Pheromone Production by the Boll Weevil (Coleoptera: Curculionidae) Fed Cotton Squares and Bolls[1]

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J. Entomol. Sci. 44(3): 209-221 (July 2009)

Abstract The boll weevil, *Anthonomus grandis* Boheman, produces pheromone on a variety of diets, but access to flower buds (squares) or small fruit (bolls) of cotton, *Gossypium hirsutum* L., is thought necessary for high levels of pheromone production. However, estimates of the pheromone emitted by weevils fed bolls are not available. We used headspace collections to determine (1) whether male weevils already emitting pheromone could sustain production on bolls, and (2) whether pheromone emission could be initiated on a boll diet. Male weevils switched to a diet of small (12 - 15 mm diam) or medium (20 - 23 mm diam) sized bolls after feeding on squares (5 - 7 mm diam) for 7 d maintained pheromone releases at levels ≥ that of weevils remaining on squares through the 13th day of adulthood. Pheromone composition did not vary substantially among the diets. When the diets were provided beginning at adult eclosion, weevils initiated pheromone emission similarly on all diets, but weevils fed small bolls released the most pheromone by day 9 of adulthood. No difference in pheromone composition was observed among the diet treatments. In addition, weevils that entered diapause by the end of the experiments produced only small amounts of pheromone. The high levels of pheromone production by weevils fed bolls may be ecologically important in ensuring that potential overwintering females are mated before emigrating from maturing cotton. Our findings also suggest that diminished competition between naturally-produced pheromone and traps is not an adequate explanation for commonly observed increases in late-season captures by pheromone traps.

Key Words boll weevil, *Anthonomus grandis*, pheromone, diet

Males of the boll weevil, *Anthonomus grandis* Boheman, produce an aggregation pheromone that is attractive to both sexes (Bradley et al. 1968, Hardee et al. 1970). Tumlinson et al. (1969) characterized this pheromone, which consists of two terpene alcohols (components I and II; (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol and *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol, respectively) and two terpene aldehydes (components III and IV; *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde and *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde, respectively). Because of the economic importance of the boll weevil, its chemical communications have been extensively studied (Hardee 1972, Hardee and Mitchell 1997). Information from these studies has led to the development and implementation of modern trap-based monitoring and detection systems that have become a core component of organized efforts to eradicate the boll weevil.

[1]Received 15 August 2008; accepted for publication 09 November 2008.

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Most studies of pheromone production by the boll weevil have relied on bioassays (Hardee et al. 1967) or the extraction of feces produced by large groups of weevils (Hedin et al. 1974, McGovern et al. 1976, McKibben et al. 1976, Dickens et al. 1988). Based on these methods, the boll weevil may produce pheromone on a variety of diets, but a high level of pheromone production requires access to flower buds (squares) or small bolls (fruit) of cotton, *Gossypium hirsutum* L. (Hardee 1970, Hardee and Mitchell 1997). Furthermore, the boll weevil exhibits a distinct seasonality in its response to pheromone traps, and competition from pheromone-producing weevils is often offered as an explanation for the low response to traps associated with actively fruiting cotton (Hardee et al. 1970, Roach et al. 1971, Rummel et al. 1977, Lopez et al. 1978, Leggett and Moore 1982, Segers et al. 1987). Although weevil response to traps increases late in the cotton production season, when bolls become the predominant food source, no estimates of pheromone production by boll-fed weevils are available from the literature. Such estimates may be useful in better understanding the chemical ecology of the boll weevil.

Recently, Spurgeon (2003) developed methodology to estimate the pheromone production of individual weevils based on headspace collections of emitted pheromone. Key findings of that work included: (1) most pheromone is released into the atmosphere; in comparison, the pheromone content of feces is negligible and of a different composition, and (2) boll weevils produce much larger amounts of pheromone and at earlier ages than was previously recognized. We used these newer methods in two experiments to evaluate the influence of adult diet (squares, two size classes of bolls) on the ability of weevils to initiate and sustain pheromone production.

Materials and Methods

Response to diet switching. Boll feeding has often been associated with the induction of adult diapause (Earle and Newsom 1964, Lloyd et al. 1967, Tingle et al. 1971), and evidence suggests that diapausing weevils produce little pheromone (Villavaso and Earle 1974). Therefore, our initial experiment examined the ability of weevils already producing pheromone to sustain production on a boll diet.

