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Learning Ability and Factors Influencing Nest Establishment of the Solitary Bees Osmia lignaria and Megachile rotundata (Hymenoptera: Megachilidae)

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LEARNING ABILITY AND FACTORS INFLUENCING NEST ESTABLISHMENT OF THE SOLITARY BEES *OSMIA LIGNARIA* AND *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE)

by

Cory A. Vorel

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ABSTRACT

Learning Ability and Factors Influencing Nest Establishment of the Solitary Bees *Osmia lignaria* and *Megachile rotundata* (Hymenoptera: Megachilidae)

by

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Utah State University, 2010

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Over the last several decades, the use of solitary bees as an alternative to honey bees (*Apis mellifera* L.) for pollination of commercial crops has increased, in part as a response to ongoing problems faced by commercial honey bee populations. Two solitary bee species have exhibited great commercial potential: the blue orchard bee, *Osmia lignaria* Say, and the alfalfa leafcutting bee, *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae). However, growth of *O. lignaria* and *M. rotundata* populations is limited in commercial systems, mainly due to low establishment of females at provided nesting sites, possibly due to mortality, dispersal, or other causes.

Rough handling of pre-emergent bees may possibly contribute to post-emergence dispersal in *O. lignaria*. The current work addressed this hypothesis by using shaking as a proxy for rough handling. However, shaken bees did not establish fewer nests than unshaken bees. Therefore, commercial fruit growers using *O. lignaria* as pollinators

should be able to remove cocoons from their nests as part of their management plan, without fear of increasing bee dispersal.

When searching for a nest site, *M. rotundata* females are known to be attracted to previously used nest materials. The current work verified the attraction of *M. rotundata* females to old conspecific nests. It also sought to determine which nest components were most attractive to females. It was found that all components were equally attractive.

It may be useful to establish these species' learning abilities in a laboratory setting. The current work attempted to design a conditioning protocol for solitary bees. Initially, a method utilizing the proboscis extension reflex was sought. However, *O. lignaria* and *M. rotundata* did not reflexively extend their proboscises upon antennal stimulation with sucrose solution. Therefore, another method of conditioning was implemented. Bees were conditioned to respond to floral odors in a feeding bioassay. Results are compared for both species, as well as for males and females.

The research completed for this dissertation may provide helpful information for commercial managers of solitary bees seeking to decrease both bee dispersal and the incidence of disease and parasites.

(139 pages)

DEDICATION

To my wonderful sons, Malaki and Ethan. You are amazing, despite me. You make me proud every day, and I love you.

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CHAPTER 1

INTRODUCTION

Over the last several decades, the use of solitary bees as an alternative to honey bees (*Apis mellifera* L.) for pollination of commercial crops has increased, in part as a response to ongoing problems faced by commercial honey bee populations and pollinators in general (National Research Council, 2007). Two solitary bee species have exhibited great commercial potential: the blue orchard bee, *Osmia lignaria* Say, and the alfalfa leafcutting bee, *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae). With its strong preference for fruit tree flowers and its tendencies to fly in cooler weather and to cross-pollinate by frequently moving between trees, *O. lignaria* is a proficient pollinator for commercial orchards (Bosch and Kemp, 2001). Since the 1960's, *M. rotundata*'s efficient handling of alfalfa flowers and straightforward maintainability has made it an important pollinator for the commercial production of alfalfa seed (Bohart, 1957; Stephen and Torchio, 1961; Richards, 1984; Torchio, 1987).

Both species construct their nests within existing cavities, such as holes left in wood by beetles. *Osmia lignaria* nests in the spring, while *M. rotundata* nests in the summer, but the two species build similar nests. Females create nests consisting of linear rows of cells. Each cell is provisioned with pollen and nectar, upon which an egg is deposited. *Osmia lignaria* females separate adjacent cells with mud partitions, and mud is also used to plug completed nests (Torchio, 1989). *Megachile rotundata*'s cells are surrounded with cut leaf pieces, and completed nests are plugged with leaf discs (Richards, 1984).

In agricultural settings, *O. lignaria* and *M. rotundata* readily nest in cavities in provided wooden or polystyrene blocks, reeds, or cardboard straws (Richards, 1984; Bosch and Kemp, 2001; Frank, 2003). Females nest gregariously, and are attracted to previously used nests (Bohart, 1962; Torchio, 1976, 1981; Fairey and Lieverse, 1986; Fairey and Lefkovitch 1993). Each female produces several nests, laying multiple female and male eggs (Richards, 1984; Bosch and Kemp, 2002). Therefore, populations should be easily sustained and possibly expanded in agricultural settings. In fact, *O. lignaria* commercial populations have been known to increase by two-fold or more each year (Torchio, 1985; Bosch et al., 2006).

However, *O. lignaria* population growth is limited in commercial systems, mainly due to low establishment of females at provided nesting sites (Bosch and Kemp, 2002). Low establishment in *Osmia* may be attributed to post-emergence mortality (Tepedino and Torchio, 1982; Bosch, 1994a; Bosch and Kemp, 2004; Bosch, 2008), but another potential cause is pre-nesting dispersal. *Osmia lignaria* populations are commonly removed from their natal nests and placed in orchards as loose cocoons, a practice that has been repeatedly shown to result in increased dispersal (Maeta, 1978; Torchio, 1985; Bosch, 1994b). Why bees released as loose cocoons tend to disperse remains a mystery, but one hypothesis is that the manipulation of removing the cocoons from the nests and the subsequent handling of the loose cocoons is more stress than the bees can tolerate.

Commercial populations of *M. rotundata* in the United States can also be difficult to maintain, and field managers are forced to supplement their populations annually. Many challenges, such as chalkbrood (fungal) disease, parasitism, emergence of a

summer generation, and immature mortality from unknown causes, must be overcome in order to maintain commercial populations (Richards, 1984; Frank, 2003). Also, far more female bees are released than actually establish nests at commercial sites (Peterson et al., 1992; Pitts-Singer, unpublished). In addition to post-emergence mortality, pre-nesting dispersal has also been implicated as a possible explanation for poor establishment (Pitts-Singer, unpublished). *Megachile rotundata* may disperse in an effort to escape overcrowded conditions. They may also perceive the commercial materials that are provided as less than suitable, and may disperse in search of more attractive natural nesting sites.

It has been well-documented that solitary bees searching for a nesting site are attracted to areas where active nesting is already occurring or where old nests exist (Michener, 1960; Cardale, 1968; Stephen et al., 1969; Michener, 1974; Eickwort et al., 1977; Buttery et al., 1981; Parker et al., 1983; Fairey and Lieverse, 1986). Although in many cases it is true that solitary bees choose to remain near the natal nest, often the criteria for nest selection is simply previous or current use of a nesting site by conspecifics. Michener (1960) and Cardale (1968) both give accounts of solitary, yet gregarious, bees being attracted to active nests and old sites. The ability of active nests or old nests to attract bees is frequently exploited for the encouragement of population establishment. Bohart (1962) advised bee managers seeking to establish *M. rotundata* at commercial domiciles to intersperse new nesting blocks with some blocks containing completed nests, while Parker et al. (1983) suggest sprinkling a few loose cells around new nesting blocks to attract females.

The fact that *M. rotundata* females are attracted to old nest materials from a previous season indicates that at least some short-range chemical (olfactory) cues persist over time. Buttery et al. (1981) identified several volatile compounds present in old *M. rotundata* cells. In preliminary tests, both caryophyllene epoxide and a mixture of caryophyllene epoxide, caryophyllene, and 2-phenylethanol were tested for their ability to attract nesting females. Buttery et al. were able to get more nesting in the new, treated nesting materials than in either new, untreated nesting materials or old nesting materials, but the results were not significant. These tests were expanded by Parker et al. (1983), who found that old cells were attractive to nesting *M. rotundata*, but neither of the compounds tested by Buttery et al. (1981) was significantly attractive. Perhaps the volatility of the compounds tested was a factor, or perhaps these compounds are simply not what the bees find attractive. More recently, the attraction to unidentified odors of old nest materials has been addressed in laboratory assays that revealed attraction to certain nest components and extracts of nest components (Pitts-Singer, 2007).

Establishment and expansion of *M. rotundata* populations could possibly be enhanced by taking advantage of their attraction to previously used nest materials. However, use of old materials may increase the incidence of parasites and pests, as well as enhance the spread of bee diseases (Bohart, 1971; Vandenberg and Stephen, 1982; Bosch and Kemp, 2001; Pitts-Singer, 2004). If the compounds responsible for the attractiveness of old nest materials could be identified, it may be possible to develop a method of treating new nest materials to make them more attractive to nesting females, thus increasing commercial populations without increasing parasites and disease.

In experiments with the solitary bee *Colletes fulgidus longiplumosus*, Dobson (1987) found that naïve bees preferred the plant species whose pollen they ate as larvae. She attributed this result to olfactory chemical conditioning of the developing bee within the natal nest. It has been reported that *M. rotundata* will nest in materials similar to those that they were reared in, even if other nesting materials are available, and that this may be a conditioned response (Stephen, 1962; Stephen et al., 1969). I believe that *O. lignaria* and *M. rotundata* may experience a form of imprinting on the olfactory cues present while they are developing in the nests, which then influences their nest choice later in life.

In the future, the possibility that the experiences of *O. lignaria* and *M. rotundata* within the natal nest influence their nesting behavior upon emergence should be addressed. However, first an important initial step is to establish their learning abilities in a controlled laboratory setting. Conditioning of honey bees has become a common way to examine their learning and cognitive abilities, including their neurophysiological and molecular attributes. Especially common is the use of respondent conditioning utilizing the proboscis extension reflex (PER) (Takeda, 1961; Bitterman et al., 1983; Giurfa, 2007). This conditioning method takes advantage of the honey bee's reflexive extension of its proboscis upon antennal stimulation with sucrose. This method has also been used to demonstrate the learning abilities of bumble bees (Laloi et al., 1999; Laloi and Pham-Delègue, 2004) and stingless bees (Abramson et al., 1999; McCabe et al., 2007). The design of a conditioning protocol for solitary bees, possibly utilizing the PER, would be useful for many reasons. First, it would help us understand the learning abilities of these

bees, paving the way for future exploration of learning during development within the natal nest. Second, it may provide useful information toward developing methods of increasing commercial population retention, such as training bees to nest in provided materials. Finally, knowledge gained from conditioning studies may be used to compare species' evolutionary and developmental pathways.

The initial focus of my dissertation research was the minimization of dispersal in *O. lignaria* and *M. rotundata*. In the second chapter, I investigate one factor that may contribute to *O. lignaria* dispersal: rough handling by growers prior to bee release. Bees were shaken as a proxy for rough handling, and nest establishment of shaken and unshaken bees was compared.

In the third chapter, I examine the role of olfactory cues present in old nests, which *M. rotundata* may use for nest selection. The attraction to old nests was verified using intact cells. The attraction to the individual components of nests was also explored, so that it could be determined if females were more attracted to some components than they were to others. Females' nest selections were compared in both field cage assays and open field assays.

The fourth and fifth chapters explore conditioning of *O. lignaria* and *M. rotundata*. First I attempted to use the PER in developing a method of conditioning *O. lignaria* and *M. rotundata*. This proved to be impossible because these species did not reflexively extend their proboscises in response to sucrose stimulation of their antennae. Therefore, I developed a novel approach to conditioning *O. lignaria* and *M. rotundata* using a feeding bioassay. Using this method, I was able to condition bees using floral

odors, exploring their learning and discriminatory capabilities. I was also able to compare the performances of males and females, as well as comparing the two species' performances.

The sixth chapter briefly summarizes the results and discusses the implications of this study, while considering possible future directions.

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CHAPTER 2

INFLUENCE OF ROUGH HANDLING ON BLUE ORCHARD BEE (*OSMIA LIGNARIA*) NEST ESTABLISHMENT[1]

Summary

The blue orchard bee, *Osmia lignaria* Say, is a promising alternative pollinator for fruit trees. Commercial *O. lignaria* populations can be sustained in agricultural settings and have been known to increase by two-fold or more each year. However, some females fail to establish at the provided nesting sites, which may be attributable to pre-nesting dispersal. Dispersal has been repeatedly found to increase when *O. lignaria* populations were placed in orchards as loose cocoons (extricated from their nests), which subjects pre-emergent bees to excessive handling. In this study we addressed the hypothesis that excessive or rough handling of pre-emergent adult blue orchard bees results in a decreased number of females that establish nests at the site from which they emerged. We tested this hypothesis by severely shaking bees (as a proxy for rough handling) and subsequently monitoring nest establishment of shaken bees, as well as unshaken bees. There was no significant difference in the number of shaken and unshaken females that nested. Based on the results of this experiment, rough handling does not discourage nest establishment. This is welcome news for *O. lignaria* mass producers who desire to control pathogens and parasites by removing healthy bees in their cocoons from their nests for winter storage.

[1] Coauthored by Cory A. Vorel and Theresa L. Pitts-Singer

Introduction

According to a recent report from a national scientific council, pollinators are imperiled and thus, the ability to commercially produce certain fruits and vegetables may be in jeopardy (National Research Council, 2007). One recommendation afforded by this council to alleviate the dependence and burden of pollination services provided by honey bees, *Apis mellifera* L., is to develop other reliable sources of pollination. For some tree fruit crops, the blue orchard bee, *Osmia lignaria* Say, is a very effective pollinator. These bees have a strong preference for fruit tree flowers and facilitate cross-pollination by frequently moving between trees. Also, they are able to forage during weather conditions that are not amenable to other bees. Compared to *A. mellifera*, fewer *O. lignaria* are required per hectare to maximize crop yield (Bosch and Kemp, 2002). Because of their superior pollination efficiency and the ongoing problems faced by commercial *A. mellifera* populations, interest in the use of *O. lignaria* as pollinators of commercial orchards has increased in recent years.

Osmia lignaria is a solitary bee that constructs nests within existing cavities, such as holes left by beetles in wood. In the spring, female bees create nests consisting of linear rows of cells. Each cell is provisioned with pollen and nectar, upon which an egg is deposited. Adjacent cells are separated by mud partitions, and mud is also used to plug completed nests (Torchio, 1989). Brood develop throughout the summer, becoming adults by late fall. They then enter winter diapause as adults in cocoons and emerge from cocoons the following spring (Torchio, 1989; Kemp et al., 2004). In agricultural settings, *O. lignaria* readily nest in cavities in provided wooden blocks (Bosch and Kemp, 2001).

Females tend to nest gregariously, and are attracted to previously used nests (Torchio, 1976, 1981). Each female produces several nests, laying an average of two to four female eggs and five to eight male eggs in each nest (Bosch and Kemp, 2002). It follows that if a minimum of 50% of the released population successfully reproduces within the orchard, then *O. lignaria* populations can be sustained in agricultural settings. In fact, commercial populations have been known to increase by two-fold or more each year (Torchio, 1985; Bosch et al., 2006). However, in each population released, some females fail to establish at the provided nesting sites, and low establishment is the main factor limiting *O. lignaria* population growth in commercial systems (Bosch and Kemp, 2002). Low establishment in *Osmia* can be in part attributed to post-emergence mortality that results from natural causes (e.g., vertebrate predation or extreme weather) or from consequences of suboptimal management that lead to bees emerging in a weakened condition (Tepedino and Torchio, 1982; Bosch, 1994a; Bosch and Kemp, 2004; Bosch, 2008).

However, an unknown fraction of poor establishment is attributable to pre-nesting dispersal. Dispersal of pre-nesting females appears to increase when populations are released in areas with inadequate bloom, i.e., floral resources (Maeta, 1978). In addition, dispersal has been repeatedly found to increase when *O. lignaria* populations were placed in orchards as loose cocoons (extricated from their nests), rather than as cocoons still in natal nests (Maeta, 1978; Torchio, 1985; Bosch, 1994b). No study has yet explained why the release of bees as loose cocoons results in their low retention at commercial

sites. The exposure of the bees to an intolerable amount of mechanical stress caused by manipulation (nest dissection and handling of loose cocoons) is one hypothesis.

