

## Effects of Three-Dimensional and Color Patterns on Nest Location and Progeny Mortality in Alfalfa Leafcutting Bee (Hymenoptera: Megachilidae)

CHRISTELLE GUÉDOT,<sup>1,2</sup> JORDI BOSCH,<sup>1,3</sup> ROSALIND R. JAMES,<sup>4</sup> AND WILLIAM P. KEMP<sup>4</sup>

J. Econ. Entomol. 99(3): 626–633 (2006)

**ABSTRACT** In alfalfa, *Medicago sativa* L., seed production where high bee densities are released, alfalfa leafcutting bee, *Megachile rotundata* (F.) (Hymenoptera: Megachilidae), females may enter several nesting holes before locating their nests. Such levels of “wrong hole” visits lead to an increase in the time spent by females locating their own nests, thereby decreasing alfalfa pollination efficiency and possibly healthy brood production. The objectives of this study were to determine the effect of different nesting board configurations in commercial alfalfa leafcutting bee shelters (separating nesting boards, applying a three-dimensional pattern to the boards, applying a color contrast pattern, or applying a combination of three-dimensional and color contrast patterns) on nest location performance, on the incidence of chalkbrood disease, and on the incidence of broodless provisions. Separating the nesting boards inside shelters improved the ability of females to locate their nests. An increase in nest location performance also occurred in boards with the three-dimensional pattern and the combined three-dimensional and color contrast pattern, compared with the uniform board (a standard configuration currently used commercially). The percentage of provisioned cells that were broodless was not statistically different between treatments, but the percentage of larvae infected with chalkbrood decreased by half in the three-dimensional board design, compared with the uniform board.

**KEY WORDS** *Ascosphaera aggregata*, color contrast, *Megachile rotundata*, nest location, three-dimensional

*Megachile rotundata* (F.) (Hymenoptera: Megachilidae), the alfalfa leafcutting bee, is native to Eurasia and was accidentally introduced to North America in the 1930s (Michener 2000). In the 1950s, *M. rotundata* was recognized as an excellent pollinator of alfalfa, *Medicago sativa* L., and has since been used commercially for alfalfa pollination (Bohart 1972, Osgood 1974, Richards 1984).

*M. rotundata* is active from June through August. In nature, females nest in preexisting cavities such as tree trunk holes or in abandoned nests of other bees or wasps. *M. rotundata* is solitary, with each female bee building and provisioning her own nest. Although solitary, *M. rotundata* is gregarious (Kukovica 1966), so it is possible to manage many individuals at the same nesting site for commercial or experimental purposes. *M. rotundata* females provision their nests with pollen and nectar by using leaf pieces as nesting material.

Because they are gregarious, nesting females have to locate their nest among nearby nesting cavities.

In alfalfa seed production, high densities of bees are commonly released, ≈20,000–60,000 *M. rotundata* females per hectare (Bohart 1972, Richards 1993, Strickler 1996). Observations at nesting boards show that *M. rotundata* females have difficulty locating their nesting cavities, that is, they often visit one or more incorrect nesting cavities before locating and entering the correct cavity (C.G., unpublished data). Furthermore, *M. rotundata* females have antagonistic interactions, such as nest usurpations and fights. This high level of “wrong hole” visits and antagonistic interactions may be due, at least in part, to confusion and nesting cavity competition in high bee densities, as suggested with the related species *M. apicalis* (Barthell and Thorp 1995). The consequence of these behaviors is an increase in the time females spend locating their nests: several seconds in low-density situations compared with several minutes in commercial situations (C.G., unpublished data), presumably decreasing alfalfa pollination efficiency and brood cell production. The design of the commercial nesting boards forces *M. rotundata* females to locate their individual nesting cavity among thousands of visually identical, closely spaced holes. Additionally, several

<sup>1</sup> Biology Department, Utah State University, Logan, UT 84322–5310.

<sup>2</sup> USDA–ARS Yakima Agricultural Research Laboratory, Wapato, WA 98951.

<sup>3</sup> Unitat d'Ecologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

<sup>4</sup> Bee Biology and Systematics Laboratory, USDA–ARS, Logan, UT 84322–5310.

nesting boards are placed adjacent to each other inside shelters (Peterson et al. 1992), probably further confusing females that are attempting to locate their nests.