Adult boll weevils were reared from 4 separate collections of oviposition-punctured squares obtained from plants in commercial cotton fields in Hill and Limestone counties of Texas between 10 August and 7 September 2004. Adults of known age were obtained using the exact procedure described by Spurgeon and Suh (2007). Also, during all experiments weevils were held under the same conditions as those used for rearing (29.4 ± 1°C, 13:11 [L:D] h photoperiod initiated at 0700 h) except during bouts of pheromone collection. Male weevils were distinguished as described by Sappington and Spurgeon (2000), and those sufficiently sclerotized to walk were weighed to the nearest 0.1 mg. Because Spurgeon (2001) found no correlation between pheromone production and weevil size for males $\geq$ 10 mg, we used only males weighing $\geq$ 10 mg.

During the 24-h periods of pheromone collection, each collection vessel contained a single weevil, a food item (square, small boll, or medium boll, with bracteoles intact), and a 4-ml glass vial filled with deionized water and closed with a cotton wick. Collection vessels (wide-mouth glass bottles each equipped with 2 adsorbent-packed glass columns) were as described by Spurgeon (2003) and Spurgeon and Suh (2007).

Sixteen collection vessels were used during each period of pheromone collection. The vessels were arranged in 2 rows in a fume hood which was sealed and sheathed with foam insulation. The pheromone collection columns of each of the 8 vessels in

a row were attached to a manifold connected to a diaphragm vacuum pump set at about 68 kPa. Airflow through each vessel was regulated at about 1 L/min by individual flow meters. The interior of the fume hood was maintained at 27 ± 2°C by a thermostatically controlled electric heater, and a photoperiod of 13:11 (L:D) h initiated at 0700 h was provided by two 40-W fluorescent bulbs controlled by an electric timer.

Because Spurgeon and Suh (2007) reported a diel pattern of pheromone emission that was influenced by the time of day during which weevils were fed, each 24-h pheromone collection period was initiated before 1000 h. At the end of each collection period pheromone was eluted from each collection column directly into GC sample vials with methylene chloride (GC grade). Final eluant volume of each sample was 1.0 ml. Samples were mixed by agitation before analysis on a Shimadzu 17A GC equipped with an AOC-20i autoinjector (Shimadzu Scientific Instruments, Columbia, MD), dual DB-5 columns (60 m × 0.32 mm i.d., J&W Scientific, Folsum, CA), and flame-ionization detectors. A duplicate 2-μL injection of each sample was analyzed on each column. The GC operating conditions, temperature program, and program run time were the same as those reported by Spurgeon and Suh (2007). Pheromone content of each sample was calculated based on an 8-point calibration curve and using the GC Real Time Analysis software (LabSolutions GCsolution Analysis, ver. 2.10, Shimadzu, Kyoto, Japan) with known concentrations of Grandlure (ISP Fine Chemicals, Columbus, OH) as external standards. Estimates from duplicate injections were averaged before statistical analysis, and total pheromone was calculated as the sum of the four components. Pheromone content of the feces was not estimated because it represents ≤5% of the total (Spurgeon 2003).

In each repetition of the experiment, 22 - 25 males which eclosed on the same day were held individually in Petri dishes (100 × 15 mm) with a fresh square (5-7 mm diam) with intact bracteoles and a section (~1 cm) of cotton wick saturated with deionized water. Squares were replaced daily before 1000 h. This procedure was intended to promote a high incidence of pheromone production and release. On day 7 of adulthood (when weevils were 6-d-old) 16 weevils were randomly selected and pheromone emitted in response to square feeding was measured to identify individuals that were not fully stimulated to produce pheromone. Also at that time, weevils were randomly assigned to diet treatments. Because the number of available collection vessels could not be fully used if treatments were equally replicated, and knowledge of pheromone production on squares was already available, sample sizes were higher for treatments that involved diet switching. Diet treatments and sample sizes were: (1) continue feeding on squares (4 weevils), (2) switched to small bolls (12 - 15 mm diam; 6 weevils), or (3) switched to medium bolls (20 - 23 mm diam; 6 weevils).