In this study we addressed the hypothesis that excessive or rough handling of pre-emergent adult blue orchard bees results in a decreased number of females that establish nests at the site from which they emerged. We tested this hypothesis by severely shaking bees as a proxy for rough handling and subsequently monitoring nest establishment of shaken bees, as well as unshaken bees.

Materials and Methods

Blue orchard bees were collected from wildlands and rural areas in Northern Utah by trap-nesting, a procedure whereby wooden blocks are placed in trees or in manmade shelters in areas where bees can easily find and use them. Each block contains several drilled holes that form nest cavities into which paper drinking straws are inserted. Bees construct nests in these straws, which can then be removed for easy monitoring and manipulation.

Bee-filled trap-nest straws collected for this study were taped to 8 x 10 plastic boards and placed in plastic storage boxes. Periodically, x-rays of the nests in straws were made to monitor bee development. One week after all of the bees reached adulthood, they were moved to a 4°C cold room for wintering, according to standard protocol (Bosch and Kemp, 2001). Over the winter months, straws were x-rayed to determine the number, location, and sex of adult bees in each nest. The outside of the straws was marked to indicate where bee cells were positioned and what sexes were

present in them. Straws were sliced longitudinally along one side to allow access to nest contents for parasite removal and later monitoring of bee emergence. Bees from all collection sites were then divided equally into two treatment groups, “shaken” and “unshaken,” and returned to plastic storage boxes kept in the cold room.

In April 2007 and 2008, 10 wooden shelters (61 cm x 61 cm x 61 cm) were placed in a River Heights, Utah apple orchard. Shelters were evenly spaced to create two rows (approx. 24 m apart) of five shelters (approx. 15-20 m apart), with all of the shelter openings facing southeast. Ten wooden nesting blocks, each having 49 – 56 drilled holes, were placed in each shelter to provide nesting sites for bees. Paper drinking straws of the same diameter as the holes were inserted into all of the nesting block holes.

Each year the progression of apple bloom was monitored to predict an appropriate time to place the wintered bee nests in the orchard (from this point forward referred to as “bee release”) and to record the number of flowers available to foraging bees (Fig. 2-1). Five trees of each variety being monitored were randomly chosen every third day throughout the experiment. Five branches on different parts of each tree were selected and the number of flowers in each of four designated categories was counted. The categories were tight bud, pink bud, open flower, and flowers with petals dropped. In 2007, three varieties were monitored (those closest to the nest shelters, covering an area of approx. 72 x 74 m). Those three varieties happened to be strongly biennial and, therefore, bloomed heavily in 2007, but sparsely in 2008. Consequently, we expanded our bloom monitoring in 2008 to include seven varieties, the three varieties monitored in 2007 plus four more varieties located further from the shelters. All trees monitored in

2007 ranged in distance from 1 to 113 m from the shelters (covering an area of approx. 140 x 170 m). Daily maximum and minimum temperatures and precipitation for Utah State University (2 km from the orchard) were downloaded from the Utah Climate Center website (http://climate.usurf.usu.edu/products/data.php, accessed 3 February 2009) (Fig. 2-1).

Ten days prior to the projected date of bee release (determined by monitoring bloom), the plastic boxes containing nests were removed from the cold room. For simulation of a single bout of "rough handling," the box containing the bees that had been designated as the "shaken" treatment group was placed in a shaker incubator (Innova 4230, Brunswick Scientific, Edison, NJ) set at 25°C and shaken at 200 rpm for 2 min. During this time, the plastic box containing the "unshaken" treatment group remained at room temperature. The boxes were then returned to the 4°C cold room. This procedure was repeated three days prior to bee release.

Bees in nests from each treatment group, shaken and unshaken, were divided between the 10 shelters, such that each shelter received a similar number of females. The nest straws of the shaken bees were placed in the top rows of holes of a wooden block situated in the top row of blocks in each shelter. The nest straws of the unshaken bees were similarly placed in a block adjacent to the shaken bees. Any remaining holes in these blocks contained new, unused straws, as did the other eight blocks in each shelter.

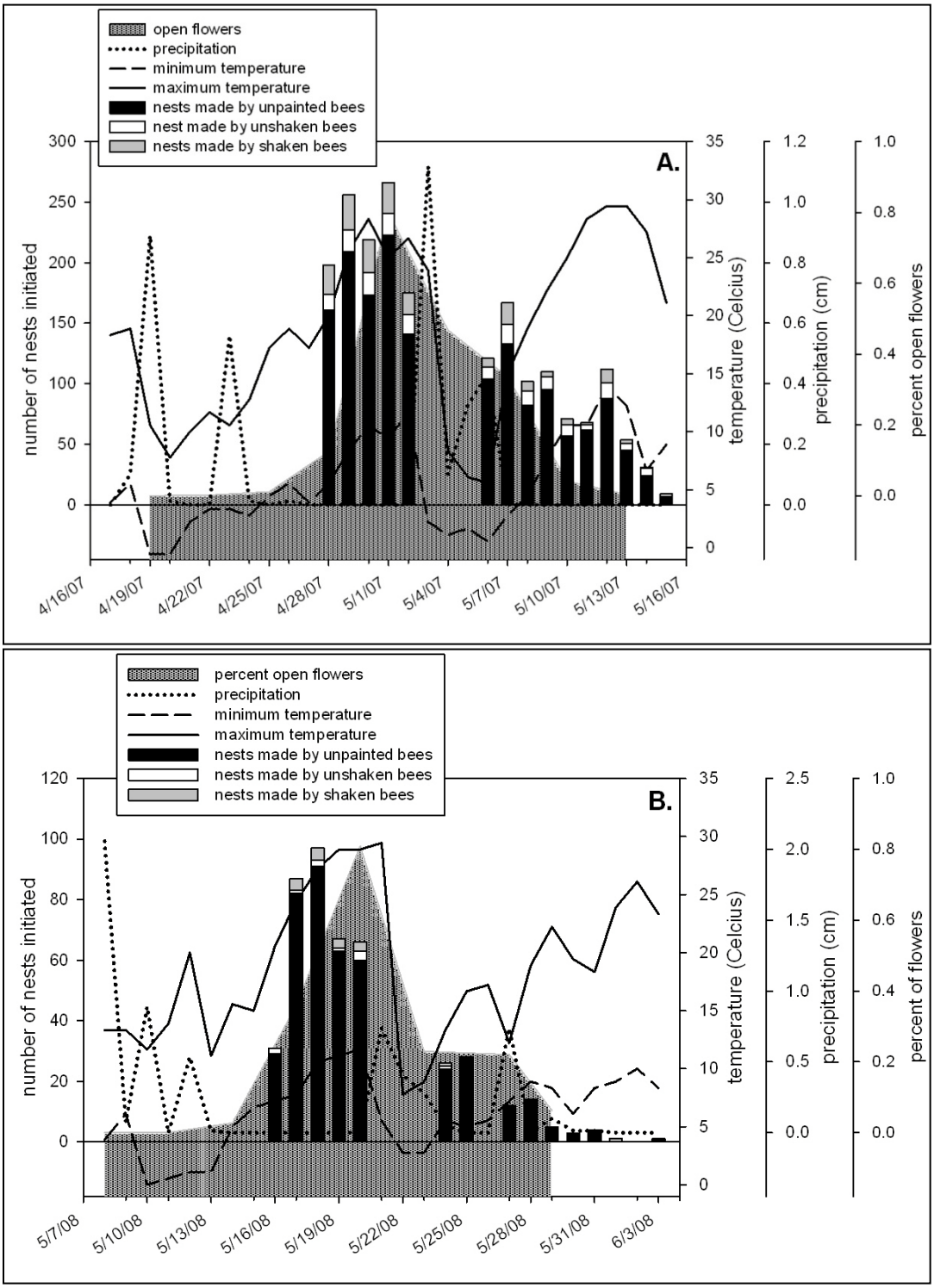

Figure 2-1. Maximum and minimum temperatures, precipitation, and proportion of open apple flowers in 2007 (A) and 2008 (B). Also, the number of nests initiated daily by shaken, unshaken, and unpainted *Osmia lignaria* females in 2007 (total no. nests initiated = 1959) and 2008 (total no. nests initiated = 442).

Beginning the day after the nest straws were placed in the shelters, bees were checked twice daily (approx. 1000 – 1200 hr and 1300 – 1500 hr MST) for emergence from the cocoon. Each nest straw was carefully removed from the block, and because the straws had previously been cut longitudinally, it was easy to peek inside and look for bees that had emerged or were in the process of emerging. If a female was chewing out of the cocoon or had already emerged, she was anesthetized with CO_2 sprayed from a hand-held Air Dr.® air blaster (Digital Innovations, Arlington Hts., IL). Two dots of paint were placed on the bee's thorax: one dot was either red (shaken bees) or green (unshaken bees); the second dot indicated the female's shelter of release. After paint-marking, each female was returned to the same cell position in the nest, which allowed her to crawl out through the straw in a natural manner when she was ready to do so. Some females emerged and left the nest undetected and, therefore, were not painted (Table 2-1).

Table 2-1. For 2007 and 2008 for each treatment group, the number of females that emerged from natal nests, the number of females that were paint-marked, the estimated number of paint-marked females that nested in the provided shelters, and the percent of established females (paint-marked bees that nested).

	2007		2008	
	unshaken	shaken	unshaken	shaken
No. females released	319	317	429	428
No. females paint-marked	238	254	339	347
No. paint-marked females established	142	144	9	15
% paint-marked females established	60%	57%	3%	4%

Painted females began investigating holes in the wooden nesting blocks, in search of a nest site, 7-10 days after the initial bee release. Observations of the shelters for nesting activity began the day after painted females were first seen checking holes. At

this time, early observations of nesting bees occurred simultaneously with the paint-marking of late-emerging bees as described above; observations continued once paint-marking was completed. To monitor nesting bees, each shelter was observed for 20 minutes in the morning (approx. 1000 – 1200 MST) and again in the afternoon (approx. 1300 -1500 MST). A bee was considered to have established a nest only if she was actively provisioning a cell with pollen. When an established bee was identified, the colors painted on the bee and the location of her nest were recorded. Nesting activity of unpainted bees was also recorded (Fig. 2-1). In 2007, nesting activity was recorded until the orchard was sprayed with insecticide, allowing for 18 days of nesting observations (Fig. 2-1A). In 2008, nesting activity was recorded until bees ceased initiating new nests, for a total of 19 days of observations (Fig. 2-1B).

Because each female bee creates multiple nests, and because bees of the same treatment and shelter received the same color paint-marks, we could not know definitively whether similarly marked bees initiating new nests had been previously observed at the same shelter. However, we were able to estimate the number of nesting females (Table 2-1). This was done by evaluating the paint-marks of the female seen creating each nest; if adjacent nests were created successively by females marked with the same colors, it was assumed that all of the nests were created by the same female. ANOVA (Proc GLM, SAS 9.2) was used to determine if treatment or year had an effect on 1) the proportion of paint-marked females released at each shelter that nested at any of the provided shelters in the orchard and 2) within each shelter, the proportion of paint-marked females released at each shelter that nested at that shelter.

Results

The maximum and minimum daily temperatures, the centimeters of daily precipitation, and the proportion of flowers that were open and available for foraging during the study period were different in timing, but not pattern, for the two years of the study (Fig. 2-1). In 2007, the trees began blooming on 25 April. In 2008, bloom came later, on 14 May. In both years, several days with warm, dry weather and many open flowers were accompanied by numerous nest initiations. The dry days were followed by several days of colder, wetter weather coinciding with a decline in the number of open flowers and as well as nesting activity. Although drier, warmer days were subsequently observed, the number of available flowers and nest initiations continued to decline. Nesting by all bees, painted and unpainted (whether released by us or naturally occurring), took place for the same amount of time in both years (Fig. 2-1).

Fewer bees were released in 2007 than in 2008, but a larger percentage of bees established nests in 2007 than in 2008, for both unshaken and shaken females (Table 2-1). In 2007, the mean proportion of shaken females nesting at any of the 10 available shelters was similar to the mean proportion of unshaken nesting females (Fig. 2-2). In 2008, fewer females were observed nesting, but the proportions of shaken and unshaken females were again similar (Fig. 2-2). A significant difference was found between the two years in the proportion of females paint-marked at each shelter that nested at any of the shelters in the orchard ($F = 55.82$, $df = 1, 37$, $P < 0.01$), but there was no treatment effect ($F = 0.05$, $df = 1, 37$, $P = 0.83$).

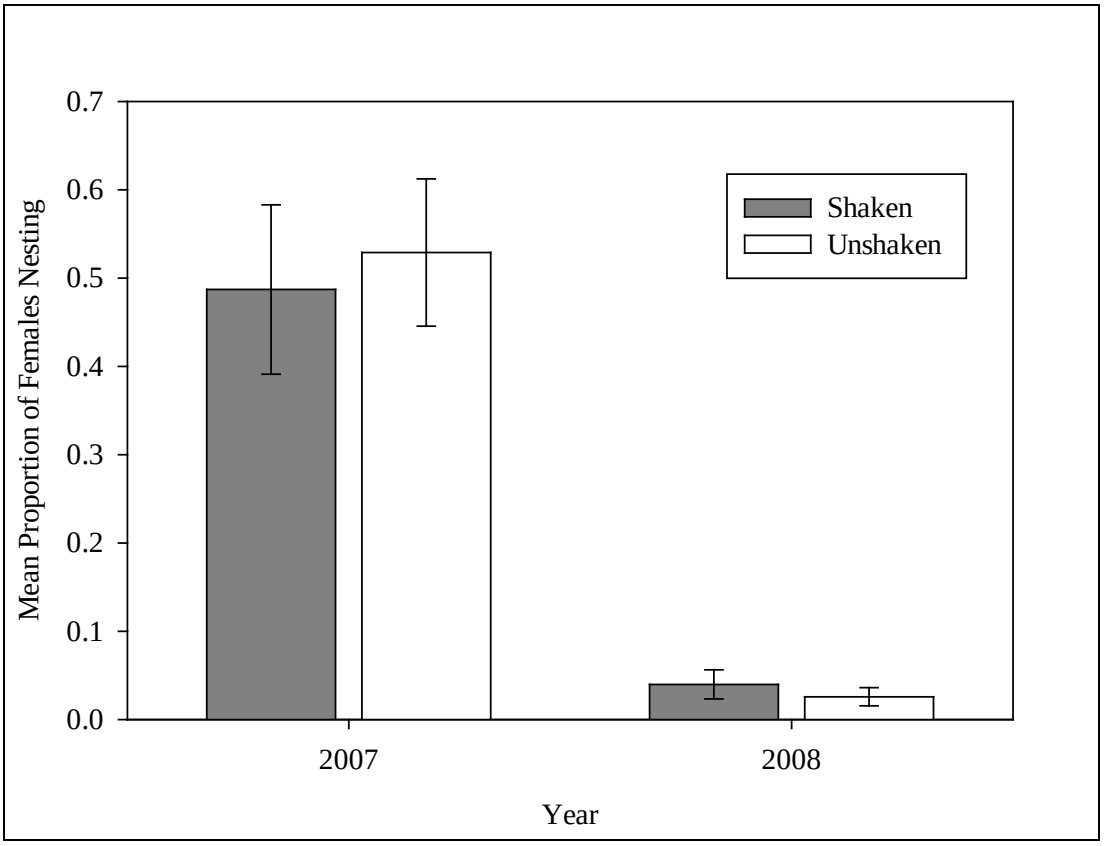

Figure 2-2. The mean proportion (±SE) of shaken and unshaken paint-marked *Osmia lignaria* females that nested at any of the shelters within an apple orchard in 2007 and 2008.

In examining the proportion of females paint-marked at each shelter that nested at the shelter from which they were released, once again shaken and unshaken females did not differ from each other in either 2007 or 2008, but the mean for both treatment groups decreased from 2007 to 2008 (Fig. 2-3). The proportion of females paint-marked at each shelter that nested at that shelter was significantly affected by year ($F = 24.93$, $df = 1, 37$, $P < 0.01$), but not by treatment ($F = 0.03$, $df = 1, 37$, $P = 0.87$).