Previous studies emphasize the importance of colors for nesting site recognition in Megachilidae (Osgood 1968, Hobbs 1977, Richards 1984, Fauria 1998). *M. rotundata* also uses three-dimensional (3D) and color contrast patterns as nest location cues (Guédot 2004). Honey bees have been shown to preferentially land on the edge of an object with a strong color contrast to the background (Lehrer 1994) or when that object is raised above a background (Srinivasan et al. 1990, Lehrer and Srinivasan 1993). Furthermore, *M. rotundata* females frequently begin nesting in the cavities located on the edges of the nesting boards (unpublished data) and are known to use the edges for nest location (Fauria et al. 2004).

In the mid-1970s, producers in alfalfa seed fields of the Pacific Northwest started to see about one live progeny for every two females released (Peterson et al. 1992). This low level of reproduction has been attributed to several causes, including, but not limited to, poor nest location time (Guédot et al. 2003, Bosch and Kemp 2005), the sudden occurrence and spread of a fungal disease called chalkbrood (Stephen et al. 1981, Peterson et al. 1992), and early immature mortality, also called "pollen ball" (Bohart 1971, Pitts-Singer 2004, Bosch and Kemp 2005).

Chalkbrood, a predominant fungal disease caused by *Ascosphaera aggregata* Skou (Ascomycota: Ascosphaeraceae) in *M. rotundata* (Vandenberg and Stephen 1982) frequently results in population losses of 50% or more in commercial situations (Peterson et al. 1992). Chalkbrood is primarily spread by females that become contaminated with spores from nestmates that died the previous season (Vandenberg et al. 1980). It was once thought that contaminated nesting materials was a primary source of disease transmission (Peterson et al. 1992), but this has since been shown not to be the case (James 2005). Spores collect on adult bees when they chew through a sporulating cadaver of infected nestmates that block their way during emergence (Vandenberg et al. 1980, Stephen et al. 1981) or when adults emerge from cells that have been removed from the boards, and tumbled (James and Pitts-Singer 2005), a common commercial practice called "loose cells." Adults emerging through chalkbrood-infected cadavers can carry from  $9 \times 10^4$  to  $8 \times 10^7$  *A. aggregata* spores (Vandenberg et al. 1980). Females emerging in the loose cell system carry an average  $3.62 \times 10^4 \pm 6.54 \times 10^3$  spores (James and Pitts-Singer 2005). Stephen et al. (1981) and McManus and Youssef (1984) demonstrated that infection occurs orally, and so the spores carried by nesting females probably get incorporated into the provision, where they are eaten by the larvae (Vandenberg et al. 1980). We hypothesize that if we reduce the number of wrong hole visits as well as contacts (antagonistic or otherwise) between nesting females, we can reduce the spread of spores between adult females.

Observations at a commercial scale also revealed a significant presence of pollen balls, or early immature mortality, defined as the provision remaining intact either because an egg was not laid or died during the early stages of development and for which the causes are not well defined (O'Neill 2004, Pitts-Singer 2004). Pollen balls may account for 60% or more of larval mortality (Bohart 1971). Collapsed eggs, dead pre-defecating larvae, dead postdefecating larvae, and broodless provisions are often associated with pollen balls (Pitts-Singer 2004, Bosch and Kemp 2005). In this study, we focused exclusively on broodless provisions, in which the provision remains intact because either the egg was removed or not laid. Overpopulation (Strickler 1996, Fauria 1998), nest usurpation (Fauria 1998), and bee aging (Tirgari 1963) could, at least partially, explain the presence of broodless provisions. Overpopulation leads to confusion and disorientation of *M. rotundata* females (Stephen 1981, Guédot et al. 2003), perhaps causing improper provisioning and nest usurpations that lead to broodless cells.

In our study, we designed two experiments using nesting boards with 3D patterns and color contrast patterns to assess the effect of these configurations on alfalfa leafcutting bee nest location performance and progeny mortality in commercial fields. We hypothesized that if we are able to improve nest location performance and reduce the number of antagonistic encounters between females via our nesting board designs, then we might also affect the successful nesting of the females by reducing the spread of chalkbrood disease and broodless provisions.