On day 8 of adulthood (when weevils were 7-d-old), diet treatments were implemented and pheromone was collected again. Additional collections of pheromone were obtained on the 10th and 13th days of adulthood. Between bouts of pheromone collection, weevils were provided their assigned diets and were held within an environmental chamber as previously described, except that weevils switched to bolls were contained in 100 × 25-mm Petri dishes. At the conclusion of the final bout of pheromone collection, weevils were dissected to determine their physiological status (reproductive, diapause) using the criteria of Spurgeon et al. (2003). The experiment was repeated 4 times, with repetitions considered as blocks.

Response to diet. Given that the initial experiment demonstrated the ability of weevils to at least temporarily sustain high levels of pheromone emission on a boll

diet, we examined the influence of diet on the initiation of pheromone release. Four distinct collections of infested squares were obtained from commercial cotton fields in Cameron Co., TX, between 2 June and 16 July 2005. Procedures for obtaining and handling experimental insects, and for estimating emitted pheromone, were exactly as described for the first experiment.

In each repetition of the experiment, 22 - 25 males which eclosed on the same day were randomly assigned to diet treatments of squares (5 - 7 mm diam), small bolls (12 - 15 mm diam), or medium bolls (20-3 mm diam). Diet treatments were implemented on the day of eclosion, and weevils were held individually in Petri dishes as previously described. On day 3 of adulthood, 6 weevils assigned to medium bolls, and 5 weevils from each the square and small boll diets, were randomly selected and assigned to pheromone collection vessels. Pheromone was collected on the 3rd, 6th, and 9th days of adulthood. Weevils that were not initially selected for pheromone collection were maintained on the assigned diets so they could be used as replacements for selected weevils that died before the 9th day of adulthood. Between bouts of pheromone collection weevils were maintained in assigned Petri dishes as in the earlier experiment. After the final day of pheromone collection weevils were dissected to determine physiological status. The experiment was repeated 4 times, with repetitions considered as blocks.

Statistical analyses. Of primary interest in the first experiment was whether weevils already emitting pheromone could sustain releases following the change in diet. Based on the results of Spurgeon (2003), most weevils provided squares produce all 4 pheromone components before the 6th day of adulthood. Therefore, in this study, weevils that did not produce all 4 pheromone components on the 7th day of adulthood were considered to represent a population other than that of normal pheromone-producing weevils and were excluded from analysis. Estimates of total pheromone emitted during each 24-h period were analyzed by repeated-measures mixed-model analysis of covariance using the SAS procedure PROC MIXED (SAS Institute 2003). The response variable was total pheromone collected. Fixed effects included diet treatment, weevil age, and their interaction. Pheromone collected on the initial day of measurement (day 7) was used as a covariate to adjust for differences existing among groups of weevils before diet treatments were implemented. Repetition of the experiment was used as a random effect. Based on preliminary analyses, a first-order heterogeneous autoregressive covariance structure [TYPE=ARH(1) option in the REPEATED statement] was used to model within-weevil correlations among the ages of pheromone estimation. Because the diet treatment × age interaction was significant, simple effects of diet treatment within weevil ages, and weevil age within diet treatments, were examined using the SLICE option of the LSMEANS statement of PROC MIXED (SAS Institute 2003). Where slices of simple effects were significant, corresponding least-squares means were compared using single-degree-of-freedom contrasts.

Effects of diet treatment and weevil age on pheromone composition were examined by multivariate analysis using the MANOVA statement of PROC GLM (SAS Institute 2003). For this analysis, pheromone composition was expressed as the respective proportions of total pheromone represented by each of the 4 components. Data for some weevils was missing for one or more ages because of death before completion of the experiment, or because no pheromone was emitted on a given day (thus precluding calculation of proportions of components). Therefore, a greater number of weevils were excluded from the analyses of pheromone composition than from

corresponding analyses of total pheromone production because the MANOVA procedure cannot accommodate missing data. Before analysis, data for each pheromone component were examined for normality and heterogeneity of variance using the Shapiro-Wilk test of PROC UNIVARIATE (SAS Institute 2003) and residual plots, respectively. Because the Shapiro-Wilk test did not indicate significant deviation from normality for most of the data, and arcsin transformation did not improve the condition of the data, the analysis used the untransformed data. The respective proportions represented by the 4 components were response variables. Model effects included experiment repetition, diet treatment, weevil age, and all possible interactions. The repetition × diet treatment × age interaction was used as the error term for testing effects of diet treatment, age, and their interaction. Where the multivariate analysis indicated a significant diet treatment × age interaction, simple effects and corresponding least-squares means were examined as described for the analysis of total pheromone.