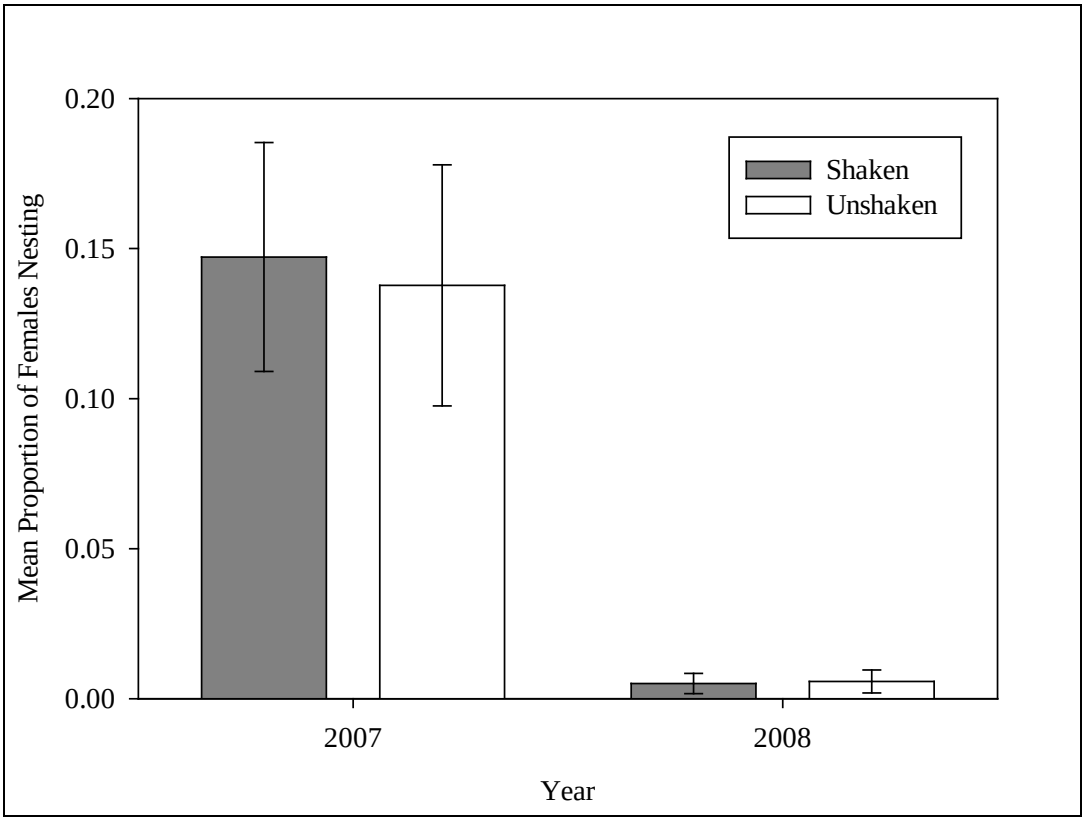

Figure 2-3. The mean proportion (±SE) of shaken and unshaken paint-marked *Osmia lignaria* females that nested at the shelter from which they were released in an apple orchard in 2007 and 2008.

Discussion

Philopatry, as defined here for female bees, is the tendency for a female bee to nest in the same area as the natal nest from which she emerged, and is believed to be an important influence on female bees during nest site selection (Malyshev, 1936; Michener et al., 1958; Yanega, 1990; Potts and Willmer, 1997; Soucy, 2002; Bischoff, 2003). It has been suggested that bees remain in natal areas to avoid a potentially costly search for resources such as suitable nest sites and mates (Michener et al., 1958; Bischoff, 2003). Some degree of philopatry is expected in *Osmia lignaria* populations. Not only are these

gregarious bees attracted to previously used nests (Torchio, 1976, 1981; Pitts-Singer, 2007), but because they spend the winter as adults in cocoons, they are able to emerge from their natal nests relatively quickly in response to improving environmental conditions in the spring. At the time of *O. lignaria* adult emergence, foraging resources and suitable pre-existing nest sites may be limited, so if these resources are available near the natal nest, blue orchard bees should tend to stay, thus conserving energy and maximizing brood production.

While *Osmia lignaria* populations have often been significantly increased in orchards, establishment of enough females to maintain commercial populations through successful brood production is considered one of the main factors limiting population growth in agricultural environments (Bosch and Kemp, 2002). Despite their predicted philopatric tendency, perhaps female bees are compelled to disperse because they sense an unacceptable level of competition for resources or peril from parasites, predators, or disease. It is also possible that some *O. lignaria* in a population are simply genetically prone to disperse. Additionally, a higher than expected level of post-emergence mortality could be mistaken for dispersal.

Our study considered a mechanical factor as a dispersal-inducing mechanism. Excessive handling of commercial populations prior to emergence, such as removal of cocoons from nests, rough handling of nests or loose cocoons, and shipment of nests or cocoons, may encourage females to disperse from the release site after emergence. Our results show that females subjected to excessive shaking prior to emergence created the same mean proportion of nests in the orchard and shelters as females that were not

shaken. Also, there was no difference between shaken and unshaken females in their likelihood to nest at the same shelter from which they emerged. Therefore, our experiment affords no evidence that rough handling of pre-emergent bees influences future nest establishment at provided shelters. An alternative explanation may be that during the process of emerging out of the natal nest and crawling through cocoons and nest debris, females are exposed to some unknown cues that affect their philopatric response, which are lost when bees are released as loose cocoons.

The similar nesting response of the bees in the two treatment groups also begs the question of whether our post-shaking methodology obscured the treatment. We subjected the shaken group of bees to what we considered an excessive amount of mishandling (two bouts of violent shaking). The shaken group undoubtedly sensed the effects of the treatment, despite the fact that both treatment groups eventually had to be handled to place nests in the orchard and to paint female bees. It is possible that the level of handling involved in setting up the experiment and in paint-marking the bees surpassed the threshold of handling that would result in bee dispersal, causing the unshaken group of bees to be just as unlikely to nest at the release site as the shaken group. The use of CO_2 to anesthetize females for paint-marking also may have influenced nest establishment. However, Guédot et al. (2009) used CO_2 to anesthetize actively nesting *O. lignaria* females for paint-marking in a homing ability experiment, and this technique did not seem to affect the ability of bees to return to their nests after being displaced up to 1200 m. Paint-marking was important in the current study; not only was it necessary for discerning shaken from unshaken bees, but also for precisely identifying which observed

O. lignaria were released for the experiment and which came to the orchard from the surrounding environment. An alternative procedure, such as allowing bees to emerge under laboratory conditions, to be paint-marked in a cold room, and then to be transported to the orchard for mass release, is known to be even more disruptive to the bees (Pitts-Singer, personal observations). Except for the treatment, our methods were designed for minimal disturbance and stress, with natural emergence from natal nests. In fact, the estimated percent establishment in 2007 was at a level considered to be sustainable; because every established female in 2007 should have laid two to four female eggs, the brood produced should have been of equal size to, or larger than, the parental stock.

The disparity between the two years of study, both in the percent *O. lignaria* establishment and in the number of wild, non-experimental bees that were present, is perplexing, especially because the environmental conditions were similar. The orchard where this research was conducted contains several varieties of fruit trees that bloom on both annual and biennial cycles. Our nest shelters happened to be directly surrounded by strongly biennial varieties of apple trees, which bloomed heavily in 2007, but sparsely in 2008. However, *O. lignaria* can forage on a variety of flowering plants (Bosch & Kemp, 2001), and bees were seen foraging on wildflowers within the orchard in addition to other fruit tree blossoms. In 2008, bees were also observed collecting pollen and nectar from distant, abundant apple and fruit trees within the orchard compound (approximately 200 x 300 m^2). Females may have had to travel further for flowers in 2008 than in 2007, but the distance was well within the 1200 m homing distance demonstrated for *O. lignaria*

(Guédot et al., 2009). Therefore, it is not likely that bees dispersed in 2008 due to lack of local floral resources or inability to orient back to their nests after foraging trips.

It is possible that fewer females established nests in 2008 than in 2007 due to increased mortality in 2008. An unexplained three-fold increase in winter mortality was observed in 2008, compared to winter mortality in 2007. If bees experienced higher mortality in winter 2008, it is possible that post-emergent bees also experienced higher mortality, resulting in fewer established nests that year. Populations in both years included the progeny of wild bees from the same orchard. Therefore, in both 2007 and 2008, our experimental populations would be similar to wild populations. This may explain why decreased nest establishment was seen in both experimental and wild populations in 2008; both may have experienced increased post-emergence mortality.

Although we cannot be sure, one potential explanation for the observed differences in winter mortality for the two years could be the different timing for the arrival of spring in the two years. Because warm temperatures came later in 2008 than in 2007, both wild and experimental bee populations emerging in 2008 would have had a 2-3 week longer overwintering period than populations emerging the previous spring, possibly increasing mortality in the 2008 experimental population.

If large scale *O. lignaria* commercialization for pollination is to be successful, it is essential to determine how to maintain and increase their populations. Based on the results of this experiment, rough handling does not discourage nest establishment. This is welcome news for *O. lignaria* mass producers who desire to control pathogens and parasites by removing healthy bees in their cocoons from their nests for winter storage.

Continued research should address the possibility that when bee cocoons are removed from their nests, they are limited in their exposure to cues within the natal nest that are important for future nest selection. Many bees use olfactory cues to locate previously used nests (Michener, 1960; Butler, 1965; Cardale, 1968; Batra, 1978; Parker et al., 1983; Pitts-Singer, 2007), and bee managers should consider how such cues are used in nest establishment and recognition.

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CHAPTER 3

ATTRACTION TO OLD NEST CUES DURING NEST SELECTION BY THE SOLITARY BEE *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE)[2]

Summary

The alfalfa leafcutting bee, *Megachile rotundata* F. (Hymenoptera: Megachilidae), is an important pollinator for the commercial production of alfalfa seed. However, poor nest establishment is an ongoing problem for bee managers. *Megachile rotundata* are solitary, yet gregarious bees that nest in pre-existing cavities. When selecting nest cavities, *M. rotundata* are attracted to previously used nests. Nests consist of a linear series of cells, each containing several components that may serve as cues for nesting females. In the current study, we sought to: a) determine if there is a preference for cells that previously held male or female conspecific bees, b) verify attraction to conspecific whole nest cells, and c) determine which individual nest components of a cell are attractive to nesting females. In a series of cage and open field experiments, *M. rotundata* females were allowed to initiate nests in blocks containing whole cells or individual cell components from old nests. Their nest choices were compared using ANOVA and REGWQ. Females were attracted to whole cells from old nests in both cage and open field studies. They were equally attracted to male and female cells. Also, they were equally attracted to whole cells from conspecifics and from another megachilid bee, *Osmia lignaria*. In cages, they were equally attracted to all cell components.

[2] Coauthored by Cory A. Vorel and Theresa L. Pitts-Singer

However, in the open field, females preferred some cell components over others. These results provide a foundation for future studies to identify potential chemical lures to aid in the retention of bee populations at commercial nest sites.

Introduction

Since the 1960's, the alfalfa leafcutting bee, *Megachile rotundata* F. (Hymenoptera: Megachilidae), has been an important pollinator for the commercial production of alfalfa seed (Bohart, 1957; Stephen and Torchio, 1961; Richards, 1984; Torchio, 1987). Alfalfa leafcutting bee managers in the United States struggle to maintain *M. rotundata* populations, resulting in the annual augmentation of these pollinators. Maintenance of bee populations is hampered by chalkbrood (fungal) disease, parasitism, emergence of a summer generation, and immature mortality resulting from unknown causes (Richards, 1984; Frank, 2003). Furthermore, field studies indicate that the number of female bees that establish nests at commercial sites falls far short of the number of female bees released (100,000-150,000 bees/ha) (Peterson et al., 1992; Pitts-Singer, unpublished). The causes of poor *M. rotundata* nest establishment have not been elucidated, but at least two explanations are possible. First, the number of bees released in a field is excessive relative to nesting and floral resources, prompting bees to leave the overcrowded commercial site. Second, commercial nesting sites are not as attractive as natural nesting sites, and bees leave to find more preferable nesting substrates. If the number of bees released in a field is appropriate for the resources available, then it would

be important to assure that those bees remain at commercial sites where they can reproduce to create a sustainable population.

Megachile rotundata is a solitary, cavity-nesting bee that nests in aggregations. Female bees construct nest cells within existing cavities, such as those left in wood by beetles or holes and grooves in manmade structures. In agricultural settings, females readily nest in cavities made in large boards made of polystyrene or wood (Richards, 1984; Frank, 2003). In the summer, females create nests consisting of linear rows of cells. Each cell is surrounded with cut leaf pieces and provisioned with pollen and nectar, upon which an egg is deposited. Completed nests are plugged with leaf discs (Richards, 1984). Some brood develop to adulthood in 6-8 weeks. Other brood develop only to the prepupal stage (fifth instar) before entering winter diapause, completing development the following summer before emerging from cocoons as adults.

When selecting a nest, *M. rotundata* may use visual orientation cues (Guédot et al., 2005a, 2006, 2007), physical properties of nesting substrates, and both long- and short-range chemical cues. It is known that *M. rotundata* females are attracted to old nest materials from a previous season, indicating that at least some short-range chemical (olfactory) cues persist over time. *Megachile rotundata* will readily reuse cavities due to this attraction (Buttery et al., 1981; Parker et al., 1983; Fairey and Lieverse, 1986). The attraction to unidentified odors of old nest materials has been addressed in laboratory assays that revealed attraction to certain nest components (Pitts-Singer, 2007). Other previous studies identified volatile compounds (caryophyllene epoxide and a mixture of caryophyllene epoxide, caryophyllene, and 2-phenylethanol) present in old *M. rotundata*

cells, which were tested for their attraction in the field but were not found to be significantly attractive (Buttery et al., 1981; Parker et al., 1983).

In addition to reusing old conspecific nests, *M. rotundata* occasionally build nests in cavities that have previously been used by other solitary bees, such as the blue orchard bee, *Osmia lignaria* (Hymenoptera: Megachilidae) (Vorel, personal observation). It is assumed that *M. rotundata*'s use of old conspecific nests is based on attraction, but the use of old *O. lignaria* nests is based on opportunity. However, this has never been formally tested.

It is possible that the reuse of nesting boards for attracting bees could aid in the retention and expansion of commercial *M. rotundata* populations. However, use of old materials may increase the incidence of parasites and pests, as well as enhance the spread of bee diseases (Bohart, 1971; Vandenberg and Stephen, 1982; Bosch and Kemp, 2001; Pitts-Singer, 2004). Rather than simply reusing old materials to lure bees to nest cavities, it may be possible to increase the attractiveness of new nesting materials in commercial sites by identifying attractive olfactory cues and using them to develop lures.

Cues could originate from several components found in old *M. rotundata* nests. A pre-nesting female may detect these cues as she explores many empty cavities before choosing one as her nest. Nest components are provided in the previous year by females that made nests and by brood that developed in them. Contributions of a female bee include the individual nest recognition cue, possibly originating in the Dufour's gland, that she applies to the inside of the nest cavity once she has accepted it (Guédot et al., 2005b) and the leaf pieces she uses as nest partitions, linings, and end caps. She also

accumulates pollen and nectar before laying an egg. The developing bee contributes feces and a cocoon to the collection of nest components. If the bee does not complete development, it leaves behind remnants of pollen-nectar provisions. Any of these nest components could contain olfactory cues that cavity-seeking females find attractive and meaningful.

Pitts-Singer (2007) found in laboratory assays that *M. rotundata* females were significantly attracted to year-old conspecific whole nest cells, as well as two of the individual nest components, leaf pieces and feces. However, laboratory bioassays may not reflect choices that would be made in the field by active, mated, nest-seeking females. The present work seeks to expand upon the results of the laboratory assay by working under settings that more accurately reflect natural conditions. Objectives were to give bees an array of choices and allow them to initiate nests to: a) determine if there is a preference for cells that previously held male or female conspecific bees, b) verify attraction to conspecific whole nest cells, and c) determine which individual nest components of a cell are attractive to nesting females. Verifying the attractiveness of certain old nest materials to female bees would provide information that directs future studies to identify potential chemical lures to aid in the retention of bee populations at commercial nest sites.