## Materials and Methods

**Experiment 1. Effect of Board Arrangement on Nest Location Performance.** *Study Sites and Nesting Materials.* This experiment was conducted in July 2002–July 2003, in commercial alfalfa seed fields, near Tremonton, UT. The nesting shelters used in experiment 1 consisted of wooden shelters (2.4 by 1.5 by 1.8 m) opened on one side and oriented southeast, for better establishment and early morning activity (Stubbs et al. 1994). Commercial polystyrene nesting boards (Beaver Plastics, Edmonton, Alberta, Canada) (60 by 30 by 9.5 cm), containing 1,770 holes each, were used as nesting material. The holes (6.3 mm in diameter) were spaced 3.5 mm apart. Five nesting boards were placed in each shelter before the release of the bees.

*Bee Populations.* *M. rotundata* cells were placed in the shelters by the bee supplier a few days before emergence in the nesting shelters. Three populations were released, one per field. In the first field,  $\approx 114,000$  bees and in the second and third field,  $\approx 124,000$  bees were released per hectare. Approximately 33% of the bees released are females (Richards 1993). Data collection was conducted no sooner than 3 wk after release, to coincide with peak activity (Bosch and Kemp 2005), to allow time for the bees to mate, and for females to select a nesting cavity and begin nest provisioning.

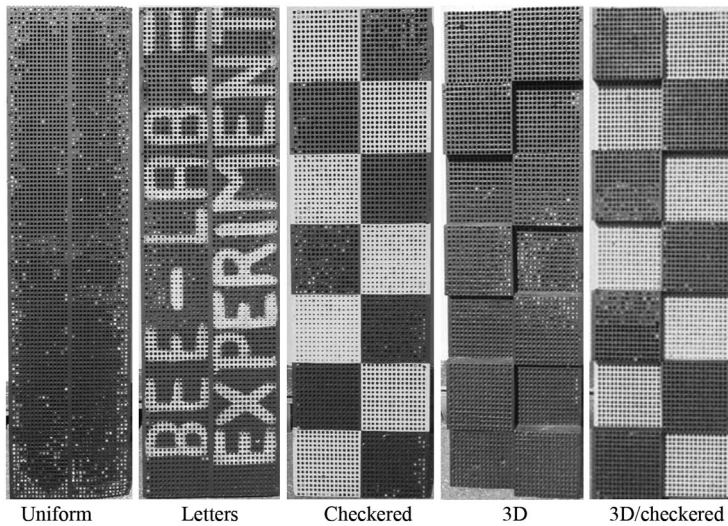


Fig. 1. Experimental board designs used in experiment 2.

**Experimental Design.** The experiment was designed to test whether applying a 3D pattern by separating the nesting boards within a nesting shelter could improve the nest location performance of *M. rotundata* females in commercial situations. In the first treatment (adjacent boards), five commercial boards were arranged next to each other on the back wall of the nesting shelter. In the second treatment (separated boards), five commercial boards were placed on the back wall of the nesting shelter, leaving 10-cm gaps between the boards, thus providing a 3D pattern against the wall. Each treatment was conducted in a separate shelter.

To control for the lack of homogeneity between fields in commercial settings, both experiments used a randomized block design, with the fields being treated as blocks. Each experiment was conducted three times, each time in a separate field. All treatments were conducted the same day to avoid a day effect, between 1200 and 1800 hours.

The percentage of wrong hole versus correct hole visits was compared for each treatment, giving an indication of the nest location performance of nesting females. Wrong and correct hole visits by returning females were counted for a total of 180 visits per treatment. During antagonistic interactions or when visiting several wrong holes, bees often dropped the leaf pieces they were carrying back to the nest. To obtain an indirect measure of antagonistic interactions and confusion, trays were attached under each experimental board to collect the leaf pieces dropped each day. The leaf pieces collected for each treatment were dried and weighed.