In the second experiment in which diet treatments were applied on the day of adult eclosion, failure to produce pheromone during the first measurement period, failure to produce all 4 pheromone components, or entry into diapause, were considered consequences of the diet treatments. Therefore, such weevils were included in the analysis of total pheromone production. However, weevils were excluded if evidence suggested they were abnormal in some manner. Weevils were considered abnormal if they produced no pheromone during the initial measurement period and died soon after, or if they died after not feeding for several days.

Estimates of total pheromone emitted during each 24-h collection period were analyzed by repeated-measures mixed-model ANOVA using the SAS procedure PROC MIXED (SAS Institute 2003). This analysis used the same response variable (total pheromone production), statistical model, and covariance structure as described for the corresponding analysis of the diet switching experiment.

Effects of diet treatment and weevil age on pheromone composition were examined by multivariate analysis using the MANOVA statement of PROC GLM (SAS Institute 2003). Pheromone composition was expressed as the proportion of total pheromone represented by each of the 4 components. Because this analysis cannot accommodate missing data, and pheromone emission was undetectable for numerous weevils during the first pheromone production period, inclusion of all of the data in a single analysis would limit sample size as well as introduce potential biases. Therefore, 2 separate analyses were conducted, one for day 3 of adulthood and one that included both days 6 and 9 of adulthood. This approach was further justified because Spurgeon (2003) demonstrated under similar experimental conditions that pheromone composition on day 3 differs from that on days 6 and 9. In addition to weevils excluded because of missing data, weevils were excluded from analysis if they entered diapause.

In both analyses, the data were examined for normality and heterogeneity of variance as previously described. Based on those examinations and failure of arcsin transformation to improve the condition of the data, the untransformed data were analyzed. Both analyses (3-d, and 6- and 9-d) used the respective proportions represented by the 4 components as response variables. Model effects for the data from day 3 of adulthood included experiment repetition, diet treatment, and their interaction. The repetition × diet treatment interaction was used as the error term for testing effects of diet. The statistical model for data from days 6 and 9 of adulthood was identical to that used for the diet switching experiment.

Results

Response to diet switching. Ten of the 64 weevils did not emit detectable quantities of all 4 pheromone components on day 7, and 3 weevils died before the completion of pheromone collection on day 8. Therefore, a total of 13 weevils were excluded from analysis. These included 2 weevils assigned to the square diet, 3 weevils assigned to small bolls, and 8 weevils assigned to medium bolls. Of the weevils surviving but not emitting all pheromone components on day 7, 5 (1 on squares, and 2 each on small and medium bolls) entered diapause. Each of the diapausing weevils emitted pheromone during at least 1 collection period, but the maximum observed daily production was only 2.2 µg. On most days total pheromone collected was 0 - 1 µg, and none of the diapausing weevils ever produced components III or IV. One weevil (assigned to medium bolls) produced no pheromone during any collection period, and exhibited a hypertrophied fat body and small testes but did not exhibit all the morphological criteria of diapause. Finally, 4 weevils (1 on squares, 3 on medium bolls) did not produce one or more of the pheromone components (either components III and IV, or component IV) on day 7, although they each produced all components (maximum total pheromone collected ranging from 18.9 - 88.4 µg) during at least one other collection period.

