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Anatomical localization and stereoisomeric composition of *Tribolium castaneum* aggregation pheromones.

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### How to cite this manuscript

If you make reference to this version of the manuscript, use the following information:

Lu, Y., Beeman, R.W., Campbell, J.F., Park, Y., Aikins, M.J., Mori, K., ...Phillips, T.W. (2011). Anatomical localization and stereoisomeric composition of *Tribolium castaneum* aggregation pheromones. Retrieved from <http://krex.ksu.edu>

### Published Version Information

**Citation:** Lu, Y., Beeman, R.W., Campbell, J.F., Park, Y., Aikins, M.J., Mori, K., ...Phillips, T.W. (2011). Anatomical localization and stereoisomeric composition of *Tribolium castaneum* aggregation pheromones. *Naturwissenschaften*, 98(9), 755-761.

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**Digital Object Identifier (DOI):** doi: 10.1007/s00114-011-0824-x

**Publisher's Link:** <http://www.springerlink.com/content/0028-1042/98/9/>

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3 ORIGINAL ARTICLE

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5 **Anatomical Localization and Stereoisomeric Composition of**

6 ***Tribolium castaneum* Aggregation Pheromones**

7

8

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26

27

28 **Abstract** We report that the abdomen and associated tissues are the predominant sources of  
29 male-produced pheromones in the red flour beetle, *Tribolium castaneum*, and for the first  
30 time describe the stereoisomeric composition of the natural blend of isomers of the  
31 aggregation pheromone 4,8-dimethyldecanal (DMD) in this important pest species.  
32 Quantitative analyses via GC-MS showed that the average amount of DMD released daily by  
33 single feeding males of *T. castaneum* was  $878 \pm 72$  ng (SE). Analysis of different body parts  
34 found the abdominal epidermis as the major source of aggregation pheromone; the thorax  
35 was a minor source, while no DMD was detectable in the head. No internal organs or obvious  
36 male-specific glands were associated with pheromone deposition. Complete separation of all  
37 four stereoisomers of DMD was achieved following oxidation to the corresponding acid,  
38 derivatization with (1*R*, 2*R*)- and (1*S*, 2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanol to  
39 diastereomeric esters, and their separation on reversed phase HPLC at  $-54^{\circ}\text{C}$ . Analysis of the  
40 hexane eluate from Porapak-Q-collected volatiles from feeding males revealed the presence  
41 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  
42 walking orientation bioassay in a wind tunnel with various blends of the four synthetic  
43 isomers further indicated that the attractive potency of the reconstituted natural blend of  
44 4:4:1:1 was equivalent to that of the natural pheromone, and greater than that of the 1:1 blend  
45 of (4*R*,8*R*):(4*R*,8*S*) used in commercial lures.

46

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

49

50 **Introduction**

51 The red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), is a major  
52 cosmopolitan pest of stored cereal grains, beans, nuts and other durable agricultural products  
53 worldwide (Weston and Rattlingourd 2000; Campbell et al. 2010). The feeding adult male  
54 secretes an aggregation pheromone, which is attractive to both sexes. This pheromone was  
55 identified as 4,8-dimethyldecanal (DMD), which has two asymmetric carbons at C-4 and C-8  
56 (Suzuki, 1980; Suzuki et al., 1984). Suzuki (1980) synthesized a mixture of all four  
57 stereoisomers of DMD and found it to be less active than the natural pheromone (Suzuki,  
58 1981; Suzuki et al., 1987). Based on bioassay of the four synthetic stereoisomers of DMD  
59 against *T. castaneum*, Suzuki and Mori (1983) inferred that the natural pheromone was the  
60 (4*R*,8*R*)-stereoisomer because it appeared to be as active as the natural pheromone and  
61 Levinson and Mori (1983) supported this claim after documenting activity to each of the four  
62 synthetic stereoisomers. Subsequently, a 4:1 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-DMD was  
63 found to be ten times more active than (4*R*,8*R*)-DMD alone, although (4*R*,8*S*)-DMD itself  
64 was inactive at lower doses (Suzuki et al, 1984). However, the absolute configuration of  
65 4,8-DMD produced by male *T. castaneum* was not further investigated, and remained  
66 unknown until the present.