**Experiment 2. Effect of 3D and Color Contrast Patterns on Nest Location Performance and Progeny Mortality. Study Sites and Nesting Materials.** This experiment was carried out during July 2002–July 2003 in commercial alfalfa fields near Nampa, ID, and in the laboratory in Logan, UT, in September–October 2002

and 2003. The nesting shelters were wooden trailers (6 by 3 by 2.5 m) opened on one side and oriented southeast. Commercial polystyrene boards (105 by 30 by 9.5 cm) containing 3,150 holes each were used as nesting material. The nesting cavities (6.3 mm in diameter) were spaced 3.5 mm apart. One experimental nesting board (treatments described below) was set up per shelter before the release of the bees. In addition, two commercial polystyrene boards (120 by 30 by 9.5 cm) were painted in black and placed on each side of each experimental nesting board to minimize the use of experimental board edges by *M. rotundata* females. All paints used for nesting boards were 100% acrylic latex exterior flat paint. Black (Olympic Premium base 5, 518-7) and yellow (Evermore, Kayak Yellow GH 103) paints were used for the experimental boards because they offer a strong contrast and because *M. rotundata* females show a strong preference toward yellow (Osgood 1968, Fauria 1998).

The cells produced by females nesting during the summer in the 15 experimental boards were collected. To do so, paper straws that could easily be removed for cell content analysis were inserted in each experimental nesting board. The tip of each straw (5.5 mm i.d.) was painted according to the color of the experimental boards.

**Bee Populations.** Three populations were released, one per field. In the first field,  $\approx 136,000$  bees; in the second field  $\approx 157,000$  bees; and in the third field,  $\approx 163,000$  bees were released per hectare.

**Experimental Design.** This experiment was designed to test the effect of 3D and color contrast patterns on the nest location performance of *M. rotundata* females. Five experimental boards were designed (Fig. 1). 1) A standard commercial nesting board uniformly painted black (uniform board; control board). 2) A standard commercial nesting board painted with the markings commonly used by alfalfa producers (black background, yellow markings) (letters board); the yellow markings

covered 44% of the nesting holes. 3) A standard commercial nesting board painted with a color contrast pattern (yellow and black) forming 14 small units (15 by 15 holes) (checkered board); the yellow markings covered 50% of the nesting holes. 4) A standard commercial nesting board provided with a 3D pattern of 4-cm depth and painted uniform black; the board was cut into fourteen small units (15 by 15 holes) that were reunited so that adjacent units were offset from each other by a depth of 4 cm (3D board). 5) A standard commercial nesting board combining the 4-cm 3D pattern described above with the checkered color contrast (black and yellow) pattern (3D/checkered board); the yellow markings covered 50% of the nesting holes.

We used five shelters per field, placing one treatment in each shelter. We repeated the experiment three times, each time in a separate field, by using a randomized block design, with the fields being treated as blocks. All treatments were conducted the same day to avoid a day effect, between 1200 and 1800 hours. In this experiment, we also recorded the percentage of wrong versus correct hole visits and the weight of leaf pieces dropped as described previously.

After the experimental boards were returned to the laboratory, they remained outside at ambient temperature for at least 21 d to permit the completion of larval development and to allow chalkbrood-infected brood to become symptomatic of the disease (Stephen et al. 1981, Kemp and Bosch 2000). Fifty straws were randomly removed from each experimental board, and radiographs were used to look at the cell contents. In numerous cases, the cells were dissected for better examination (as described by Pitts-Singer 2004). The following conditions were noted for each cell: 1) *A. aggregata* infected, 2) broodless with provision, or 3) other (healthy and all other conditions). Chalkbrood-infected larvae were identified by the hard, chalky appearance of the dead larva (Vandenberg and Stephen 1982), with or without spores. Broodless cells

were identified by the presence of an intact provision with no egg or developing larvae attached to it.

**Statistical Analysis.** We used SAS Version 8 (SAS Institute 1999) for all analyses. Analysis of variance (ANOVA) (PROC MIXED) was used to determine whether treatments had a significant effect on the proportion of wrong hole visits, the mass of leaf pieces dropped, the proportion of larvae infected with chalkbrood, and the proportion of broodless provisions. All proportions were arcsine transformed, and the mass of leaf pieces dropped were log transformed to normalize the distributions (Zar 1999). When more than two treatments were tested and a treatment effect was found, a Tukey pairwise comparison was used (Zar 1999).