Materials and Methods

Nest Initiation in Field Cages

Studies were conducted during the summers of 2007 and 2008 in field cages. Nests of *M. rotundata* were obtained from Logan, Utah populations. Bees from the previous year had nested in holes containing removable paper straw inserts. These nest-filled straws were collected and x-rayed (Stephen and Undurraga, 1976). By referring to the x-rays, it was possible to see where individual cells were in the nest, allowing each nest-filled straw to be cut into segments, each containing one bee cell. These cells were individually placed into vials and kept at 4°C from early October until June. In June, the cells were incubated at 29°C until the bees completed development and emerged from their cocoons. Because each cell was in a vial, it was possible to keep vacated male and female cells separated for later use in experiments.

Four field cages (length x width x height = 6.1 m x 6.1 m x 1.8 m) were erected in a blooming alfalfa field in Logan, Utah. A simple shelter (Fig. 3-1) made of plywood and plastic pipes was placed in each cage, facing southeast to capture the warmth of the early morning sun. Polystyrene blocks (width x height x depth = 7 cm x 8 cm x 9 cm) (Beaver Plastics, Edmonton, Canada) with holes (0.5 cm diameter, 9 cm depth) in them were attached with Velcro (Velcro USA, Inc., Manchester, NH) in a horizontal line across the face of the shelter, approximately 10 cm apart. Each block was covered entirely with aluminum tape, except for 16 holes (4 x 4) in the face of the block. A paper straw (0.5 cm diameter, 9 cm length) was inserted into each hole; each straw contained a cue as assigned by the experimental design (described below).

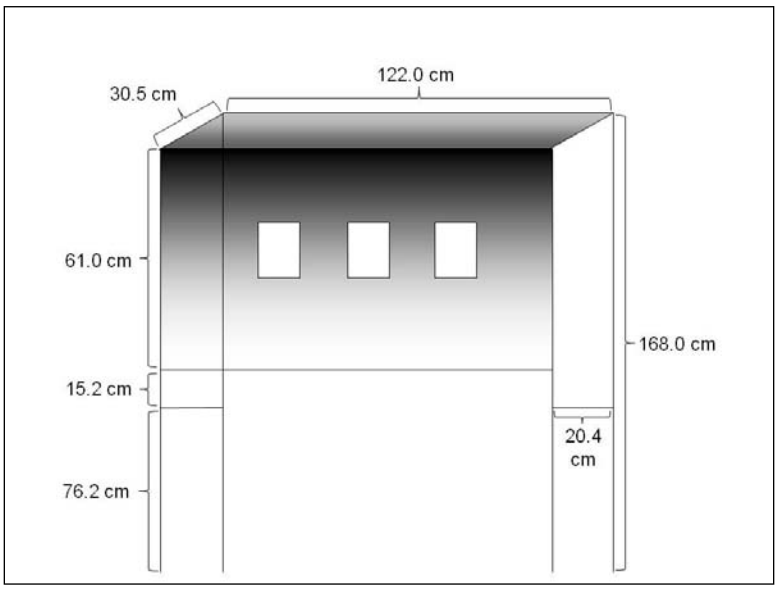

Figure 3-1. Shelter used for *M. rotundata* nesting cue attraction studies. The back panel and the roof were made of plywood. Legs and cross-pieces were made of plastic pipes. Polystyrene nesting blocks (small rectangles in center of panel) were attached with Velcro. The number of nesting blocks was three or six, depending on the experiment.

Trials were conducted by placing a different nesting cue in each block, with the same cue in the back of every hole (straw) of a block. Bees were released inside the cage, and nest initiation was recorded as described below. When one trial had ended, the same field cage could be used for another trial, but with a different arrangement of cues and a different set of released bees.

Attraction to Whole Female and Male Conspecific Cells

These trials were conducted to determine if nesting females have a preference for cells that previously held male or female conspecifics, whilst verifying attraction to conspecific whole nest cells in a closed system. The cues used for these trials were entire individual *M. rotundata* cells, which would include the segment of paper straw surrounding the cell and bearing a nest recognition cue, leaf pieces, a cocoon, and feces.

The cells were collected the previous year, and it was known whether a male or a female bee had emerged from each cell. For these trials, three blocks were attached to each shelter. One block had a female cell placed in the back of each hole before adding new paper straws to the holes, in front of the cell. A similar arrangement of male cells and straws was made in another block. The final block only had paper straws in the holes; there were no bee cells (hereafter referred to as 'blank'). Twelve trials were conducted. The three blocks were arranged differently for each trial, so that each of the six possible arrangements was used twice.

Fifteen females were paint-marked with enamel paint at room temperature (approximately 22ºC). All females were marked with the same color, and a different color was used for each trial. Thus, we were ensured that observed females were from the current trial and not from a previous trial in the same cage or unintentionally introduced as we were entering and exiting the cage. Paint-marked females and 15 males were released in each cage where they could mate and forage freely on the blooming alfalfa. Usually 2-3 days after bee release, females had commenced nest-building at the blocks. Each nest hole was examined daily with an otoscope to look for evidence that a female had begun to build a nest. Because females occasionally initiate a nest by lining a hole with leaf pieces but then they abandon it, a female was considered to have chosen a nest only when she began provisioning her first cell with pollen and nectar. When a provisioned nest was identified, its location was recorded. Once the female creating this nest returned after a foraging bout, she was caught and removed from the cage. The nest also was removed and replaced with another straw of the same type (male cell, female

cell, or blank). Nesting activity was checked 2-3 times daily (1000 – 1800 hrs MST), until every female had chosen a nest hole or until no new nests had been initiated for two days. Frequent nest monitoring and prompt removal of nesting females were performed in an effort to reduce the gregarious nesting behavior of these bees as much as possible.

Attraction to Individual Nest Components in Field Cages

These trials were conducted to determine, in a closed system, which individual nest components are attractive to nesting females. The cues used for these trials were individual components of *M. rotundata* nests, representing every cue that a bee could experience throughout its development in the natal nest. Nest components used were: female cocoon, feces, nest straw, pollen-nectar provision (hereafter referred to as 'provision'), and leaf pieces. Nest components had been collected in the previous year after the adults had emerged from the nest, with the exception of provisions, which were collected from the previous year's cells in which eggs had not been deposited or the eggs failed to hatch. Each cue was used in an amount equal to one cell's worth, on average, of that nest component (Table 3-1). Cues were attached to corks using hot glue, and the corks were placed in the back of paper straws, which were then inserted into nesting blocks. Six nesting blocks were used for each trial. Five blocks each contained a different nest component. Inserted into each hole of the sixth block was a paper straw with a cork in the back. The cork had a dab of hot glue placed on it, but no nest component (hereafter referred to as 'blank'). Fourteen trials were completed, with the blocks placed in a different arrangement for each trial.

As in the above trials, 15 paint-marked females and 15 males were released in each cage and monitored for nest-building, the location of nest initiation was recorded, and newly nesting bees and their nests were removed. Once straws with nests were removed, they were replaced with straws containing cues of the same type.

Table 3-1. Amount of each nest component used in *M. rotundata* cue attraction studies.

Component	**Amount**
Leaf Pieces	0.030 ± 0.005 g
Female Cocoon	1 cocoon
Feces	0.010 ± 0.005 g
Provision	1 provision
Straw Pieces	0.025 ± 0.005 g

Nest Initiation in an Open Field

The following studies, using either whole cells or individual cell components as nesting cues, were conducted in Logan, Utah, in an open alfalfa field setting; i.e., bees were not held in cages. *Megachile rotundata* were not released *en masse* for these experiments. Approximately 100 females, managed as described above, were released slowly over the course of each experiment. The majority of nesting females immigrated from the surrounding farming area, and as such their management history was not known.

Attraction to Whole Cells of Conspecifics and of Another Megachilid Species

To verify attraction to conspecific whole nest cells in an open system, in 2007 six shelters (Fig. 3-1) were aligned north to south and facing southeast on the edge of an alfalfa field in Logan, Utah. Three polystyrene nesting blocks, as described above, were attached to each shelter. One block had an entire *M. rotundata* female cell (from which a

female had emerged in the previous year) placed behind a new paper straw in each hole. One block had an entire *O. lignaria* female cell (from which the bee had emerged) placed behind a new paper straw in each nest hole. One block had a new paper straw, but no bee cell, in each nest hole. Each shelter had a different arrangement of blocks, such that each of the six possible configurations was used.

Nesting activity was checked twice daily (approx. 1000 – 1200 hrs and 1600 – 1800 hrs MST). If a bee had begun provisioning a nest hole with pollen and nectar, the date and location of the nest were recorded, and the straw and cue, if applicable, were replaced. Nest-building was monitored daily from 25 June to 13 August 2007.

Unlike the field cage experiments, only the newly initiated nests (but not the nesting females) were removed in an effort to reduce the effect of *M. rotundata's* predisposition for aggregative nesting. Because nesting females were not removed from the experiment, they tended to repeatedly initiate nests in the same hole, even after their nest was replaced with a new straw. To account for this in data analysis, consecutive instances of nesting in a hole were scored as the effort of only one nesting bee. If a hole was vacant for 2 days and then reoccupied, this was scored as the effort of a new bee.

Attraction to Individual Nest Components in an Open Field

This experiment was conducted to determine which individual nest components of a cell are attractive to nesting females in an open system. In 2008, six shelters (Fig. 3-1) were aligned north to south and facing southeast on the edge of the same alfalfa field in Logan, Utah that was used in 2007. Six polystyrene nesting blocks were attached to each

shelter, as described above. The cues within the blocks were as described in the "attraction to individual nest components" cage study above. Each shelter had a different arrangement of blocks, randomizing as much as possible so that two types of blocks were not next to each other more than twice.

Monitoring of nest-building activity and data compilation took place from 8 July – 25 July 2008 and were as described above in the "attraction to whole cells" field study.

Statistical Analysis

For each trial in a cage or for each shelter in the open field, the proportion of nests initiated for each cue choice (whole cell or individual component) or blank was calculated (i.e., the number of nests made in a particular block in comparison to the total number of nests made in that cage or shelter). For cage studies, ANOVA (Proc GLM, SAS 9.2) was used to determine if the arcsine square root-transformed proportion of nests initiated was affected by nest cues present in the chosen nesting block, position of the block on the shelter, and trial (i.e., was there a difference between the different trials?). For field studies, ANOVA was used to determine if the arcsine square root-transformed proportion of nests initiated was affected by nest cues present in the chosen nesting block, position of the block on the shelter, and position of the shelter within the field. REGWQ was used for post hoc comparisons among factors determined to be significant by ANOVA (Proc GLM, SAS 9.2).

Results

Nest Initiation in Field Cages

Attraction to Whole Female and Male Conspecific Cells

The presence of a cue (either male or female) in the nesting block had a significant effect on the mean proportion of nests initiated ($n = 176$, $F = 6.40$, d.f. = 2,16, $P < 0.01$). The position of the block at the shelter did not have a significant effect, nor did the interaction between cue and position of the block. On average, bees initiated more nests in blocks containing female cells than blank blocks ($P < 0.05$) and also initiated more nests in blocks containing male cells than in blank blocks ($P < 0.05$) (Fig. 3-2). However, there was no significant difference in the mean proportion of nests initiated in blocks containing female or male cells (Fig. 3-2). The 12 trials were not significantly different from each other.

Attraction to Individual Nest Components in Field Cages

The mean proportion of nests initiated in each block was significantly different depending on the position of the block on the shelter ($n = 143$, $F = 4.29$, d.f. = 5,35, $P < 0.01$). Post hoc comparison with REGWQ found that the mean proportion of nests made in the western-most block (0.06) was significantly less than the mean proportion of nests made in the two eastern-most blocks (0.55 and 0.50) ($P < 0.05$). Although the holes containing feces and provisions had the most initiated nests, the cue present in the block had no significant effect on the mean proportion of nests made (Fig. 3-3), nor did the

interaction between the cue present and the position of the block have a significant effect. The results of the 14 trials were not significantly different from each other.

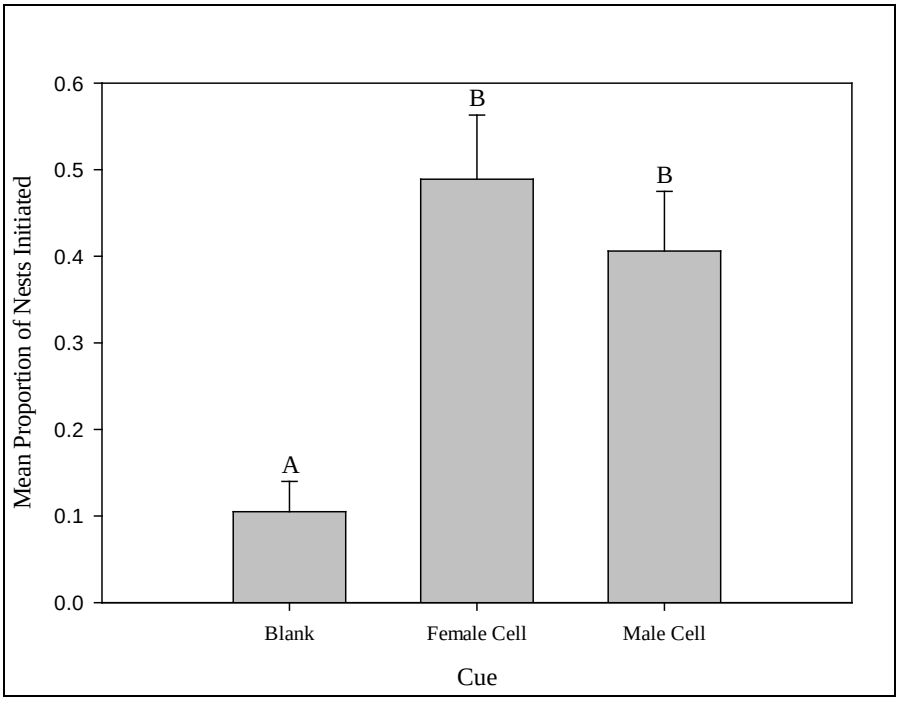

Figure 3-2. From experiments in field cages, mean proportion (±SE) of nests initiated by *M. rotundata* females in blank blocks, blocks containing female cells, and blocks containing male cells serving as cues. Bars with different letters indicate significant difference at $\alpha = 0.05$.

Nest Initiation in an Open Field

Attraction to Whole Cells of Conspecifics and of Another Megachilid Species

The whole cell cue present in the block had a significant effect on the mean proportion of nests initiated ($n = 280$, $F = 10.81$, d.f. = 2,4, $P < 0.03$). Nest selection was not affected by the position of the block on the shelter, the position of the shelter in the field, or the interaction between the cue present in the block and the position of the block on the shelter. Bees were more likely to initiate a nest in any block containing old bee nests of

either species than in a blank block ($P < 0.05$), but there was no difference in nesting between blocks containing *O. lignaria* cells and blocks containing *M. rotundata* cells (Fig. 3-4).

Attraction to Individual Nest Components in an Open Field

A significant difference was found in the mean proportion of nests that *M. rotundata* females initiated in response to the individual nest component cue present in the chosen block ($n = 737$, $F = 3.95$, d.f. = 5,20, $P < 0.02$). The position of the block on the shelter did not affect nest selection, nor did the position of the shelter within the field. Post hoc comparisons with REGWQ showed that the mean proportion of nests initiated in blocks containing pieces of used straw was significantly less than the mean proportions of nests initiated in blocks containing feces or female cocoons (Fig. 3-5).