67

68 The chemical synthesis of the four stereoisomers of DMD at high enantiomeric purity and a  
69 derivatization and HPLC separation method that gave baseline resolution of all four  
70 stereoisomers were recently described (Akasaka et al. 2011). The primary objective of the  
71 research reported here was to determine the stereoisomeric composition of naturally  
72 produced DMD from male beetles and to compare the attraction of natural and artificial  
73 blends to that of the synthetic pheromone blend used in commercial lures [a 1:1 mixture of  
74 (4*R*,8*R*)- and (4*R*,8*S*)-DMD]. Although male-specific production of DMD has been  
75 demonstrated, there has been some confusion regarding the location of tissues or glands in  
76 males that might produce and/or release the pheromone. The male-specific exocrine glands  
77 beneath the setiferous patches on the ventral side of the profemora were considered the site  
78 of production by Faustini et al. (1981), but Bloch-Qazi et al. (1998) demonstrated that males

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

85

## 86 **Materials and methods**

87 *Insect cultures.* *Tribolium castaneum* from the GA-1 strain (Haliscak and Beeman 1983),  
88 which originated nearly 30 years ago from a field site in the state of Georgia, USA, was used  
89 for pheromone collection and the KS-1 strain (Romero et al. 2009), recently collected from a  
90 commercial flour mill in Kansas, USA, was used for the wind tunnel walking bioassay.

91 Beetles were reared on a mixture of whole-wheat flour and brewer's yeast (95:5) at 27°C and  
92 60% RH and a 16:8 (light-dark) photoperiod. To obtain males for pheromone collection or  
93 extraction, pupae were collected, segregated by sex, and maintained separately on flour until  
94 needed for experiments.

95

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

103 28°C and under 24 hr of constant light, to elicit maximum pheromone production and release  
104 (Hussain 1993), provided by a 40 W incandescent light bulb. The incoming air flow rate was  
105 200 ml/min and the air was humidified by passing through a flask of distilled water. Volatiles  
106 were collected on small glass columns packed with Porapak-Q (Alltech Assoc., Deerfield,  
107 IL); columns were changed daily for four days. Columns were each eluted with  
108 approximately 500 µl of HPLC-grade hexane and the eluate spiked with 555 ng of  
109 *n*-dodecane in 5 µl of hexane (111 ng/µl) as an internal standard. Extracts were stored in 1.5  
110 ml glass vials with Teflon-lined septum caps at -80°C pending analysis.

111

112 *Analyses of tissues containing pheromone.* Male beetles used for tissue extracts were 7-14  
113 days old. The virgin males were placed individually into glass vials with 0.5 g cracked wheat  
114 mixed with wheat flour for feeding until analyzed. Dissection protocols were modified from  
115 Olsson et al. (2006). Briefly, the beetles were sedated with CO<sub>2</sub> and then were mounted  
116 ventral-side-up onto double-stick cellophane tape affixed to the bottom of a glass Petri dish.  
117 Head, thorax and abdomen were separated from the body with forceps and a group of 5 of  
118 each body part was extracted in 1 ml HPLC-grade hexane for 30 min. Five such groups of  
119 each body section were separately extracted and an internal standard of 555 ng of *n*-dodecane  
120 was added directly to each extract before further processing. Extracts were concentrated to  
121 approximately 500 µl under a gentle stream of N<sub>2</sub> at room temperature. After we confirmed  
122 that abdomens were the primary source of DMD, the abdomens of additional males (five  
123 groups of five abdomens each) were further dissected into two parts; the cuticle with any  
124 adhering tissues, and all other tissues, which included the digestive system (including the

125 hindgut, posterior midgut and Malpighian tubules), reproductive system and fat body. The  
126 two abdominal tissue groups were extracted as described above for the three body regions.  
127 All extracts were stored at -20°C for no longer than 48 h before chemical analyses.  
128  
129 *Chemical analyses.* Samples from all aeration and tissue extraction experiments were  
130 subjected to quantitative analysis by coupled gas chromatography-mass spectrometry  
131 (GC-MS) with electron impact ionization (EI, 70eV) using a Shimadzu GC-MS QP5050A  
132 (Kyoto, Japan) equipped with a J&W Scientific DB-1 capillary column (30 m × 0.25 mm ×  
133 0.25 μm) in splitless mode, with helium as carrier gas. The injector oven was set at 250°C  
134 and the heated transfer line to the MS was set at 250°C. Oven temperature was programmed  
135 at 40°C for 0.5 min, then increased 10°C/min to 200°C and was held for 1 min, then  
136 increased to 240°C at 20°C/min, and held at 240°C for 1 min. Initial studies were conducted  
137 with the MS in the full scan mode, recording mass fragments from 35 to 350 amu. A 1:1  
138 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-DMD was analyzed for retention time and mass spectrum,  
139 which matched the spectrum published by Suzuki (1981). In order to maximize detection  
140 sensitivity for DMD in the experiments described above, the MS was subsequently operated  
141 in the multiple ion detection mode (MID) in which only the characteristic fragment ions  $m/z$   
142 = 41 and  $m/z$  = 57 were detected. These fragment ions are common to both the internal  
143 standard dodecane, and DMD. The quantity of DMD in each sample was determined by  
144 comparison of the peak area of the internal standard, representing 555 ng in the initial  
145 solution, and that of DMD from the MID chromatogram. Accuracy of our internal standard  
146 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<sub>4</sub> were added. The mixture was stirred at room temperature for 1 h. After  
157 oxidation, 0.5 ml of 10% NaHSO<sub>3</sub> 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<sub>2</sub>SO<sub>4</sub>, 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.