## Results

**Experiment 1. Effect of Board Arrangement on Nest Location Performance.** *M. rotundata* nesting females improved their nest location performance, resulting in a decrease in the percentage of wrong hole visits with the separated board arrangement compared with the adjacent board arrangement ( $F = 192.38$ ;  $df = 1, 2$ ;  $P = 0.005$ ) (Fig. 2). The mass of the dropped leaf pieces decreased with the separated treatment compared with the adjacent treatment (Table 1), but this effect was not statistically significant ( $F = 10.05$ ;  $df = 1, 2$ ;  $P = 0.087$ ).

**Experiment 2. Effect of 3D and Color Contrast Patterns on Nest Location Performance and Progeny Mortality.** Treatment had a significant effect on the percentage of wrong holes visited by *M. rotundata* females ( $F = 5.41$ ;  $df = 4, 8$ ;  $P = 0.02$ ) (Fig. 3). Females visited fewer wrong holes when nesting in the 3D (Tukey adjusted  $P = 0.01$ ) and 3D/checkered (Tukey adjusted  $P = 0.04$ ) boards compared with the uniform board (Fig. 3). The boards painted with checkered marks or letters were not significantly different from

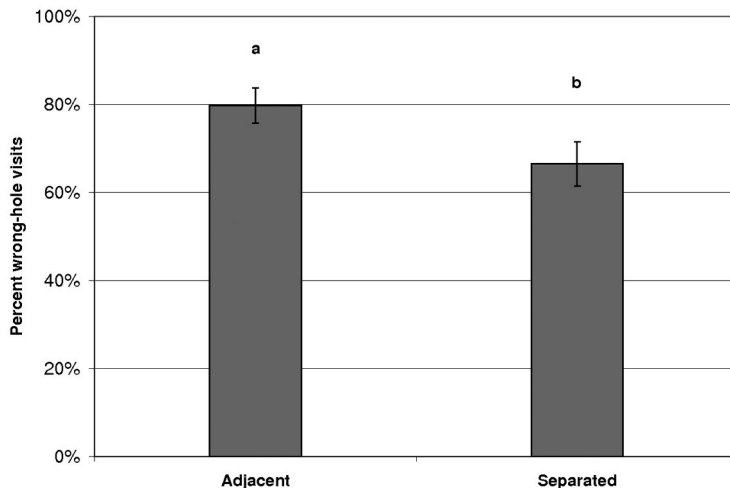


Fig. 2. Mean percentage of wrong hole visits by *M. rotundata* in two board arrangements (adjacent and separated). Treatments with different letters above them are significantly different statistically ( $P \leq 0.05$ ).

**Table 1.** Mean mass of leaf pieces dropped in 1 d by *M. rotundata*, for experiment 1 (adjacent and separated board arrangements) and experiment 2 (uniform, letters, checkered, 3D, 3D/checkered); and significance parameters for ANOVA analysis between treatments for each experiment

Exp	Treatment	Mean leaf pieces (mass [g] ± SE)	Significance
1	Adjacent	20.68 ± 7.4	df = 1, 2; $F = 10.05$ ; $P = 0.087$
1	Separated	11.65 ± 3.3	
2	Uniform	3.08 ± 1.8	df = 4, 7; $F = 0.52$ ; $P = 0.73$
2	Letters	3.13 ± 2.4	
2	Checkered	3.41 ± 2.7	
2	3D	1.88 ± 1.5	
2	3D/checkered	1.51 ± 1.0	

the other treatments, obtaining intermediate percentages of wrong hole visits between the uniform and the two 3D boards. Treatments did not show an effect on the mass of leaf pieces ( $F = 0.52$ ;  $df = 4, 7$ ;  $P = 0.73$ ) (Table 1).

The percentage of *A. aggregata* chalkbrood-infected larvae present in *M. rotundata* cells revealed a statistical difference between treatments ( $F = 4.23$ ;  $df = 4, 8$ ;  $P = 0.04$ ) (Fig. 4). The percentage of larvae infected with chalkbrood was lower in the cells produced in the 3D board compared with the uniform board (Tukey adjusted  $P = 0.027$ ). The percentage of broodless provisions present in *M. rotundata* cells was not statistically different between the five treatments ( $F = 0.64$ ;  $df = 4, 8$ ;  $P = 0.68$ ) (Fig. 5).