The analyses did not indicate significant overall effects of diet treatment (F=2.20; df=2, 46.6; P = 0.12) or weevil age (F=2.55; df=2, 73.4; P = 0.09), but the significant covariate (pheromone production on day 7 of adulthood; F=41.30; df=1, 55.1; $P < 0.01$) indicated within-weevil correlations among quantities of pheromone collected at different ages. Because the diet treatment × weevil age interaction was also significant (F=3.34; df=4, 80.3; P = 0.01) main effects of diet treatment and weevil age could not be interpreted without reference to the time of pheromone collection. Effect slices examining the influence of weevil age within diet treatments indicated pheromone releases changed over time for small (F=5.80; df=2, 73.5; $P < 0.01$) and medium bolls (F=3.69; df=2, 73.5; P = 0.03), but remained relatively constant for weevils maintained on squares (F=0.56; df=2, 73.2; P = 0.57). Weevils switched from squares to small bolls emitted more pheromone on day 8 of adulthood than on day 13, whereas weevils switched to medium bolls emitted more pheromone on day 10 than on day 8 (Table 1). Significant differences in pheromone emissions were also observed among the diet treatments, but only on the day on which diets were switched (day 8, F=7.93; df=2, 45.3; $P < 0.01$; day 10, F=2.01; df=2, 46.2; P = 0.15; day 13, F=0.39; df=2, 48.8; P = 0.68; Table 1). On the day on which diet treatments were implemented (day 8 of adulthood), pheromone released by weevils switched from squares to small bolls was higher than for weevils maintained on squares or switched to medium bolls (Table 1).

A total of 18 weevils was excluded from the analyses of pheromone composition (4 weevils assigned to squares, 4 assigned to small bolls, and 10 assigned to medium bolls). Thirteen of these were the same weevils excluded from the analysis of total pheromone production. Of the additional 5 weevils excluded, 1 (on medium bolls) did not emit pheromone on day 13, and 3 (2 on squares, 1 on small bolls) died during the experiment. One weevil (on medium bolls) entered diapause after emitting a maximum of 7.7 µg of pheromone on day 7, including all pheromone components. However, pheromone emissions subsequently diminished to 1.2 µg by day 13, when only components I and II were produced.

Multivariate analysis of pheromone composition indicated significant effects of diet treatment (Wilk Lambda=0.0076; F=39.14; df=8, 30; $P < 0.01$) and weevil age (Wilk

Table 1. Daily pheromone emissions (μg, mean ± SE) at three different ages for male boll weevils fed for 7 d on squares and then maintained on squares or switched to small or medium bolls

Day of adulthood	Squares*	Small bolls*	Medium bolls*
8	68.4 ± 6.5aB	91.8 ± 5.3aA	63.4 ± 6.1bB
10	60.2 ± 10.1aA	80.7 ± 8.1abA	86.2 ± 9.3aA
13	59.3 ± 8.9aA	65.3 ± 6.9bA	69.5 ± 7.9abA

* Squares were 5 - 7 mm diameter, small bolls were 12 - 15 mm diameter, and medium bolls were 20 - 23 mm diameter. All food types were provided with intact bracteoles.
Least-squares means in the same column followed by the same lower-case letter, or in the same row followed by the same upper-case letter, are not significantly different ($P > 0.05$).

Lambda=0.0240; F=10.30; df=12, 39.978; $P < 0.01$). However, the diet × age interaction (Wilk Lambda=0.1286; F=1.79; df=24, 53.539; $P = 0.04$) also suggested that either temporal changes in pheromone composition were influenced by weevil diet, or that differences in pheromone composition among diet treatments were dependent on weevil age. Univariate analyses of individual pheromone components indicated significant effects of weevil diet for all components (component I, F=13.16; df=2, 18; $P < 0.01$; component II, F=16.00; df=2, 18; $P < 0.01$; component III, F=4.49; df=2, 18; $P = 0.03$; component IV, F=80.10; df=2, 18; $P < 0.01$). Differences among weevil ages in the proportions of total pheromone represented by each component were detected for components I, III, and IV (component I, F=45.83; df=3, 18; $P < 0.01$; component III, F=3.58; df=3, 18; $P = 0.03$; component IV, F=15.87; df=3, 18; $P < 0.01$), but not for component II (F=1.34; df=3, 18; $P = 0.29$). The diet treatment × age interaction was not significant for any pheromone component ($P = 0.16$ - 0.65).

When the proportions of total pheromone represented by each component were compared among diet treatments, adjusting for multiplicity using the Tukey-Kramer method, an effect of diet was detected only for component IV (Table 2). The proportion of component IV was statistically smaller for the square diet compared with either small boll or medium boll diets. However, these differences appeared too small to have biological significance. Similar comparisons among weevil ages, when adjusted for multiplicity, failed to detect differences for any pheromone component (Table 2).