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

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

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

194 All four stereoisomers of 4,8-DMD were synthesized as previously described (Akasaka  
195 et al. 2011); chemical purities: (4*R*,8*R*)-isomer at 92.2%, (4*R*,8*S*)-isomer at 95.3%,  
196 (4*S*,8*R*)-isomer at 91.7% and (4*S*,8*S*)-isomer at 94.4%; stereoisomeric purities of all four  
197 stereoisomers were 97% ee at C-4 and over 99% ee at C-8. We compared the behavioral  
198 responses of beetles to the following treatments in bioassays: 0.1 ng, a very small yet  
199 biologically relevant amount determined to give an acceptable positive response from among  
200 a arrange of concentrations (unpublished data), of a 1:1 blend of synthetic stereoisomers  
201 (4*R*,8*R*)- and (4*R*,8*S*)-DMD, which mimics the blend used in commercial pheromone lures; a  
202 1:1:1:1 blend of all four synthetic stereoisomers (4*R*,8*R*)-, (4*R*,8*S*)-, (4*S*,8*R*)-, and  
203 (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  
204 (4*S*,8*S*)-DMD, which mimics the natural blend of stereoisomers produced by male beetles  
205 (reported below); naturally collected DMD, which included other potential semiochemicals  
206 in the complete eluate collected on Porapak-Q during aeration of feeding males; and a  
207 solvent control. The percentage of beetles in a given test group contacting the odor source  
208 within two minutes (i.e., percent of positive response out of 20 beetles) was subjected to  
209 arcsine square root transformation to normalize distribution and then analyzed using ANOVA.  
210 Differences among means were determined using the Student-Newman-Keuls (SNK) Test  
211 with SAS software (SAS Institute, 2001).

212

213 **Results**

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

225 Enantioselective GC analysis on a chiral stationary phase column (details in Akasaka et  
226 al. 2011) enabled good separation of (4*R*,8*R*)-DMD and (4*R*,8*S*)-DMD, but did not resolve  
227 the (4*S*,8*R*)-DMD from the (4*S*,8*S*)-DMD stereoisomers, which co-eluted as one peak (Fig.  
228 1). Complete separation of the four stereoisomers of 4,8-DMD was achieved following  
229 oxidation with  $\text{KMnO}_4$  to the corresponding acid, its derivatization with (1*R*,2*R*)- and  
230 (1*S*,2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanol to diastereomeric esters, their  
231 analysis on the reversed-phase HPLC column immersed in a cooling bath at  $-54^\circ\text{C}$  (Fig. 2),  
232 and the absolute configuration of naturally produced DMD was revealed. The natural ratio of  
233 the four stereoisomers of 4,8-DMD was approximately 4:4:1:1  
234 [(4*R*,8*R*):(4*R*,8*S*):(4*S*,8*R*):(4*S*,8*S*)] , measured by calculating the area under each stereoisomer

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

237 The behavioral responses of *T. castaneum* to the four synthetic stereoisomers of  
238 4,8-DMD at the naturally produced ratio ( $[(4R,8R)-(4R,8S)-(4S,8R)-DMD = 4 : 4 : 1 : 1]$ )  
239 and the natural blend of pheromones and other semiochemicals collected from virgin feeding  
240 males were similar and significantly greater than the response to the (4*R*,8*R*)-DMD :  
241 (4*R*,8*S*)-DMD = 1 : 1 (as used in commercial pheromone lures) or the (4*R*,8*R*)- : (4*R*,8*S*)-:  
242 (4*S*,8*R*)- : (4*S*,8*S*)-DMD =1:1:1:1 ratio treatment (Fig. 3). All treatments with stereoisomers  
243 of DMD elicited significantly higher responses than the solvent control in the walking  
244 bioassay (Fig. 3). The results confirmed that a synthetic blend of DMD stereoisomers in the  
245 same relative ratio as the natural aggregation pheromones is more attractive to *T. castaneum*  
246 males and females than the ratio of stereoisomers currently used in commercial pheromone  
247 lures.