## Discussion

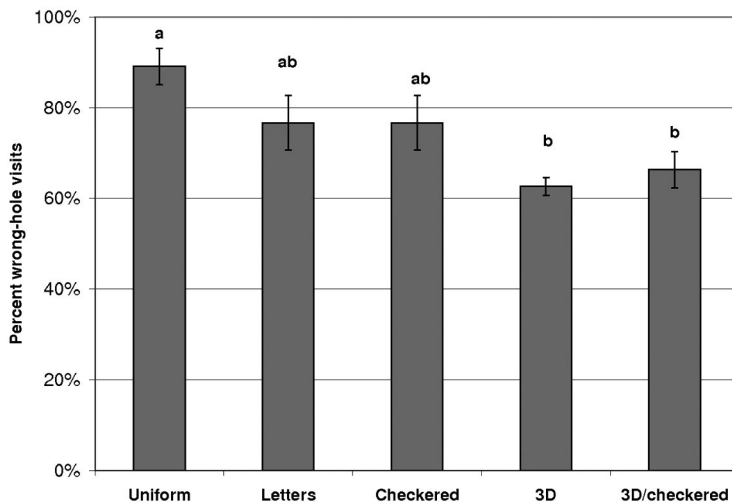
Our results indicate that separating the boards in nesting shelters improved nest location performance, but the mass of dropped leaf pieces (indicating antagonistic interactions and confusion) was not significantly lower. Separating the boards added a 3D con-

figuration, with more edges, and thus more orientation cues to returning females (Fauria et al. 2004), whereas the five adjacent boards formed one large nesting surface with very few orientation cues. Our results are consistent with Fauria (1998) where *M. rotundata* showed an improved nest location performance in commercial situations when one nesting board was attached on a shelter wall versus several boards.

*M. rotundata* females improved their nest location performance when nesting in the two 3D board treatments (3D and 3D/checkered), suggesting that a 3D pattern could be a better nest location cue than a standard color contrast pattern. Indeed, the differences between the two 3D boards were not significant; thus, the color pattern present on the 3D/checkered board did not improve nest location. The lack of significant differences among uniform, checkered, and letters designs could indicate that a color contrast pattern did not improve nest location, but these results do not concur with previous reports for *M. rotundata* (Fauria 1998). Perhaps, with lower densities of bees, differences in nest location might be seen between uniform board and those with various color patterns.

Applying a 3D pattern by separating the boards or designing 3D boards added edges to the nesting boards, offering more orientation cues to be used by *M. rotundata* females. Future studies should investigate separating 3D boards within nesting shelters, juxtaposing two levels of 3D cues to further improve nest location of *M. rotundata* females.

The 3D boards had only about half the chalkbrood levels as the uniform boards. These results are consistent with the decrease in the percentage of wrong hole visits we observed for the same boards, providing support for our hypothesis that wrong hole visits could facilitate the dissemination of *A. aggregata* spores, thus spreading chalkbrood disease. However, the treatment that combined color contrast with the 3D pat-



**Fig. 3.** Mean percentage of wrong hole visits by *M. rotundata* females nesting in five board designs (uniform, letters, checkered, 3D, and 3D/checkered). Treatments with different letters above them are significantly different (Tukey test, adjusted  $P \leq 0.05$ ).

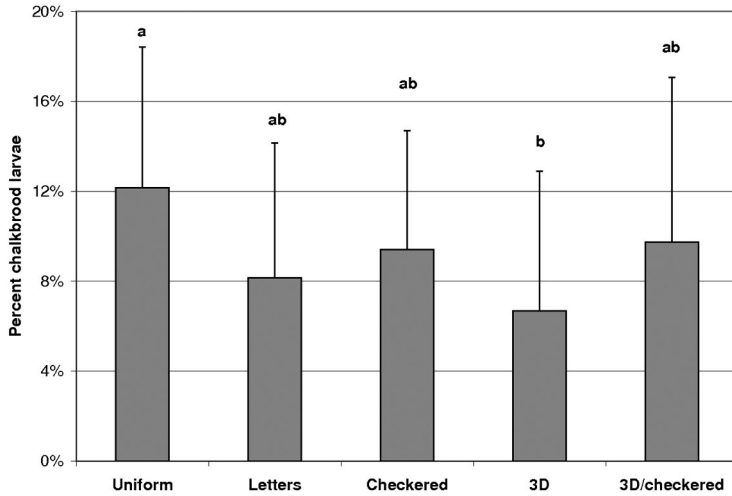


Fig. 4. Mean percentage of chalkbrood-infected larvae present in *M. rotundata* cells in five board designs (uniform, letters, checkered, 3D, and 3D/checkered). Treatments with different letters above them are significantly different (Tukey test, adjusted  $P \leq 0.05$ ).

