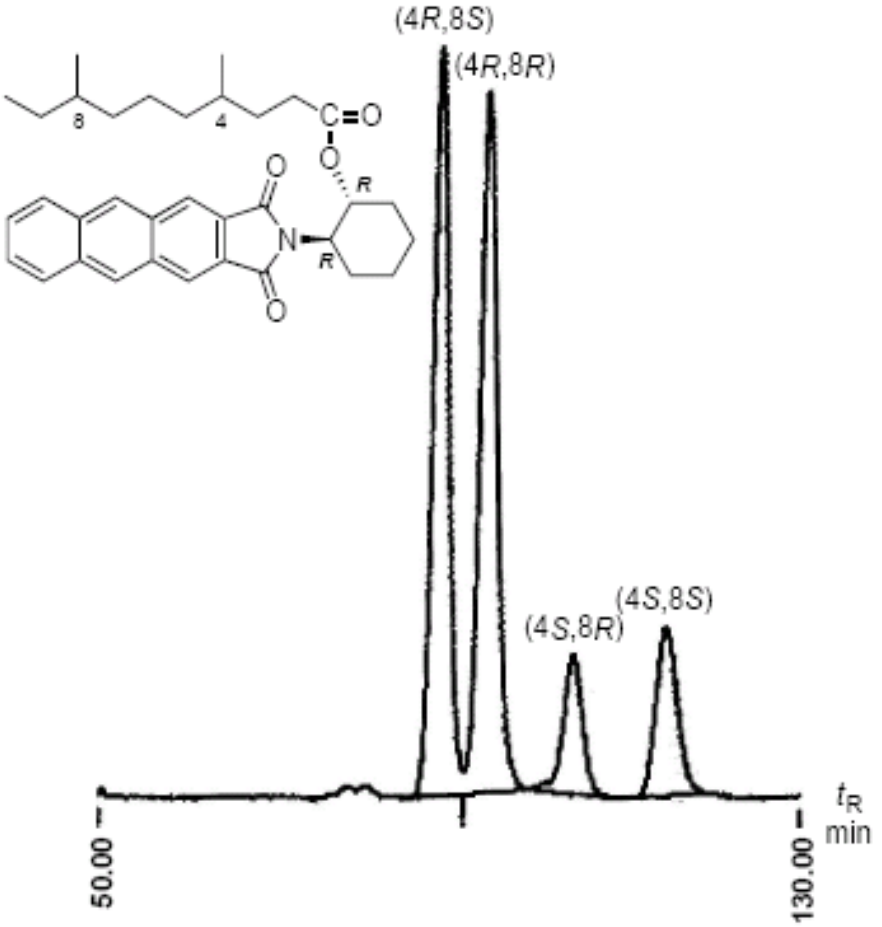

Fig. 2 HPLC separation at –54°C of the aggregation pheromone derivatives (structures shown) prepared from the naturally occurring pheromone from Porapak-Q aerations of feeding males. See Akasaka et al. (2011) for details. All four isomers were resolved. (4*R*,8*S*)-:(4*R*,8*R*)-:(4*S*,8*R*)-:(4*S*,8*S*)- isomers = 4:4:1:1 (peak area).