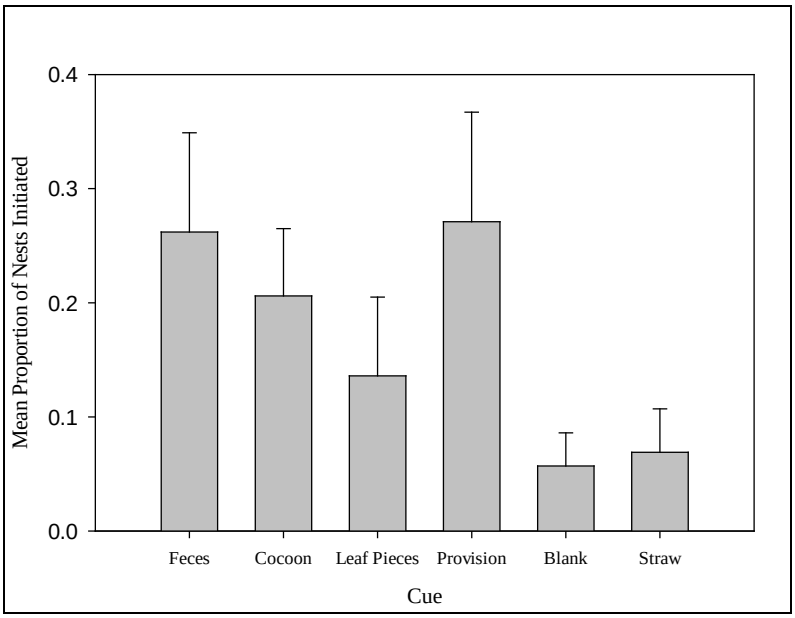

Figure 3-3. From experiments in field cages, mean proportion (±SE) of nests initiated by *M. rotundata* females in blocks containing different nest components serving as cues. No significant difference was found at $\alpha = 0.05$.

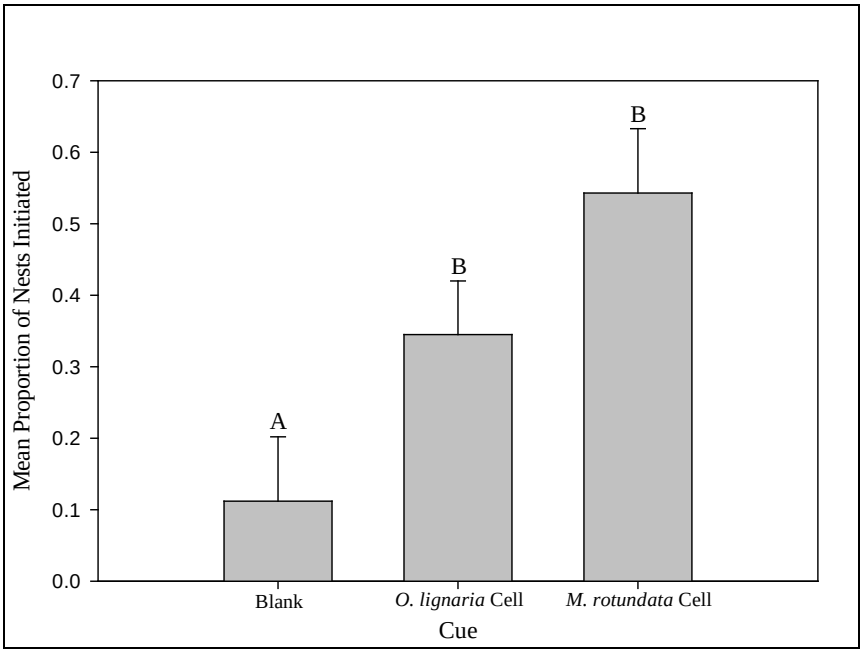

Figure 3-4. From experiment in open field, mean proportion (±SE) of nests initiated by *M. rotundata* females in blocks containing *M. rotundata* female cells, blocks containing *O. lignaria* female cells, and blank blocks. Bars with different letters indicate significant difference at $\alpha = 0.05$.

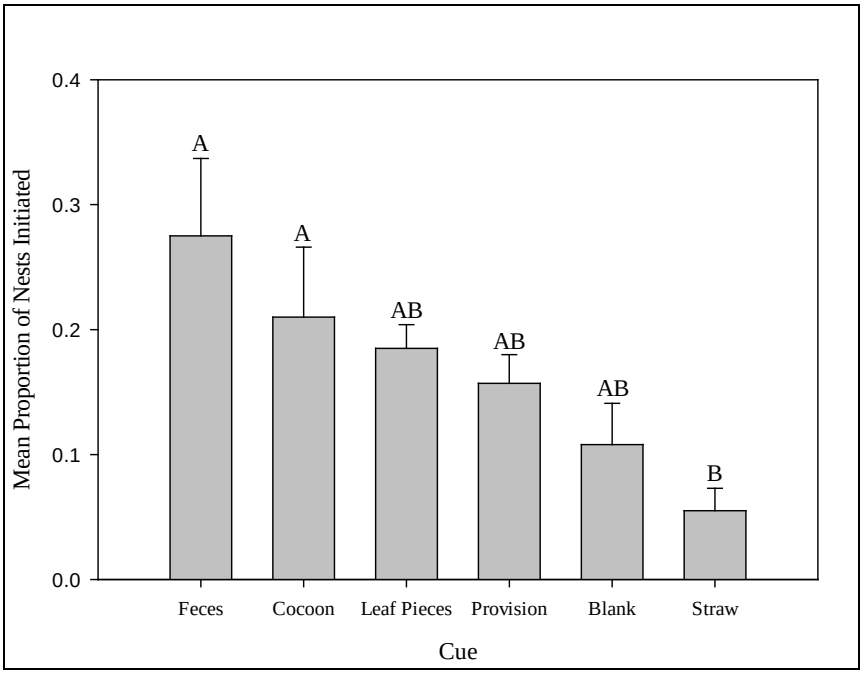

Figure 3-5. From experiment in open field, mean proportion (±SE) of nests initiated by *M. rotundata* females in blocks containing different nest components serving as cues. Bars with different letters indicate significant difference at $\alpha = 0.05$.

Discussion

This study was designed to determine what olfactory cues stimulate nest initiation by an individual *M. rotundata* female, and not what cues influence aggregation among members of a nesting population. This is a difficult question to ask in the midst of other bees and other cues. Therefore, our data were analyzed and results were interpreted while keeping in mind the impact of uncontrollable and unavoidable stimuli. We also wanted to verify the general attraction of bees to old nest materials, which is known from long-standing anecdotes and results of tests that were less controlled than the ones we devised. We found that, whether free-flying or enclosed in cages, *M. rotundata* females were more likely to nest in holes that contained old nest cells than in holes containing no cues. While caged, the bees did not reveal a statistically significant preference for any particular nest component, but the caged bees' preferences were still similar to those observed in an open field situation, where a preference for some of the individual nest components was revealed. Although evidence from a previous cue attraction study performed under strict laboratory conditions yielded more definitive results, the evidence from free-nesting conditions underscores the complexity of bee behavior in the field.

Interestingly, *M. rotundata* females nested equally in response to old conspecific cells and cells of another megachilid species, *O. lignaria*. This contrasts with the results of a similar study, in which *O. lignaria* females were allowed to choose between old *O. lignaria* cells, old *M. rotundata* cells, and new nests (Vorel and Pitts-Singer, unpublished). *Osmia lignaria* chose to nest in old conspecific nests significantly more often than in old *M. rotundata* nests. They also were more likely to nest in new nests

than in the old *M. rotundata* nests (Vorel and Pitts-Singer, unpublished). It is possible for both species to encounter each other's old nests in their environment. However, due to size differences, *M. rotundata* can easily nest in cavities used by *O. lignaria*, and often do, but the reverse is not true. This may help explain why *O. lignaria* would be more selective than *M. rotundata*. The presence of an old nest, not necessarily conspecific, could signal a suitable nest site to *M. rotundata*; *O. lignaria*'s size limits its options for nest sites.

Furthermore, Guédot et al. (2007) found that upon returning to the nest site, *M. rotundata* is more reliant on visual cues for nest location than *O. lignaria*. If the attraction to old nests found in the current study represents a response to an olfactory cue, the difference in degree of species-specificity for *M. rotundata* and *O. lignaria* could indicate that *O. lignaria* relies more heavily on olfactory cues when selecting a nest, and *M. rotundata* is more reliant on visual cues.

The differences in selectivity in these two bee species may be attributed to the duration of nesting season and abundance of resources available to bees during their nesting cycles. Thus, biotic factors may influence female bees' level of urgency and discrimination in choosing nest holes for optimizing their reproductive success. Orchard bloom and spring weather experienced by *O. lignaria* are more ephemeral and less reliable than alfalfa bloom and summer weather experienced by *M. rotundata*. That is, *M. rotundata* may not have to be as choosy as *O. lignaria* because they have a relatively long nesting season.

Megachile rotundata females were just as likely to nest in holes containing male conspecific cells as those containing female conspecific cells. Only components produced by the developing bee, i.e., feces and cocoon, would potentially contain compounds that may differ depending on gender. The fact that nesting females were not differentially attracted to male and female cells implies that the key compounds are present in cells of both sexes and are equally attractive upon detection, or that the attractive compounds are found in several nest components, including feces and cocoon.

When old bee cells were divided into separate components that were tested as nesting cues, caged *M. rotundata* did not preferentially nest in response to any component(s). However, in the field study, free-flying *M. rotundata* were more likely to nest in blocks containing feces or female cocoons than in blocks containing pieces of old nest straws. Guédot et al. (2005b) found that a nesting *M. rotundata* female will mark the sides of her chosen nest, enabling her to distinguish it from among thousands of other nest holes. This may explain why females in the current field study were least likely to choose a nest hole containing old straw pieces; they may have detected the nest recognition cue left by other bees (that created the nests from which the straw pieces were cut) and interpreted the hole as already being occupied. In contrast, the presence of old feces or cocoons may have indicated to a nesting female that successful nesting had previously taken place in that hole, and therefore, the hole was suitable for new nesting.

Results for attraction to nest components in the cage study may have differed from the results in the companion field study because of sample sizes as well as the gregarious nature of these bees. Only 15 females were released in each of the cage trials,

and in most trials, not all of the released females initiated nests. Therefore, the combined total of initiated nests for all 14 trials of the cage study was 143. Additionally, in the cages there was a significant effect of the position of the cue at the shelters that may have diminished the revelation of a cue preference. In the open field, a total of 737 nests were initiated, and this large number of data points may have made cue choice differences more apparent, especially because no effect of cue position was detected. Also in the cage study, we carefully managed against aggregative nesting by removing females when they first started a nest; in the open field it was very difficult to control gregarious behavior. In the absence of the confounding effect of gregarious behavior, nest choices made within the cages may have better represented females' responses to nest initiation cues than choices made in the field. Conversely, in the open field bees' choices may have been influenced by the presence of other bees, and these results are more likely to represent a natural or commercial situation, where bees select nests in response to many stimuli. Furthermore, nest initiations of only a few bees were examined in the cages for only a few days, while the choices of many bees were examined in the field over several weeks.

In a previous study, predominant compounds from *M. rotundata* whole cell extracts were identified (Buttery et al., 1981), but in a follow-up field study did not prove attractive to nest-seeking *M. rotundata* females (Parker et al., 1983). The identified compounds, caryophyllene epoxide and a mixture of caryophyllene epoxide, caryophyllene, and 2-phenylethanol, are common plant components. Our study corroborated the results of Parker et al. (1983); bees were not attracted to plant chemicals

(i.e., leaf pieces in our experiment). Because leaf pieces were not more attractive to the bees than any of the other components tested in our study, it is unlikely that these plants compounds play an important role in nest initiation. Another laboratory bioassay, however, found that old leaf pieces and feces were attractive to *M. rotundata* females (Pitts-Singer, 2007). Our study did agree with the previous laboratory study in the attractiveness of feces, which was very often chosen by females along with cocoons.

The current study sought to separate the various components present in *M. rotundata* nests and compare their relative attractiveness. Successful identification of feces and cocoon as attractive nest components in an open field situation has narrowed the scope of future attempts to identify attractive compounds that may serve as nesting cues. It would seem that, in a natural nesting situation, the cue that *M. rotundata* females find attractive may be a particular suite of components rather than any individual compound. In the continued search for attractive compounds, chemical analysis of all individual cues will be performed and may provide a chemical concoction that can be applied to new or clean nesting boards to enhance establishment at commercial sites and minimize the exposure to disease and parasitism.

No study has yet attempted to answer why bees are attracted to certain cues during nest selection. Are the recognition and discrimination of cues innate, or are they learned? If they are learned, then when and how might this occur? If learning occurs in the nest as an immature or as a pre-emerged adult, then bees would be exposed to all odors and textures of these nest components. If the bees are removed from the nest cavity, as occurs in the commercial management system designed for *M. rotundata*, then

the chance to learn important cues may be disrupted or lost. If we knew the importance of odor cues and their chemical identities, bee managers could provide cues for bees to encounter and learn at appropriate developmental times so that adult bees are able to orient more effectively and reliably at commercial nesting sites where those cues are made available.

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CHAPTER 4

EVALUATION OF THE PROBOSCIS EXTENSION REFLEX IN HYMENOPTERA OF VARIOUS SOCIAL LEVELS UNDER LABORATORY CONDITIONS[3]

Summary

Elicitation of the proboscis extension reflex (PER) is a tool used to further the understanding of the cognitive processes of social bees, such as honey bees, stingless bees, and bumble bees. We were interested in answering questions about the cognitive processes of solitary bees by using the same laboratory procedures that are used for honey bees. We investigated the use of various PER-elicitation procedures with several Hymenoptera representing different levels of sociality and domestication. We predicted that eusocial Hymenoptera would be most likely to extend their proboscises in response to a reward stimulus touched to their antennae. By fully or partially restraining their bodies but not their heads, we attempted to elicit the PER from honey bees, yellowjackets, bumble bees, sweat bees, blue orchard bees, alfalfa leafcutting bees, and sunflower leafcutting bees. The eusocial bees and wasps responded to a drop of sucrose touched to their antennae by extending their proboscises and glossae, respectively. The communal and solitary species never responded with the PER. Consequently, for answering questions about the cognitive processes of solitary bees, an alternative conditioning technique will have to be used and perhaps a different behavioral response will have to be sought.

[3] Coauthored by Cory A. Vorel and Theresa L. Pitts-Singer

Introduction

A honey bee will reflexively extend its proboscis in response to antennal stimulation with sucrose solution (Bitterman et al., 1983). Elicitation of the proboscis extension reflex (PER) from honey bees (*Apis mellifera* L.) has been used for decades as a tool to further the understanding of bees' cognitive processes, such as learning and memory (Takeda, 1961; Bitterman et al., 1983), and revealing their neural and molecular foundations (reviewed in Giurfa, 2007). The PER has also been used to demonstrate the learning abilities of bumble bees (Laloi et al., 1999; Laloi and Pham-Delègue, 2004) and stingless bees (Abramson et al., 1999; McCabe et al., 2007).

We were interested in answering questions about the cognitive processes of solitary bees, especially blue orchard bees (*Osmia lignaria* Say) and alfalfa leafcutting bees (*Megachile rotundata* Fabricius), and were encouraged to use the PER for this purpose. We assumed that the same basic laboratory procedures that are used for honey bees (e.g., Bitterman et al., 1983) could be used to elicit a response from solitary bees. However, our attempts to use these procedures with blue orchard bees failed, although honey bees readily responded. We thus questioned whether the technique used to elicit the PER from honey bees can be applied universally to all bees, or if the success of the technique is influenced by procedural factors such as method of bee restraint (Robin L. Foster, personnal communication). We also wondered if success could be influenced by species-specific factors, such as level of sociality.

We investigated the use of various PER-elicitation procedures, and applied these procedures to several Hymenoptera representing different levels of sociality. We

predicted that eusocial Hymenoptera would be most likely to extend their proboscises in response to a reward stimulus touched to their antennae.

Materials and Methods

All trials were performed at the USDA-ARS Bee Biology and Systematics Laboratory (BBSL) in Logan, Utah, and only female bees and wasps were used (Table 4-1). All "wild caught" bees and wasps were captured while they were foraging within a 0.5 km radius of the BBSL.