## 248 249 **Discussion**

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

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

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

265 Chiral specificity in pheromones or allelochemicals as single isomers or in precise ratios  
266 is essential for chemical communication in numerous insect species (Silverstein 1979, Mori  
267 2007). The two asymmetric carbons of DMD potentially provide for four different  
268 stereoisomers, and it has been hypothesized that the isomeric composition may differ among  
269 species of *Tribolium* (Suzuki et al., 1984, 1987; Arnaud et al., 2002, Verheggen et al., 2007).  
270 Enantiomers typically are identical in various physical properties except the direction of  
271 optical rotation, such that they can not be separated by conventional and achiral  
272 chromatographic methods. However, the method of Akasaka et al. (2011) provided a means  
273 of separating all four stereoisomers of 4,8-DMD.

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

280 2007). Here we found that feeding *T. castaneum* males produce all four stereoisomers of  
281 DMD at a specific ratio of approximately 4:4:1:1, and that this blend has greater activity as  
282 an attractant than either racemic DMD or the commercial blend. Although (4*R*,8*R*)- +  
283 (4*R*,8*S*)-blends of stereoisomers used for commercial pheromone lures in traps have high  
284 biological activity, as does the pure (4*R*,8*R*)-stereoisomer alone (unpublished data), the  
285 natural blend of four stereoisomers reported here proved to be more active than any of the  
286 other blends tested, which is a relatively rare situation in insects (reviewed in Mori 1997 and  
287 Mori 2007). Despite the natural production of all four stereoisomers by male beetles in our  
288 study it is possible that all four stereoisomers are not absolutely required for response, which  
289 future work will need to address. We note also that the total amount of DMD eliciting the  
290 positive responses in our walking bioassays was very small, at 0.1 ng applied to filter paper,  
291 or approximately 1/10,000<sup>th</sup> the amount produced by a single male in one day, which points  
292 to the extremely high level of biological activity DMD has for orientation of these beetles.  
293 Further, since the summed amount of all DMD isomers tested in any bioassay replicate was  
294 0.1 ng applied to the paper, the amount of the most active (4*R*,8*R*)-isomer differed by a  
295 considerable 4-fold amount among the blends tested. Thus the relative effects of dose versus  
296 blend ratio could not be unambiguously separated in this experiment. Additional details of  
297 the effects of individual stereoisomers of DMD, and more concentrations and combinations  
298 of them, will be reported separately.

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

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

314

315 **Acknowledgements** The authors thank Alain VanRyckeghem, Insects Limited, Westfield, IN  
316 for providing an authentic standard of DMD as a GC standard, and David Hagstrum, Lee  
317 Cohnstaedt and Jim Throne for constructive reviews. Partial funding for this research came  
318 from the Kansas Agricultural Experiment Station and the USDA Risk Avoidance and  
319 Mitigation Program. Mention of trade names or commercial products in this publication is  
320 solely for the purpose of providing specific information and does not imply recommendation  
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323