Response to diet. Only 2 of the 64 weevils were excluded from analysis of total pheromone production. One of these weevils (assigned to small bolls) did not emit pheromone on day 3 and died the following day, and the other weevil (assigned to medium bolls) died on day 7 after not feeding for four of the preceding 6 d.

Analyses indicated that both diet treatment (F=4.56; df=2, 63.8; $P = 0.01$) and weevil age (F=99.14; df=2, 72.4; $P < 0.01$) influenced the amount of pheromone collected. However, the diet treatment × age interaction (F=2.77; df=4, 82.2; $P = 0.03$) suggested that differences among the diets varied with weevil age. Examination of slices of the diet treatment indicated temporal changes in the amount of pheromone emitted on each diet (squares, F=29.60; df=2, 73.4; $P < 0.01$; small bolls, F=52.87; df=2, 72.5; $P < 0.01$; medium bolls, F=19.98; df=2, 71.1; $P < 0.01$). Based on contrasts among weevil ages within diet treatments, pheromone emission increased with each increment of age for weevils fed either small or medium bolls (Table 3). A similar pattern was observed for weevils fed squares, but statistical differences between

Table 2. Pheromone composition (mean ± SE, proportion of total pheromone represented by each component*) corresponding to univariate analyses of treatment effects (diet, day of adulthood) for male boll weevils fed for 7 d on squares and then maintained on squares or switched to small or medium bolls

Treatment effect	Component I	Component II	Component III	Component IV
Diet**				
squares	0.436 ± 0.002	0.435 ± 0.004	0.028 ± 0.002	0.101 ± 0.001b
small bolls	0.438 ± 0.002	0.427 ± 0.003	0.028 ± 0.001	0.106 ± 0.001a
medium bolls	0.436 ± 0.002	0.427 ± 0.003	0.032 ± 0.002	0.106 ± 0.001a
Day of adulthood				
7	0.438 ± 0.002	0.429 ± 0.004	0.028 ± 0.002	0.104 ± 0.001
8	0.439 ± 0.002	0.431 ± 0.004	0.027 ± 0.002	0.103 ± 0.001
10	0.432 ± 0.002	0.430 ± 0.004	0.031 ± 0.002	0.106 ± 0.001
13	0.438 ± 0.002	0.428 ± 0.004	0.031 ± 0.002	0.104 ± 0.001

* Component I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; component II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; component III, *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; component IV, *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde.

** Squares were 5 - 7 mm diameter, small bolls were 12 - 15 mm diameter, and medium bolls were 20 - 23 mm diameter. All food types were provided with intact bracteoles.

Least-squares means in the same column followed by the same lower-case letter are not significantly different ($P > 0.05$, Tukey-Kramer test).

pheromone releases on days 6 and 9 of adulthood were not demonstrated (Table 3). Examinations of slices by weevil age indicated differences in pheromone emissions among diet treatments only on day 9 of adulthood (day 3, F=0.66; df=2, 52.1; P = 0.52; day 6, F=2.89; df=2, 60.7; P = 0.06; day 9, F=5.05; df=2, 66.3; $P < 0.01$). Contrasts comparing least-squares means of the diets on day 9 indicated weevils fed small bolls emitted more pheromone than did weevils fed other diets (Table 3).

Because the multivariate analysis cannot accommodate missing observations, data for only the 35 weevils that emitted detectable quantities of pheromone were

Table 3. Daily pheromone emissions (μg, mean ± SE) at three different ages for male boll weevils fed squares, small bolls, or medium bolls

Day of adulthood	Squares*	Small bolls*	Medium bolls*
3	2.1 ± 2.6aA	6.1 ± 2.8aA	4.1 ± 2.4aA
6	50.6 ± 9.8bA	70.4 ± 10.1bA	37.6 ± 9.4bA
9	67.1 ± 9.4bA	96.8 ± 9.6cB	56.6 ± 8.9cA

* Squares were 5 - 7 mm diameter, small bolls were 12 - 15 mm diameter, and medium bolls were 20 - 23 mm diameter. All food types were provided with intact bracteoles.