In honey bees, the concentration of sucrose solution that will elicit the PER can be influenced by factors such as age, foraging behavior, and foraging experience (Page et al., 1998; Pankiw et al., 2001). As a preliminary step, we tested several bumble bees, blue orchard bees, honey bees, sweat bees, and yellowjackets, using the method described in Question 1 below, with sucrose concentrations of 25%, 40%, 50%, 60%, and 70% w/v to determine which would be most likely to successfully elicit the PER. This range corresponds to the average sugar concentrations of floral resources normally foraged upon by the solitary bees (Free, 1993) and the sucrose concentrations used at the BBSL for lab-rearing solitary bees (25%) and bumble bees (50%). Also, this is a range of concentrations similar to those commonly used in the literature (Bitterman et al., 1983; Abramson et al., 1997; Buckbee and Abramson, 1997; Laloi et al., 1999; Erber et al., 2000; Sandoz et al., 2000; Déglise et al., 2003; Laloi and Pham-Delègue, 2004; Rueppell et al., 2006). In these preliminary attempts, only honey bees and yellowjackets responded with the PER, and based on their responses, we decided to use 25% and 50%

sucrose solution for our bioassays. We then performed the following series of experiments in an effort to find a universally successful PER technique.

Insect Preparation

The following pre-testing procedure was used for all trials, unless otherwise noted. Hymenoptera were held individually in a small, covered paper cup for 30 min. They were then restrained by anesthetizing them with CO_2 and inserting them into modified, inverted centrifuge tubes. The modifications made to the tubes differed between some methodological treatments, and are described below. During earlier experiments, insects were left in their restraints prior to testing until some died (possibly from starvation or dehydration). In later experiments, holding times were adjusted to minimize mortality. In general, insects were left in their restraints unfed for 15-22 hours, except honey bees, which were restrained and unfed for 3 hours because they experienced high mortality when restrained for longer time periods.

PER Bioassay

To eliminate the possibility that insect response was due to thirst, a drop of water delivered from the needle tip of a 1 ml hypodermic syringe was touched to an antenna. (In our study, none of the test insects extended their proboscis in response to water.) A syringe needle was then used to touch a drop of 25% or 50% sucrose solution to an antenna (Fig. 4-1A). If the insect extended its proboscis (or glossa, in the case of yellowjackets), then the drop was placed on the proboscis as a reward (Fig. 4-1C), and a positive response was recorded.

Fig. 4-1. Bee restraint variations: A) "Mummy" - Honey bee is restrained in an inverted centrifuge tube with the tip cut out; Teflon tape below the head secures the bee. Sucrose solution touched to an antenna elicits the PER. B) "Belt" - *Bombus griseocollis* female is restrained by cutting all but a narrow strip from the tip of an inverted centrifuge tube. Elastic string secures the bee's petiole to the strip, and the string is taped in place. C) "Intermediate" - *Bombus nevadensis* female is restrained in a centrifuge tube that has a wide slot cut into it; adhesive tape secures the bee. The bee's proboscis is extended and the bee is rewarded with sucrose solution.

Eliciting the PER

Methodological Question 1: Will fully restrained Hymenoptera of several social levels exhibit the PER?

This series of trials, using bees and yellowjackets, was conducted in May and June 2006 (Table 4-1). The number of individuals of each species tested and the sucrose concentrations used are shown in Table 4-2. Yellowjackets, honey bees, bumble bees, and sweat bees were wild caught. Standard protocols were followed for overwintering blue orchard bees (Bosch and Kemp, 2001), alfalfa leafcutting bees (Richards, 1984), and sunflower leafcutting bees (Pitts-Singer, 2007). These bees emerged from their cocoons in an incubator (22°C for blue orchard bees, 29°C for leafcutting bees). They were then allowed to forage in a greenhouse (blue orchard bees) or a field cage (leafcutting bees) for 1-2 weeks before testing.

To create restraints, the tips were cut out of inverted centrifuge tubes, so that when the insects were inserted, only their heads were exposed and able to move. The

insects were secured by wrapping Teflon tape around them, just below their heads, such that the tape overlapped with the centrifuge tube (Fig. 4-1A). From this point on, we refer to this method as the "mummy" restraint.

The preparation and PER bioassay proceeded as described above, except when the insects were first brought into the laboratory, they were fed 25% or 50% sucrose solution *ad libitum* for 30 min while they were held in small cups (sucrose concentration fed corresponds to concentration used for testing).

Table 4-1. Hymenoptera tested, their social level, and experiments in which they were used.

Common Name	Species	Social Level	Question(s)
Blue orchard bee	*Osmia lignaria* Say	solitary, aggregating	1, 2, 5
Alfalfa leafcutting bee	*Megachile rotundata* Fabricius	solitary, aggregating	1, 2, 5
Sunflower leafcutting bee	*Megachile pugnata* Say	solitary, aggregating	1, 2
Sweat bee	*Agapostemon* spp., possibly *A. nasutus* Smith, *A. virescens* Fabricius, or *A. coloradinus* Vachal	communal	1
Bumble bee	*Bombus appositus* Cresson *B. centralis* Latrielle *B. fervidus* Fabricius *B. griseocollis* DeGeer *B. huntii* Greene *B. nevadensis* Cresson	primitively eusocial	1, 2, 3, 4
Yellowjacket	*Vespula pensylvanica* Saussure *V. germanica* Fabricius	eusocial	1
Honey bee	*Apis mellifera* Linnaeus	highly eusocial	1

Methodological Question 2: Would a restraint that allowed more movement increase the likelihood of the PER in bumble bees and solitary bees?

This series of trials was conducted in June 2006, and focused on bumble bees and solitary bees (Table 4-1). Bees were acquired as described above. Honey bees and yellowjackets were not included in this series of trials, because they readily responded

when the mummy restraint was used. Sweat bees were not included because wild sweat bees were not flying during this time of year.

As in the previous trials, these bees were fed *ad libitum* for 30 min prior to testing. All bees were fed and tested with 25% sucrose solution, except for nine bumble bees that were fed, and subsequently tested with, 50% sucrose solution. Restraints for this method were inverted centrifuge tubes cut such that they became a circular base with a long, narrow strip extending upward. A piece of elastic string was tied around the bee's petiole and the narrow strip, to bind the bee to the strip. The string was then secured with adhesive tape (Fig. 4-1B). Bees restrained in this manner were only secured at the petiole so that they could move their heads, wings, and legs. Hereafter, we refer to this method as the "belt" restraint.

Table 4-2. From Question 1, showing Hymenoptera that responded with proboscis extension while restrained by the mummy method.

Hymenoptera Tested (n)	Sucrose Concentration	No. Exhibiting PER	Percent PER
Osmia lignaria (30)	25%	0	0%
Osmia lignaria (2)	50%	0	0%
Megachile rotundata (15)	25%	0	0%
Megachile pugnata (15)	25%	0	0%
Agapostemon spp. (22)	25%	0	0%
Bombus spp. (38)	25%	2	5%
Bombus spp. (5)	50%	1	20%
Vespula spp. (14)	25%	10	71%
Apis mellifera (32)	25%	26	81%

Methodological Question 3: Would an intermediate level of restraint increase the likelihood of the PER in bumble bees?

This series of trials was conducted in June 2007. The bumble bees tested were wild *B. griseocollis* DeGeer, *B. rufocinctus* Cresson, *B. fervidus* Fabricius, *B. huntii*

Greene, and *B. bifarius* Cresson. Unlike the previous trials, these bees were kept in Plexiglas cages (length by width by height: 26.2 by 26.2 by 30.5 cm) in the laboratory for 1-6 days and fed a diet of 25% sucrose solution before testing.

R. L. Foster (personal communication) successfully elicited the PER from *B. huntii* using a restraint that allowed more movement than the mummy restraint, but less than the belt restraint. Therefore, the restraints for this method were made by cutting a slot in each centrifuge tube, such that the bee's wings would be exposed but not its legs. The bee was placed high enough in the tube so that only her forelegs were able to move freely. Each bee was then secured with a thin strip of adhesive tape affixed anterior to the tegulae and another strip affixed under her wings, posterior to the tegulae but before the petiole (Fig. 1C). From this point on, we refer to this method as the "intermediate" restraint. Ten bumble bees were tested with 25% sucrose solution, exclusively. Sixteen bumble bees were tested with 25% sucrose solution, and then after a waiting period of 10 min, were tested with 50% sucrose solution.

Methodological Question 4: Would maintaining bumble bees in the laboratory for an extended period of time increase the likelihood of the PER?

Intermediate restraint: These tests were conducted in June 2007. A colony of *B. centralis* Cresson was brought into the laboratory for rearing under standard laboratory conditions (Plowright and Jay, 1966). Testing occurred over a period of 17-28 days after the colony was brought into the laboratory. The 21 workers tested from this colony may have been adults that were brought into the lab as part of the original colony, or they may have eclosed after the colony was maintained in the lab. In addition, we tested two *B.*

bifarius queens and one *B. fervidus* queen that had been in the lab for 19 days and 23 days, respectively. Bees were restrained using the intermediate restraint, as in Experiment 3. They were tested with 25% sucrose solution and, after a 10 min waiting period, tested again with 50% sucrose solution. After testing, a dot of paint was placed on each bee's thorax before returning her to the colony, to avoid the possibility of any bee being tested twice.

Belt restraint: These tests were conducted in July 2007. All eight bumble bees used in this experiment were *B. centralis* from the colony described above, which by this time had been in the lab for 39-41 days. They were tested with both 25% and 50% sucrose solutions, as described above. They were restrained with the belt restraint, as described in Experiment 2.

Methodological Question 5: Would maintaining solitary bees in the laboratory and using an intermediate restraint increase the likelihood of the PER?

Twenty-two blue orchard bee females and 34 alfalfa leafcutting bee females were overwintered and then incubated for laboratory emergence in July 2007. Once emerged, the bees were kept in Plexiglas cages, along with males, and fed 25% sucrose solution *ad libitum* for one week (blue orchard bees) or 3-4 weeks (alfalfa leafcutting bees). The bees were restrained for testing using the intermediate restraint. They were tested with 25% sucrose solution and, after a 10 min waiting period, 50% sucrose solution.

Results

Using Methodology 1, honey bees and yellowjackets had a high rate of proboscis extension, 71% and 81%, respectively, which was not observed in any of the other bees tested (Table 4-2). We saw very little response from bumble bees (Table 4-2). No communal or solitary bees responded using this method (Table 4-2).

The results from testing with Methodology 2 are shown in Table 4-3. The single response was from a bumble bee tested with 50% sucrose solution. None of the solitary bees that were tested responded.

Table 4-3. Results from Question 2, showing number and percent Hymenoptera that responded with proboscis extension while restrained by the belt method. Bees were tested with 25% sucrose solution, except for nine bumble bees, which were tested with 50%. The single response was to 50% sucrose solution.

Hymenoptera Tested (n)	No. Exhibiting PER	Percent PER
Osmia lignaria (10)	0	0%
Megachile rotundata (15)	0	0%
Megachile pugnata (15)	0	0%
Bombus spp. (12)	1	8%

Using Methodology 3, the PER was exhibited by one out of 10 bumble bees that were tested with 25% sucrose solution only. Of the 16 bumble bees tested using Methodology 3 and tested with both 25% and 50% sucrose solutions, one bee responded to both 25% and 50%, and two bees responded to 50% only. In total, four of the 26 bumble bees (15%) tested using Methodology 3 responded with the PER.

When we tested lab-reared bumble bees using Methodology 4, 12 out of 21 (57%) that were held in the intermediate restraint responded positively. Of these, six responded only to 25%, four responded to both concentrations, and two responded to only 50%.

When the belt restraint was used in conjunction with Methodology 4, six out of eight (75%) held in the belt restraint responded with the PER; five responded to both sucrose concentrations and one responded only to 50%.

Methodology 5 mimicked conditions under which bumble bees had responded with the PER. However, none of the solitary bees in this series of trials responded positively, regardless of sucrose concentration.

The restraint used with bumble bees, as well as the length of time that they were held in the lab, influenced their response. There was a progressive increase in positive responses of bumble bees across trials (Fig. 4-2).

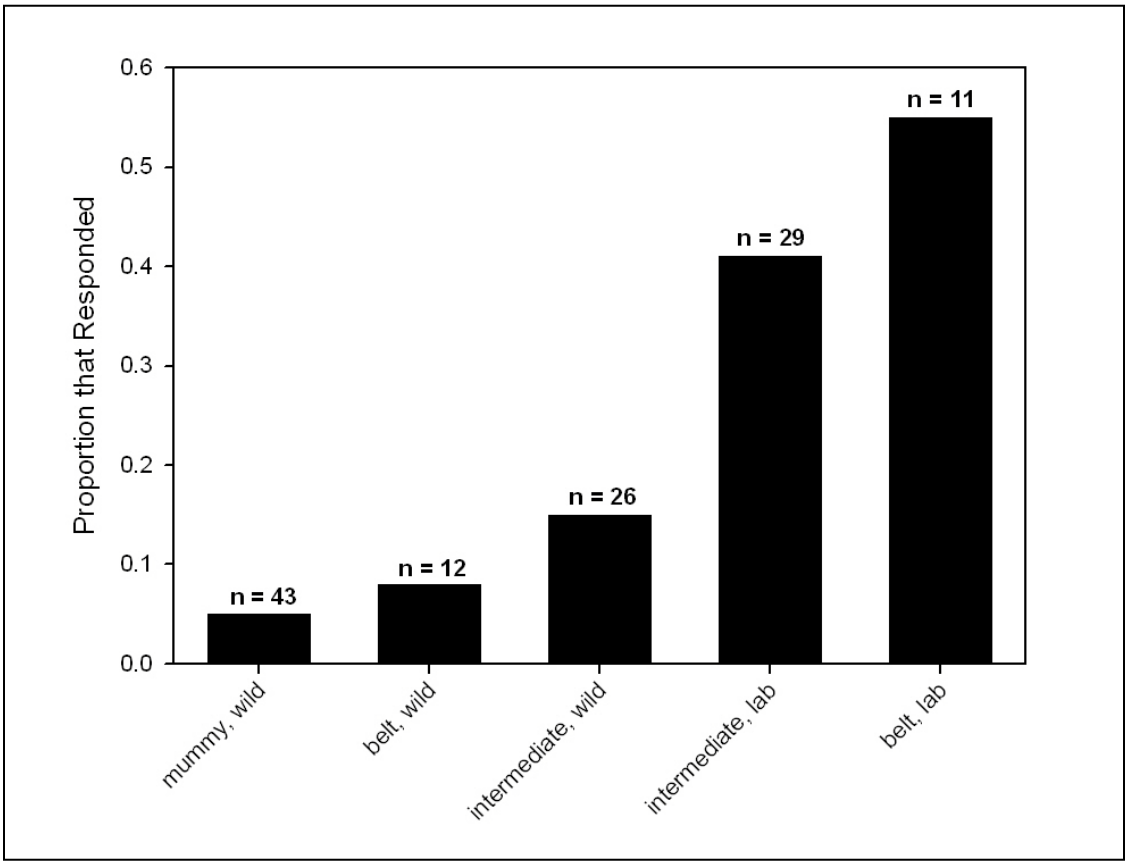

Figure 4-2. Proportions of bumble bees that responded positively with the PER to either 25% or 50% sucrose solution, grouped according to restraint and source.

Discussion

As we had predicted, the PER was more readily elicited from eusocial species. The presence of the proboscis extension reflex in honey bees has been well documented, so it is not surprising that honey bees readily responded to the sugar stimulus using the traditional mummy restraint (Methodology 1). Although yellowjackets do not actually have a proboscis, many of those tested clearly extended their glossa when an antenna was touched with a drop of sucrose solution, demonstrating an oral response quite similar to that of honey bees. These results, coupled with the complete lack of proboscis extension in all of the solitary and communal species tested, seem to suggest that the basic technique used for eliciting the PER in honey bees (Bitterman et al., 1983) cannot be universally employed for all bees.