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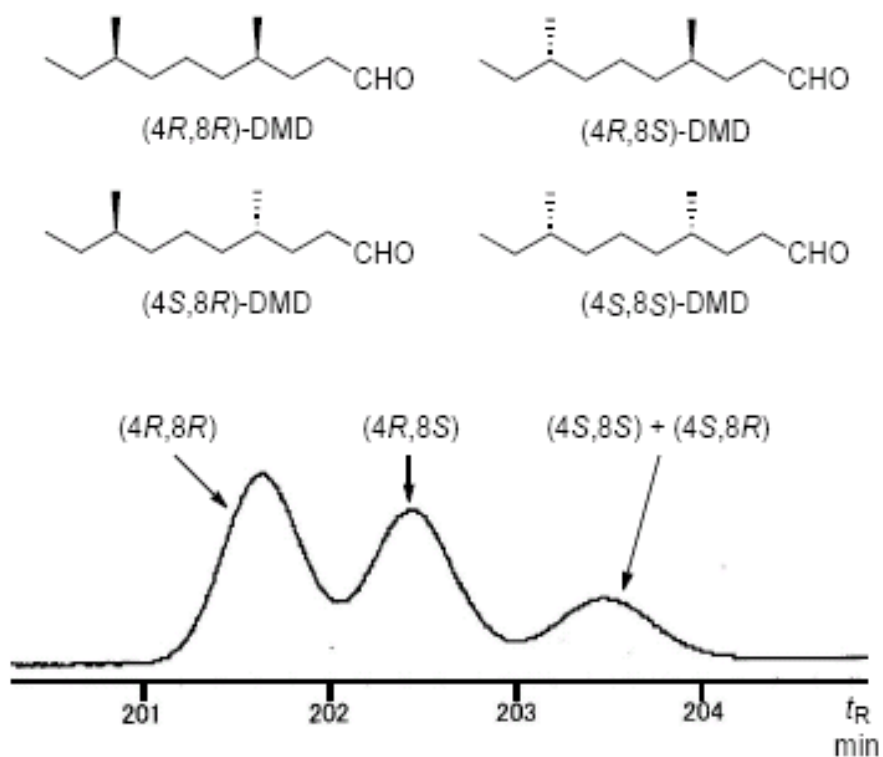
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Fig.1 Separation of naturally collected aggregation pheromone stereoisomers on a

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cyclodextrin-based chiral stationary phase column (50% MOMTBDMSGCD) via GC-flame

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ionization detection of volatiles from Porapak-Q aerations of feeding males. The *RR* and *RS*

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stereoisomers resolve but the *SS* and *SR* stereoisomers co-elute as one broad peak.