tern resulted in decreased wrong hole visits, but they did not result in a significant decrease in chalkbrood. Also, we found no treatment effect on the mass of leaf pieces, our measure of the level of antagonistic interactions and confusion. It is difficult to draw any firm conclusions regarding whether females spread the disease during antagonistic interactions because we did not observe a significant difference in such interactions.

The percentage of broodless provisions found in our study averaged  $25.34 \pm 3.45\%$ , contrasting with lower percentages reported by Pitts-Singer (2004) and Bosch and Kemp (2005). We suspect that there may be a great deal of variability in the occurrence of this condition, depending on various management and weather conditions.

Confusion due to overpopulation, nest usurpation, and bee aging could explain the presence of broodless provisions. Overpopulation leads to confusion and disorientation when *M. rotundata* females attempt to locate their nest (Stephen 1981, Guédot et al. 2003), with some females occasionally abandoning their nests after extensive searches (unpublished data). Providing a limited number of nesting cavities leads to competition among females and nest usurpation, with females often removing the larva present in the cell (Gerber and Klostermeyer 1972). Third, pollen balls, including broodless provisions, were found predominantly in the cells produced late in the season (O'Neill 2004), supporting observations showing that older (>30 d) females do not always lay an egg after completing the provision (Tirgari 1963).

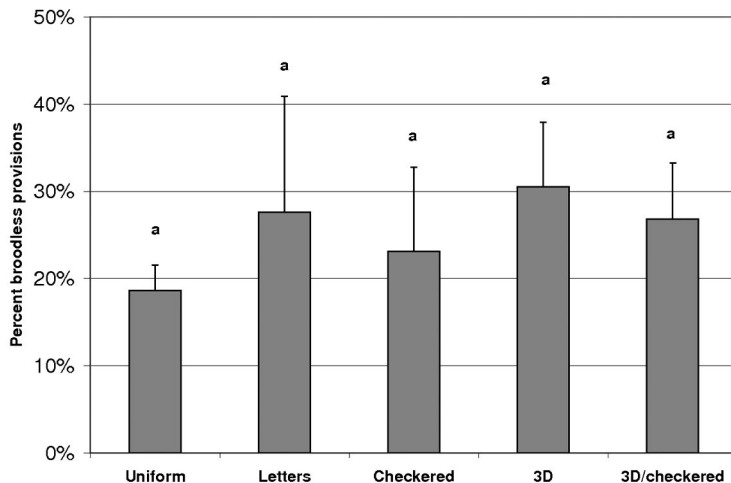


Fig. 5. Mean percentage of broodless provisions present in *M. rotundata* cells in five board designs (uniform, letters, checkered, 3D, and 3D/checkered). Treatments with different letters above them are significantly different (Tukey test, adjusted  $P \leq 0.05$ ).

Our study suggests that providing 3D configurations to commercial nesting boards, by either separating the boards or by implementing 3D boards, allowed *M. rotundata* females to improve their nest location performance. Furthermore, the percentage of chalkbrood-infected larvae found in *M. rotundata* cells seemed to decrease with the 3D board design. These results could have important implications on pollination efficiency and brood production. Indeed, improving nest location performance of *M. rotundata* females leads to a decrease in the time spent locating the nest, thereby allowing females to spend the extra time pollinating alfalfa flowers.

### Acknowledgments

Special thanks go to T. Pitts-Singer for fruitful conversations and S. Durham for great help with the statistics. We also thank G. Trostle, F. Sgolastra, and S. Crouzet for essential help throughout the study. We are grateful to our collaborators. Funding was provided in part by the Northwest Alfalfa Seed Grower Association.

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*Received 2 August 2005; accepted 10 February 2006.*

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