We presumed that bumble bees have a proboscis extension reflex that can be elicited in a similar manner as for honey bees, but our results show that the protocol that works for honey bees was ineffective for bumble bees. Other studies have had success in conducting conditioning experiments that train bumble bees using the PER as the response (Laloi et al, 1999; Laloi and Pham-Delegue, 2004; R. L. Foster, personal communication). The difficulty we had in eliciting the PER in wild caught bumble bees using either a mummy or a belt restraint (Methodologies 1 and 2) was perplexing. By changing the restraint as suggested by R. L. Foster (personal communication) and by maintaining the bees for extended time periods in the laboratory prior to testing, however, we were able to get positive responses using less restrictive restraints than were used for honey bees (Methodologies 3 and 4). Nevertheless, when similar protocol changes were

made for alfalfa leafcutting bees and blue orchard bees, these bees still failed to display the PER.

In eusocial species, the PER could possibly function in feeding. Many eusocial bees and wasps distribute food to larvae and adults via trophallaxis (Wilson, 1971; Michener, 1974). In fact, trophallactic interactions are a key feature of honey bee societies, where trophallactic behaviors include antennal stimulation followed by proboscis extension (Wilson, 1971). It has been demonstrated that associative learning via trophallaxis is important for the dissemination of olfactory information throughout honey bee colonies (Farina et al., 2005; Gil and De Marco, 2005; Grüter et al., 2006; Farina et al., 2007), so it is logical that the PER could be used to readily condition honey bees in the laboratory.

Trophallaxis is not seen in bumble bees or solitary bees, so the PER would not play a role in feeding for these bees. Adult bumble bees do have other interactions and methods of communication (Goulson, 2003). It may be that the PER can be elicited from bumble bees in the laboratory, under the right conditions, because of their social lifestyle and similarities to the highly eusocial bees. However, solitary bees have no adult-larva and very few adult-adult interactions, so antennal stimulation might not be expected to have any relevance for them. These differences in feeding and conspecific interactions may explain why Hymenoptera of different social levels would differ in their propensities to exhibit the PER in the laboratory.

Abramson et al. (1999) found that the stingless bee *Melipona scutellaris* Latreille would not exhibit the PER in response to sucrose solution. However, when the protocol

was changed such that *M. scutellaris* honey was touched to the antennae, the PER was elicited from these bees. We were able to increase our success in eliciting the PER from bumble bees by changing the basic (i.e., honey bee) protocol. Therefore, the possibility remains that the PER can be elicited from communal and solitary bees, but further protocol changes are needed.

We remain interested in answering questions about the cognitive processes of blue orchard bees and alfalfa leafcutting bees. We have developed an alternative technique involving a more passive protocol for the learning of olfactory cues, which we are currently using to explore solitary bees' learning capabilities. Although the PER has proven to be an immensely useful tool for studying learning in eusocial bees, the PER was not elicited in solitary bees using any of the protocol variations that we tried in this study.

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CHAPTER 5

OLFACTORY CONDITIONING OF THE SOLITARY BEES *OSMIA LIGNARIA* AND *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE)[4]

Summary

For decades, scientists have been using conditioning experiments to explore the cognitive processes of honey bees, as well as the neurophysiological and molecular mechanisms underlying those processes. Few studies have utilized conditioning to further understand learning and cognition in solitary bees. In this study, two species of solitary megachilid bees, *Osmia lignaria* Cresson and *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae) were conditioned to respond to, and discriminate between, particular floral odors during feeding bioassays in the laboratory. As expected, both species were able to learn and to discriminate between floral odors in the laboratory bioassays, although O. lignaria performed better in the discrimination test. Also, for some of the odor pairs tested, one sex responded better than the other sex, although the better performances were not consistently associated with either males or females.

Introduction

For nearly half of a century, scientists have been conditioning honey bees in an effort to explore their sensory abilities (e.g., von Frisch, 1950) and cognitive processes such as learning and memory (Bitterman et al., 1983; reviewed in Giurfa, 2007). In the laboratory, conditioning has become a useful tool for examining the neurophysiological

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and molecular mechanisms (e.g., Menzel et al., 1974; Farooqui et al., 2003) that underlie cognitive processes. In the field, conditioning is a useful tool for examining the role of learning and other cognitive processes in daily life, including insect-insect interactions and insect-environment interactions. Much information has been gleaned from honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), conditioning studies, but one cannot assume that the honey bee represents all Hymenoptera. Differences in ecological requirements and constraints, as well as differences in physiology and in life histories (such as social systems), could lead to differences in learning ability and other cognitive processes (Gould and Marler, 1984; Heinrich, 1984). Thorough understanding of learning and cognition can be gained only through studies in a variety of Hymenoptera.

Few studies have utilized conditioning to further understand learning and cognition in solitary bees. Solitary bees do not live in a colony, but instead every female creates her own nest and produces her own progeny. For solitary bees, previous research has been done in controlled field conditions, such as field cages (Dukas and Real, 1991; Campan and Lehrer, 2002; Amaya-Márquez et al., 2008), that may lack the rigid control afforded under laboratory conditions. Examination of the cognitive abilities of bees that represent different evolutionary histories would allow us to learn more about the differences and similarities in cognitive processes, neurophysiology, and molecular workings between species. A phylogenetic comparison of learning ability in Hymenoptera, comparing different hymenopterans' learning abilities for different tasks and contexts, may also help answer questions about the evolution of sociality.

Conditioning typically takes one of two forms, respondent conditioning or operant conditioning (Gould and Marler, 1984; Pierce and Cheney, 2004). Respondent conditioning, often referred to as classical conditioning, takes advantage of reflex behaviors. An unconditioned stimulus elicits an unconditioned response (reflex). A conditioned stimulus is paired with the unconditioned stimulus, and together they elicit the unconditioned response. Subsequently, the conditioned stimulus presented alone will elicit the same response, which is now known as the conditioned response. Pavlov's work with salivating dogs is the most well-known example of respondent conditioning.

Operant conditioning, sometimes called trial-and-error learning, requires some active behavior (as opposed to an involuntary reflex) by the subject being conditioned. An operant behavior is reinforced, with either reward or punishment, until the point where the subject's behavior is modified. A cue, such as a light or an odor, may be employed for a more complex form of operant conditioning, in which the reinforcement only occurs in the presence of the cue. An example of operant conditioning in nature is a bee learning how to recognize a newly encountered species of flower and what maneuvers are needed to efficiently handle that particular flower for retrieving its rewards. The bee learns to respond to the visual and olfactory cues associated with the most rewarding flowers.

In the laboratory, the conditioning of honey bees often employs the proboscis extension reflex (PER), an example of respondent conditioning (Bitterman et al., 1983; Farooqui et al., 2003; reviewed in Giurfa, 2007). However, the PER cannot be elicited in the laboratory from some solitary bees (Vorel and Pitts-Singer, Ch. 3). Without the PER

bioassay for solitary bees, a different approach is needed in order to assess learning through conditioning in a controlled manner in the laboratory.

The work presented here employs operant conditioning with two species of solitary megachilid bees, *Osmia lignaria* Cresson and *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae). We attempted to condition bees to respond to particular floral odors during feeding bioassays in the laboratory. If conditioning were successful, then the bees would respond by "choosing" (the operant behavior) the feeder bearing the odor that was previously associated with a sucrose reward. We hypothesized that these solitary bee species could be conditioned to choose the scented feeder when given a choice between a scented feeder and 1) an unscented feeder and 2) a feeder scented with a second odor.

Materials and Methods

All experiments were conducted at the USDA-ARS Bee Biology and Systematics Laboratory (BBSL) in Logan, Utah. Two species of solitary, cavity-nesting bees were used. The spring-flying blue orchard bee, *Osmia lignaria*, is being increasingly used as a pollinator of orchard crops. The alfalfa leafcutting bee, *Megachile rotundata*, flies in the summer and is widely used to pollinate alfalfa for seed production. Both bee species are available commercially and are readily maintained in the laboratory. *Osmia lignaria* originated from Northern Utah populations (3 B Sales and Service, North Logan, UT, USA). *Megachile rotundata* were from Manitoba, Canada (JWEM Leafcutters, Inc.). Both species were purchased in the fall and maintained at the BBSL following standard

protocols (*O. lignaria*: Bosch and Kemp, 2001; *M. rotundata*: Richards, 1984) throughout the winter. Bees emerged from their cocoons stored in Petri dishes in an incubator (22°C for *O. lignaria*, 29°C for *M. rotundata*) before being used in conditioning experiments as follows.

Three conditioning experiments were carried out. In the first experiment, bees were conditioned to associate sucrose solution with an odor and to associate water with the absence of odor. In the second and third experiments, bees were conditioned to associate sucrose solution with one odor (the positive odor) and to associate water with a second odor (the negative odor). The odors used for conditioning were citral, geraniol, and phenylacetaldehyde (PAA). These odors are components of floral aromas and were chosen because they have been used extensively by other researchers to condition honey bees.

Experiment One - Simple Conditioning

For this experiment, *O. lignaria* and *M. rotundata* were conditioned to select the scented feeder when given a choice between a scented feeder and an unscented feeder. *Osmia lignaria* were conditioned and tested April - June 2007. *Megachile rotundata* were conditioned and tested June - July 2007.

Two groups of bees were designated, a conditioned group and an unconditioned group. The unconditioned bees were treated and tested exactly as were the conditioned bees, as described below, but odor was never applied to any of their feeders, thus they were never exposed to an odor during the conditioning phase. The unconditioned bees did not encounter an odor until the testing phase.

Osmia lignaria were conditioned and tested in a room with overhead lighting at 22-23°C; artificial lighting was kept on an 11:13 day:night schedule. *Megachile rotundata* were conditioned in a temperature-controlled greenhouse (~18-38°C) under natural lighting (i.e., sunlight). *Megachile rotundata* were tested in a room with overhead lighting. The room temperature was ~22-23°C; extra heat was provided by placing testing arenas on heating pads (Kaz, Inc., Southborough, MA, USA; Sunbeam Products, Inc., Baton Rouge, FL, USA) with the dial set to "medium." The extra warmth was needed because the temperature of the room was inadequate for sustaining *M. rotundata* activity.

Conditioning took place in well-ventilated Plexiglas cages (length x width x height: 26.2 cm x 26.2 cm x 30.5 cm). Two feeders were placed in each cage, which served as the method of odor delivery. Each feeder consisted of a small plastic 5.0 ml cup with a lid (height x diameter: 2.5 cm x 2.3 cm) (Nalgene, Rochester, NY, USA) (Fig. 5-1). The lid had a wick (4.0 ± 0.5 cm) made from clean cigarette filter. A 2.3 cm ring of filter paper (Whatman International, Ltd., Maidstone, England) surrounded the wick and was secured from underneath using white medical tape (Fisher Scientific, Pittsburgh, PA, USA). An odor, either 0.50 µl geraniol (MP Biomedicals, Inc., Solon, OH, USA) or 0.25 µl PAA (Sigma-Aldrich Co., St. Louis, MO, USA), was dripped onto the filter paper ring using a Hamilton syringe.

All bees that emerged from their cocoons by noon on a given day were placed in a Plexiglas cage together in a male:female ratio of approximately 1:1. The bees were given scented feeders filled with 25% sucrose solution. These feeders were alternated with

unscented feeders (no odor placed on the filter paper ring) filled with filtered water. The scented and unscented feeders were alternated over two days according to the schedule shown in Table 5-1. On the third day, the bees were tested.

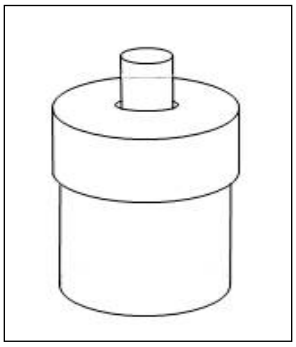

Figure 5-1. Feeder used to deliver conditioning odors. Feeder is a covered 5.0 ml plastic cup, with a hole drilled through the lid. Feeder and lid combined height is 2.5 cm; diameter is 2.3 cm. A wick (4.0 ± 0.5 cm) made of a segment of cigarette filter is inserted into the hole. A filter paper ring (2.3 cm diameter) surrounds the wick and is affixed to the lid with a small piece of white medical tape.

Table 5-1. Schedule of feeder changes and testing for Experiments One and Two.

Day 1	
1200 MST	Scented, sucrose solution-filled feeder
1700 MST	Unscented, water-filled feeder
Day 2	
0800 MST	Scented, sucrose solution-filled feeder
1100 MST	Unscented, water-filled feeder
1400 MST	Scented, sucrose solution-filled feeder
1700 MST	Unscented, water-filled feeder
Day 3	
0900 MST	Testing

Testing arenas were made from 1.42 L (48 fl. oz.) reusable plastic bowls (height x diameter: 8.5 cm x 16.5 cm) with lids (Western Family, Portland, OR, USA) (Fig. 5-2). For ventilation and to facilitate observation during testing, the entire center of each lid was replaced with mesh screen. Three 5.0 cm x 3.0 cm holes were cut in the sides of

each bowl and covered with mesh screen. Also, a circular hole was cut in the side of each bowl; the circular hole was exactly large enough for the mouth of a 20.0 ml glass scintillation vial to be inserted with a fit tight enough to prevent the vial from falling out of the hole. This circular hole served as the entry point for the bees. A rectangle of aluminum foil (length x width = 4.0 cm x 9.0 cm ± 0.5 cm) was placed in the bottom of each bowl. Two "faux feeders" were placed on the aluminum foil, approximately 2.5 cm apart and equidistant from the entry hole. The foil allowed for swapping of the positions of the faux feeders in between each individual test bee. The faux feeders were made of a 2.3 cm diameter circle of filter paper and a small length (1.0 – 2.0 cm) of cigarette filter wick, which was held in place by a thumbtack on the underside of the filter paper circle. The wicks of both faux feeders were saturated with filtered water. One faux feeder was scented with the same odor used for conditioning, either 0.25 µl geraniol or 0.50 µl PAA; the other faux feeder was unscented.

On Day 3, a bee was removed from the Plexiglas holding box using a 20.0 ml glass scintillation vial, and the vial was attached to the entry hole of the arena. As soon as the bee left the vial, a timer was started. The bee was given 10 min to choose between the two faux feeders. A feeder choice was recorded if the bee extended its proboscis to drink from a faux feeder. A small number of bees tested never actually extended their proboscises, but they circled one of the faux feeders with their heads down, intensely probing with their antennae. If they continued this behavior for more than 10 sec, it was recorded that the bee had chosen that particular faux feeder. We also recorded if the bee did not choose either feeder during the 10 min period.

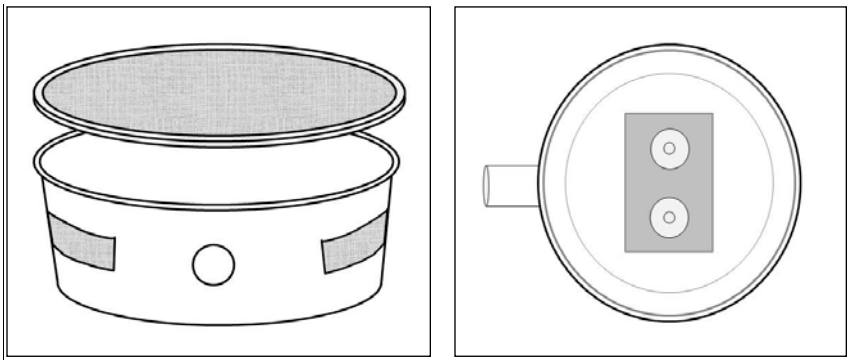

Figure 5-2. Testing arena made from a 1.42 L plastic bowl (height x diameter: 8.5 cm x 16.5 cm) with mesh windows and a mesh lid. A bee enters the arena from a vial attached to the side. Once inside, the bee chooses between two faux feeders, which are filter paper circles with small segments of cigarette filter attached from below by thumbtacks. The faux feeders rest on a rectangle of aluminum foil.

Experiment Two – Discriminatory Conditioning, Protocol A

For this experiment, *O. lignaria* and *M. rotundata* were conditioned to select the positively-scented feeder when given a choice between a positively-scented feeder and a negatively-scented feeder. *Osmia lignaria* were conditioned and tested June 2008. *Megachile rotundata* were conditioned and tested July - August 2008. Unfortunately, on 25 June, an incubator failure resulted in the premature emergence of most of the male *O. lignaria* being held at 4ºC until needed for the experiment. As a result, there were fewer bees than intended in many of the groups of males that were tested.