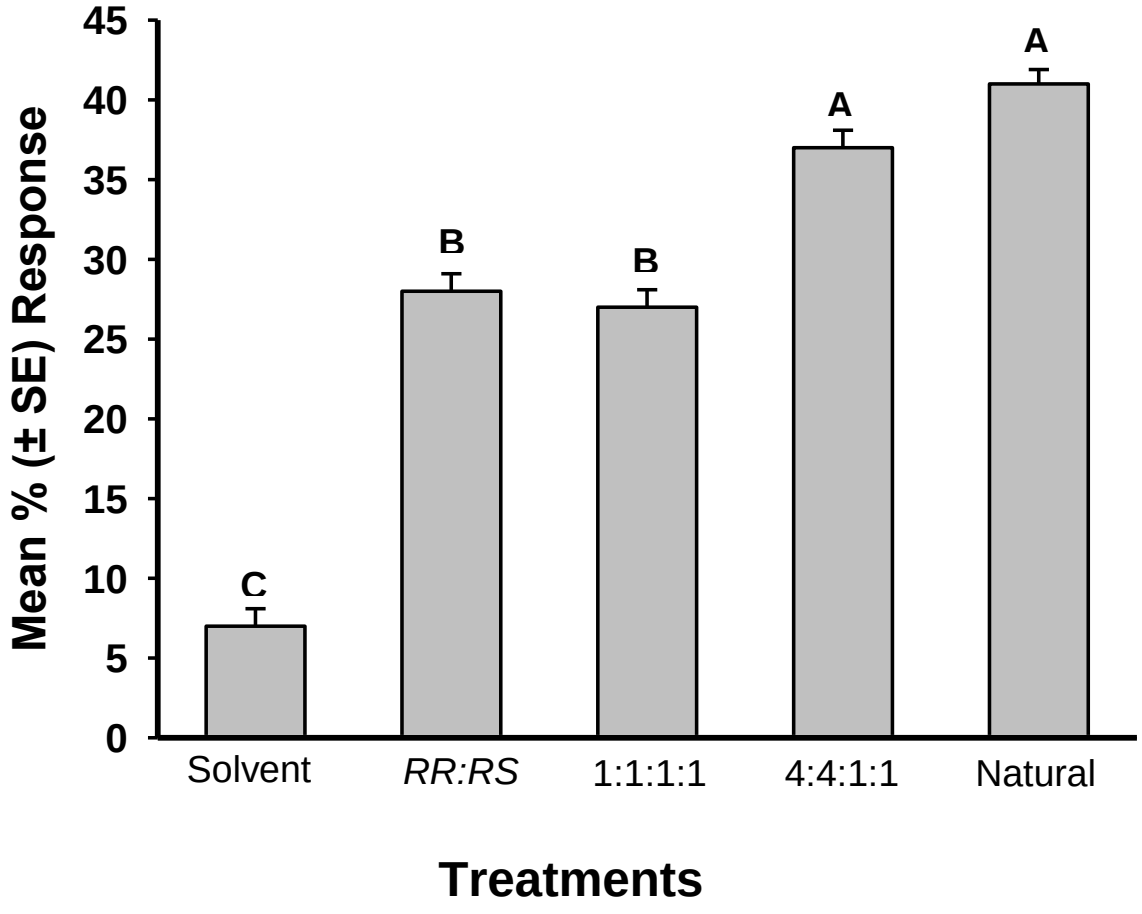

Fig. 3 Mean upwind walking responses (and SEs) of mixed-sex *T. castaneum* adults to different 4,8-DMD treatments in a wind tunnel bioassay. Solvent Control was 20µL hexane only, *RR*:*RS*=1:1 was a 1:1 ratio of (4*R*,8*R*)-DMD:(4*R*,8*S*)-DMD; 1:1:1:1 was an equal mixture of (4*R*,8*R*)-DMD:(4*R*,8*S*)-DMD:(4*S*,8*R*)-DMD:(4*S*,8*S*)-DMD; 4:4:1:1 was a mixture of (4*R*,8*R*)-DMD:(4*R*,8*S*)-DMD:(4*S*,8*R*)-DMD:(4*S*,8*S*)-DMD that mimics the naturally produced blend of pheromone stereoisomers; Natural Pheromone was the Porapak-Q hexane eluate collected from multiple groups of 5 feeding males with cracked wheat and wheat flour. Total amount of DMD in any given treatment was 0.1ng on filter paper. Means with different letters are significantly different; ANOVA ($F_{4,20}$=118.57, P< 0.001) followed by means comparison with the SNK test (P<0.01, n=5).