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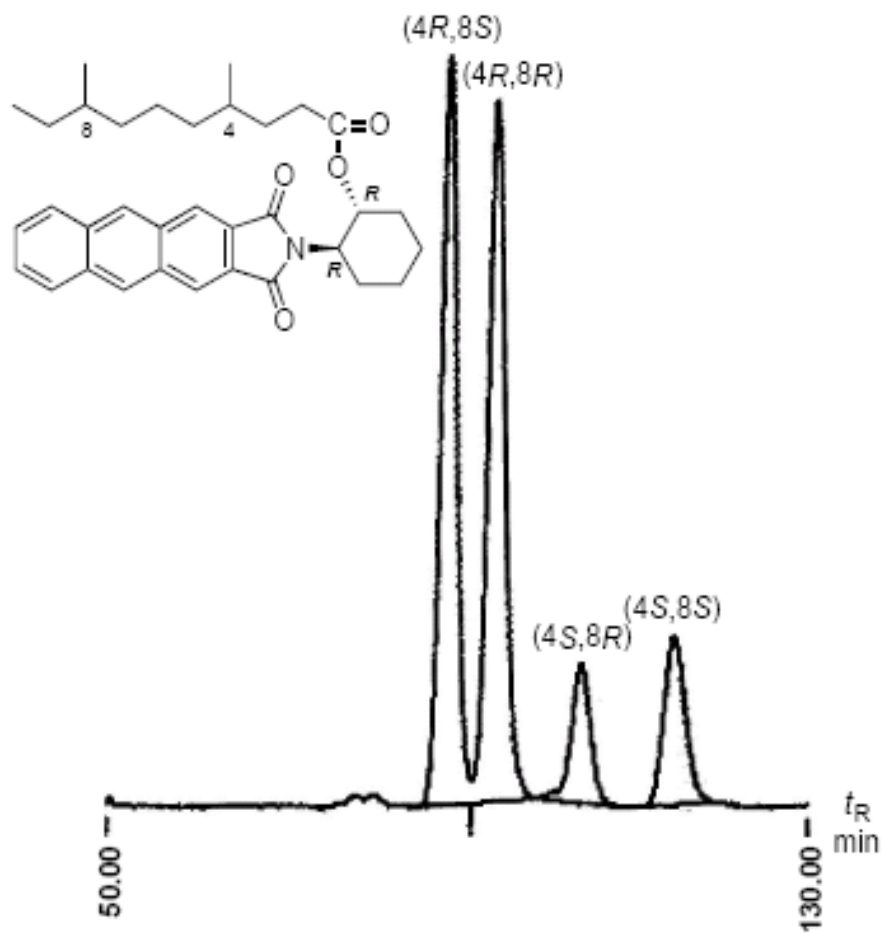
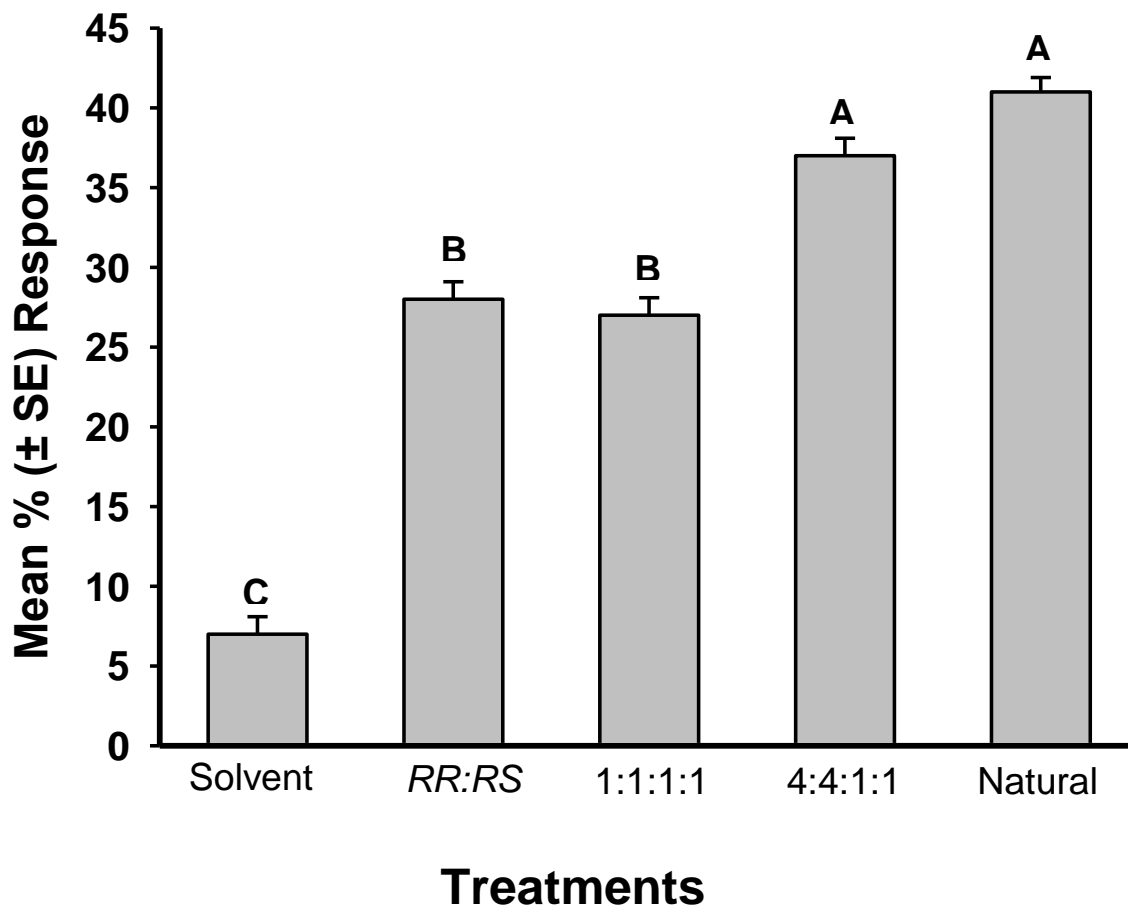


Fig. 2 HPLC separation at  $-54^{\circ}\text{C}$  of the aggregation pheromone derivatives (structures shown)

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).



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438 Fig. 3 Mean upwind walking responses (and SEs) of mixed-sex *T. castaneum* adults to  
 439 different 4,8-DMD treatments in a wind tunnel bioassay. Solvent Control was 20µL hexane  
 440 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  
 441 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  
 442 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  
 443 produced blend of pheromone stereoisomers; Natural Pheromone was the Porapak-Q hexane  
 444 eluate collected from multiple groups of 5 feeding males with cracked wheat and wheat flour.  
 445 Total amount of DMD in any given treatment was 0.1ng on filter paper. Means with different  
 446 letters are significantly different; ANOVA ( $F_{4,20}=118.57$ ,  $P < 0.001$ ) followed by means  
 447 comparison with the SNK test ( $P < 0.01$ ,  $n=5$ ).