Least-squares means in the same column followed by the same lower-case letter, or in the same row followed by the same upper-case letter, are not significantly different ($P > 0.05$).

Table 4. Composition (mean ± SE, proportion of total pheromone represented by each component*) of pheromone produced on day 3 of adulthood for male boll weevils fed squares, small bolls, or medium bolls

Diet treatment**	Component I	Component II	Component III	Component IV
squares	0.551 ± 0.045	0.405 ± 0.043	0.004 ± 0.003	0.041 ± 0.014
small bolls	0.474 ± 0.054	0.462 ± 0.051	0.008 ± 0.003	0.055 ± 0.017
medium bolls	0.537 ± 0.041	0.418 ± 0.039	0.003 ± 0.002	0.042 ± 0.013

* Component I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; component II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; component III, *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; component IV, *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde.

** Squares were 5 - 7 mm diameter, small bolls were 12 - 15 mm diameter, and medium bolls were 20 - 23 mm diameter. All food types were provided with intact bracteoles.

included in the analysis of pheromone composition on day 3 of adulthood. The analysis did not indicate a significant influence of adult diet on pheromone composition (Wilk Lambda=0.2475; F=0.76; df=8, 6; P = 0.65; Table 4).

Analysis of pheromone composition at days 6 and 9 of adulthood included data from 55 weevils that emitted at least one pheromone component on both days. Four of the excluded weevils (1 on small bolls, 3 on medium bolls) entered diapause after emitting a daily maximum of 1.06-1.99 µg of pheromone. None of the diapausing weevils emitted components III or IV. Neither diet treatment (Wilk Lambda=0.3489; F=0.52; df=8, 6; P = 0.81) nor weevil age (Wilk Lambda=0.0927; F=7.34; df=3, 4; P = 0.07) were shown to influence pheromone composition (Table 5). Furthermore, the diet treatment × age interaction (Wilk Lambda=0.0744; F=2.00; df=8, 6; P = 0.21)

Table 5. Pheromone composition (mean ± SE, proportion of total pheromone represented by each component*) corresponding to univariate analyses of treatment effects (diet, day of adulthood) for male boll weevils fed squares, small bolls, or medium bolls for 6 and 9 d

Treatment effect	Component I	Component II	Component III	Component IV
Diet**				
squares	0.440 ± 0.005	0.444 ± 0.006	0.019 ± 0.002	0.097 ± 0.004
small bolls	0.443 ± 0.005	0.440 ± 0.006	0.020 ± 0.002	0.097 ± 0.004
medium bolls	0.449 ± 0.005	0.436 ± 0.006	0.021 ± 0.002	0.094 ± 0.004
Day of adulthood				
6	0.448 ± 0.004	0.442 ± 0.004	0.020 ± 0.002	0.090 ± 0.003
9	0.440 ± 0.004	0.438 ± 0.004	0.020 ± 0.002	0.102 ± 0.003

* Component I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; component II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; component III, *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; component IV, *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde.

** Squares were 5 - 7 mm diameter, small bolls were 12 - 15 mm diameter, and medium bolls were 20 - 23 mm diameter. All food types were provided with intact bracteoles.

was not significant. Although the *P* value for weevil age (0.07) suggested a possible influence by this effect, numerical differences between weevil ages for the various components were too small to be biologically significant.

Discussion

Our results demonstrate that, with the exception of individuals committing to diapause soon after eclosion, the male boll weevil is capable of both initiating and sustaining high levels of pheromone emission on bolls of the sizes we examined. Our observations also confirm that weevils exhibiting the morphological characters of diapause suggested by Spurgeon et al. (2003) emit only small amounts of pheromone. However, we observed that weevils possessing hypertrophied fat bodies but lacking a degree of gonadal atrophy typical of diapause were capable of producing and releasing large amounts of pheromone. Such weevils were often classified as intermediate diapause by previous investigators (McCoy et al. 1968, Tingle and Lloyd 1969, Tingle et al. 1971).

Where differences in pheromone emissions among the tested diets were observed, releases tended to be higher on small bolls than on either squares or medium bolls. Although this observation suggests that a diet of small bolls may be superior to squares in terms of pheromone production, the observed differences may have been artifacts. During pheromone collection, the flow of dry air through the collection vessels tends to remove moisture from the vessel contents. By virtue of the smaller size of squares, their deterioration from weevil feeding and loss of moisture is greater than for bolls. Spurgeon and Suh (2007) suggested similar factors were responsible for differences in daily patterns, in both the amounts and composition of pheromone produced, corresponding to weevils fed at different times of day. Therefore, it may be erroneous to conclude that the statistically higher amounts of pheromone emitted on small bolls signifies their superiority as a food source in comparison with squares.

Relative to earlier reports, the consistency in pheromone composition we observed, both within and between studies, was remarkable. Previous reports of pheromone composition, based on fecal extractions, have varied widely. For example, Tumlinson et al. (1970) reported component ratios of 52.4:39.3:4.1:4.1, whereas Hedin et al. (1974) reported a composition of 6:6:2:1 (I:II:III:IV). McGovern et al. (1976) combined components III and IV and reported a composition of 46:10:44 (I:II:III+IV). Component ratios estimated in our studies were about 44:43:3:10 (I:II:III:IV) for weevils $\geq$ 6 d of age. These estimates are consistent with those obtained by Spurgeon (2003) and Spurgeon and Suh (2007), who used similar methodology. The consistency in results among the studies using the methods of Spurgeon (2003) suggests a higher degree of reproducibility than was provided by earlier methods relying on fecal extractions. Also, the similarities in pheromone composition among studied diets suggest it is unlikely that weevils rely on altered pheromone composition to signal changes in fruiting phenology of the cotton host. Although the component ratios we observed were similar to those observed by Spurgeon (2003) and Spurgeon and Suh (2007), they differed substantially from the composition of the commercial lure (30:40:15:15; I:II:III:IV; Leggett et al. 1989). Therefore, it is possible that reformulation of the commercially available boll weevil pheromone lure could provide improved detection efficiency in eradication and maintenance programs. Because the weevil responds to a wide range of component

ratios (Leggett 1980), field testing will be required to determine if such changes would be advantageous.

Our results have additional ecological implications. Although the boll weevil is known to overwinter in a reproductive diapause, many overwintering females are mated (Brazzel and Newsom 1959, Beckham 1962, Taft et al. 1963, Roach et al. 1984). This implies some mechanism to facilitate mating of weevils destined to diapause. Diapausing males are known to be fertile, and therefore capable of mating (Villavaso 1981, Spurgeon and Raulston 1996, 1998). Although Villavaso and Earle (1974) reported that weevils in diapause, or destined for diapause, are deficient in pheromone production, we observed high levels of emitted pheromone by weevils exhibiting morphological characters consistent with those described by Spurgeon et al. (2003) for males entering diapause. Furthermore, these findings are consistent with observations from the field. Hand collections of about 10,000 weevils from a maturing cotton field, for a dispersal study reported by Westbrook et al. (2000), revealed marked clumping of the weevil population on individual bolls and a sex ratio of about 75:25 (♂:♀) (D.W.S., unpubl. observ.). Such aggregation behavior could result from high levels of pheromone emission, and would likely facilitate mating by recently emerged weevils before they left the field for overwintering quarters. Our observations also suggest the late-season increase in captures by pheromone traps cited by many authors (Hardee et al. 1970, Roach et al. 1971, Rummel et al. 1977, Lopez et al. 1978, Leggett and Moore 1982, Segers et al. 1987) is not a simple consequence of reduced competition from males in the field.

Finally, bioassays performed by Hardee (1970) suggested the boll weevil continues to emit pheromone for a short time after food is removed. Suh and Spurgeon (2004) used direct estimates of emitted pheromone to confirm continuation of releases for at least the first 3 d of starvation. These observations suggest the potential for captured males to confound field studies of lure formulations. Because we found that boll-fed males are capable of producing large amounts of pheromone, this concern may be particularly relevant late in the cropping season when large numbers of weevils are captured in traps. In fact, in field studies we have frequently observed captured males with accessory glands consistent with those described by Spurgeon (2003) for pheromone-producing weevils (D.W.S, unpubl. data). This information may lead to improved experimental designs for field-testing lure formulations, and provides insights that may be useful in interpreting or explaining results of lure evaluations.

Acknowledgment

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

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