As above, there were two groups of bees, a conditioned group and an unconditioned group; the unconditioned bees did not encounter an odor until the testing phase. Conditioning and testing protocols for both species were the same as described above, except water-filled feeders now had an odor applied to the filter paper ring.

The odors used for conditioning and testing during this experiment were 0.25 µl geraniol, 0.25 µl PAA, or 0.25 µl citral (Sigma-Aldrich, St. Louis, MO, USA). Odors

were applied to the filter paper rings with a micropipetter. The odors were paired such that every combination of odors was tested (Table 5-2).

Table 5-2. Odor pairings used in Experiments Two and Three.

Positive Odor (Paired with Sucrose Solution)	Negative Odor (Paired with Water)
Geraniol	PAA
PAA	Geraniol
Geraniol	Citral
Citral	Geraniol
PAA	Citral
Citral	PAA

Experiment Three – Discriminatory Conditioning, Protocol B

For this experiment, *O. lignaria* and *M. rotundata* again were conditioned to select the positively-scented feeder when given a choice between a positively-scented feeder and a negatively-scented feeder, as in Experiment Two. However, some changes were made to the conditioning and testing procedures to determine if increasing the time the bees had with the feeders present enhanced their performance in the bioassay. *Osmia lignaria* were conditioned and tested April - June 2009. *Megachile rotundata* were conditioned and tested July - September 2009.

Again, there were two groups of bees, a conditioned group and an unconditioned group. The conditioning schedule was modified so that conditioned bees were exposed to each odor/feeder pairing (positive odor + sucrose solution or negative odor + filtered water) for a longer period of time; therefore, conditioning occurred over 4 days instead of 2 days (Table 5-3). Bees were placed in a Plexiglas cage at 0800 MST on Day 1 and feeder changes took place at 0800 MST on subsequent days (Table 5-3). Other than the

schedule change, *O. lignaria* conditioning was as previously described. Testing of *O. lignaria* was also as previously described, except that all bees made a choice by extension of the proboscis to drink from a faux feeder.

Table 5-3. Schedule of feeder changes and testing for Experiment Three. All feeder changes took place at 0800 MST.

Day 1	Positively-scented, sucrose solution-filled feeder
Day 2	Negatively-scented, water-filled feeder
Day 3	Positively-scented, sucrose solution-filled feeder
Day 4	Negatively-scented, water-filled feeder
Day 5, 0900-1200 MST	Testing

Conditioning of *M. rotundata* was as previously described, except for the aforementioned schedule change. However, during testing of *M. rotundata*, two protocol changes were made. First, testing occurred in the same greenhouse as conditioning and no heating pads were used to provide additional heat. Testing occurred under natural lighting at ~26-32°C. Second, as with *O. lignaria*, choices were only recorded when a bee extended its proboscis to drink.

As in Experiment Two, the odors used for conditioning and testing during this experiment were 0.25 µl geraniol, 0.25 µl PAA, or 0.25 µl citral, and they were applied with a micropipetter. The odors were paired such that every combination of odors was tested (Table 5-2).

Statistical Analysis

Bees were grouped according to species and gender for statistical analyses. Statistical analyses addressed five questions. First, were conditioned bees more likely than unconditioned bees to choose a faux feeder during testing? Second, were

conditioned bees more likely than unconditioned bees to choose the reward-associated faux feeder during testing? Third, did male bees perform differently than female bees, in terms of likelihood of making a choice and in terms of likelihood of choosing the reward-associated faux feeder? Fourth, did bees have a bias toward the left or the right during testing? Fifth, did unconditioned bees exhibit an odor preference during testing? A two-tailed Fisher's exact test (Zar, 1999; Proc Freq, SAS 9.2) was used to answer the first, second, and third questions. The fourth and fifth questions were answered using a binomial test (Zar, 1999; Proc Freq, SAS 9.2).

Results

Experiment One - Simple Conditioning

Were conditioned bees more likely than unconditioned bees to choose a faux feeder during testing?

In all cases, conditioned bees made a choice more often than unconditioned bees, but only *O. lignaria* females were significantly more likely than unconditioned bees to make a choice (Table A-1).

Were conditioned bees more likely than unconditioned bees to choose the reward-associated faux feeder during testing?

In all cases, a higher proportion of conditioned bees than unconditioned bees chose the scented feeders over the unscented feeders, but, in some cases, the results were not significant (Table A-2). Geraniol-conditioned *O. lignaria* males, geraniol-conditioned *M. rotundata* females, and PAA-conditioned *M. rotundata* males were not

significantly more likely than unconditioned bees to choose the reward-associated (i.e., scented) faux feeder (Table A-2).

Did male bees perform differently than female bees, in terms of likelihood of making a choice and in terms of likelihood of choosing the reward-associated faux feeder?

Of all groups tested, only one group exhibited a difference between males and females in their likelihood of making a choice: PAA-conditioned *O. lignaria* females were more likely to choose than PAA-conditioned *O. lignaria* males (Table A-3). Males and females from all groups performed similarly in terms of their likelihood of choosing the scented faux feeder (Table A-4).

Did bees have a bias toward the left or the right during testing?

Only PAA-conditioned *M. rotundata* females were more likely to choose the faux feeder on the right (Table A-5). No other groups of bees displayed a bias.

Did unconditioned bees exhibit an odor preference during testing?

Only unconditioned *M. rotundata* males were significantly more likely to choose unscented faux feeders than geraniol-scented faux feeders (Table A-6).

Experiment Two - Discriminatory Conditioning, Protocol A

Were conditioned bees more likely than unconditioned bees to choose a faux feeder during testing?

The citral/geraniol-conditioned females were the only conditioned *O. lignaria* significantly more likely than unconditioned *O. lignaria* to make a choice (Table A-7). Both sexes of the citral/PAA-conditioned *M. rotundata* were significantly more likely than the unconditioned *M. rotundata* to choose either faux feeder (Table A-7).

Were conditioned bees more likely than unconditioned bees to choose the reward-associated faux feeder during testing?

Females and males of both species that were conditioned with the geraniol/PAA pairing were significantly more likely than the unconditioned bees to choose the positively-scented (i.e., geraniol-scented) feeder (Table A-8). There was also a significantly higher proportion of PAA/geraniol-conditioned *M. rotundata* females that chose the positively-scented feeder than their unconditioned counterparts (Table A-8).

Did male bees perform differently than female bees in terms of likelihood of making a choice and in terms of likelihood of choosing the reward-associated faux feeder?

For all of the groups of *O. lignaria* tested, females and males were equally likely to choose a faux feeder (Table A-9). Unconditioned *M. rotundata* males tested with geraniol/PAA, geraniol/citral, and citral/geraniol pairings were significantly more likely to make a choice than females tested with the same odors (Table A-9).

For all groups, no significant differences in the likelihood of choosing the positively-scented faux feeder were found between *O. lignaria* females and males (Table A-10). For conditioned *M. rotundata* tested with PAA/ geraniol pairing, as well as unconditioned *M. rotundata* tested with PAA/ citral pairing, males were significantly more likely to choose the positively-scented faux feeder than females.

Did bees have a bias toward the left or the right during testing?

None of the groups of bees tested in Experiment Two were more likely to choose the left or right faux feeder during testing (Table A-11).

Did unconditioned bees exhibit an odor preference during testing?

Unconditioned *O. lignaria* males were significantly more likely to choose PAA when given a choice between citral and PAA (Table A-12), although the number of bees that chose for this odor pairing was very low. Unconditioned *M. rotundata* males were significantly more likely to choose PAA in both cases where PAA was one of the choices tested (Table A-12).

Experiment Three – Discriminatory Conditioning, Protocol B

Were conditioned bees more likely than unconditioned bees to choose a faux feeder during testing?

For all of the *O. lignaria* groups tested, the conditioned bees were significantly more likely to make a choice than unconditioned bees, with only two exceptions: males tested with the citral/geraniol pairing and females tested with the PAA/citral pairing (Table A-13). For only half of the *M. rotundata* groups tested were the conditioned bees significantly more likely to make a choice than the unconditioned bees (Table A-13).

Were conditioned bees more likely than unconditioned bees to choose the reward-associated faux feeder during testing?

Geraniol/citral-conditioned males were the only *O. lignaria* group not significantly more likely than their corresponding unconditioned group to choose the positively-scented faux feeder (Table A-14). In contrast none of the *M. rotundata* groups of conditioned bees were significantly more likely than the unconditioned groups to choose the positively-scented faux feeder except for males conditioned with the PAA/citral pairing (Table A-14).

Did male bees perform differently than female bees in terms of likelihood of making a choice and in terms of likelihood of choosing the reward-associated faux feeder?

Unconditioned *O. lignaria* males tested with geraniol and citral were significantly more likely to make a choice than their female counterparts, as were conditioned *M. rotundata* males tested with PAA and citral (Table A-15).

Osmia lignaria females conditioned with geraniol and PAA and females conditioned with citral and PAA were significantly more likely than males from these same groups to choose the positively-scented faux feeder (Table A-16). Male and female *M. rotundata* were equally likely to choose the positively-scented faux feeder for all groups tested (Table A-16).

Did bees have a bias toward the left or the right during testing?

None of the groups tested in Experiment Three were more likely to choose the left or right faux feeder during testing (Table A-17).

Did unconditioned bees exhibit an odor preference during testing?

The only group of unconditioned bees tested that exhibited an odor preference was the *O. lignaria* males tested with the geraniol/PAA paring; they were significantly more likely to choose PAA (Table A-18).

Discussion

The conditioning experiments described here involving females and males of two related solitary bee species yielded both expected and surprising results. As expected,

both species were able to learn and to discriminate between floral odors in the laboratory bioassays, although surprisingly *O. lignaria* responded more often to the bioassay and better exhibited learning through conditioning than did *M. rotundata*. Differences in the bioassay responses between conspecific males and females also were particularly interesting.

Determining whether unconditioned bees were similar to conditioned bees in bioassay performance gave insight into whether any choice responses were on account of bee behaviors or characteristics unrelated to the conditioning experience. It is important to note that no right/left bias was exhibited by unconditioned or conditioned bees, except in Experiment One where PAA-conditioned *M. rotundata* females were more likely to choose the feeder on the right for unknown reasons (Table A-5). Finding no consistent positional bias ruled out the influence of physical or environmental factors in the experimental process or design. By design in Experiment One, *O. lignaria* and *M. rotundata* females and males were conditioned to associate either geraniol or PAA with sucrose solution and to associate the absence of odor with water (Table A-1). Only conditioned *O. lignaria* females were more likely to respond than unconditioned bees. Otherwise, this simple conditioning did not result in a behavior modification in that conditioned bees were not more likely than unconditioned bees to make a choice during the testing phase. Little evidence of behavior modification also was revealed when the bees were conditioned by exposing them in short time intervals (< 5 hrs during daytime) to two odors in Experiments Two. Under such circumstances, only the unconditioned *O. lignaria* females exposed to one of the six odor pairings (citral/geraniol) and both sexes

of unconditioned *M. rotundata* in one odor pairing group (citral/PAA) were more likely to make a choice in the bioassay (Table A-7). In Experiment Three, however, when bees were conditioned by twice exposing them for an entire day to each odor, all groups of the conditioned *O. lignaria* females and most of the males were more likely to choose than unconditioned bees (Table A-13). Half of the *M. rotundata* conditioned groups responded more often than the unconditioned bees. Therefore, when odor discrimination was involved, the behavior of the conditioned bees seemed to have been modified, especially in *O. lignaria*, when an adequate amount of time was allowed for passive learning.

If the bees made a choice in the bioassays, then the conditioned bees were more likely (although not always significantly) than the unconditioned bees to make a choice that indicated an effect of the conditioning experience by choosing the reward-associated ("positive") odor (Tables A-2, A-8, A-14). This was especially clear in Experiment Three. The extra time allowed for the *O. lignaria* to find and feed from the scented feeders influenced not only the percentage of bees that chose, but the percentage of bees that chose the positive feeder. It was quite surprising that, with only one exception, the *M. rotundata* conditioned groups were not more likely than unconditioned groups to choose the positive odor even with the increased exposure time. There is an apparent difference between the ability, or perhaps the propensity, of the two solitary bees to respond to this conditioning experience.

The use of common floral odors raised concern that conditioning could be influenced by bees' innate biases. Interesting choices indeed were made in the bioassay

by some groups of unconditioned *O. lignaria* and *M. rotundata* males that seemed to have an innate preference to PAA (Tables A-12, A-18). PAA was likely to be chosen by males, although not always with statistical significance, even when it had not been paired with a reward. Otherwise, no innate preference for an odor was prevalent in any of the groups of unconditioned bees tested, except for the unconditioned *M. rotundata* males that chose the unscented faux feeder every time they were given a choice between geraniol-scented and unscented feeders, as if they were repelled by geraniol.

It is possible that *O. lignaria* and *M. rotundata* have an innate, hard-wired preference for certain odors, but a preference may also be the result of previous experience during the bees' development. Dobson (1987) found a preference for certain flower, pollen, and pollenkitt odors in inexperienced *Colletes fulgidus longiplumosus*, another solitary bee. Dobson believed that these bees were not acting on an innate preference for which they were hard-wired, but rather they had been conditioned or imprinted on floral odors while developing in the nest. All three of the odors that were used for conditioning in this study are common components of floral aromas, including the aromas of many Rosaceae and Leguminosae, which are the preferred floral resources of *O. lignaria* and *M. rotundata*, respectively. Therefore, *O. lignaria* and *M. rotundata* may have been exposed to PAA during development within the nest, but they may have also been exposed to geraniol and citral. Without additional information about the pollen consumed during development by the bees used in this study, it cannot be determined if the innate preference observed was due to hard-wiring, previous exposure to PAA, or some other cause. However, regardless of the origination of a pre-existing preference,

the preference should not preclude future behavioral modification as a result of experience or conditioning (Heinrich, 1984; Dobson, 1987; Amaya-Márquez et al., 2008). Indeed, the current study includes several examples of male bees that were successfully conditioned to choose a geraniol- or citral-scented feeder, even when a PAA-scented feeder was also a choice. Further research concerning innate preferences for, or avoidance of, certain floral odors in more context specific bioassays may lead to interesting species- or sex-specific results.

Two experimental factors influenced the statistical outcome of the results in this study. For *O. lignaria* males, the sample size for some groups was quite low due to the failure of an incubator that caused the entire stock of bees to begin the incubation process *en masse* rather than in small sample sizes over time. Also, the number of bees that made a choice in some groups was very low. These small groups lacked statistical power, and results may have differed if large sample sizes had been possible.

Another adjustment to protocol was necessary in the conditioning process performed in cages where mating, but no nesting, could occur. Because all visits to feeders during training were for adult sustenance, and not for larval provisioning, the bees were not compelled to frequently visit the feeders. In Experiment Three, the conditioning schedule was adjusted to allow bees more time to learn each association. The schedule change resulted in a great improvement in *O. lignaria* performance, with all but two groups of conditioned bees being more likely than their unconditioned counterparts to choose between the two faux feeders during testing; in Experiment Two, only one conditioned group was more likely to choose than the unconditioned group.

However, *M. rotundata* did not show as marked an improvement in discrimination, with six groups of conditioned bees not being any more likely than unconditioned bees to make a choice (as compared to 10 groups in Experiment Two).

Although *O. lignaria* and *M. rotundata* are both solitary-nesting, aggregating bees, there are some differences in their life histories. *Osmia lignaria* emerge as adults, mate, and nest in the spring. Their offspring develop into adults by fall, and therefore, they spend the winter as adults in cocoons. *Osmia lignaria* populations forage during the early spring at times when floral resources may be sparse and ephemeral. In contrast, *M. rotundata* emerge in the summer, mate, and then nest into late summer. Their offspring only develop to the fifth instar, prepupal stage by fall. They remain in this stage for the winter, completing development over the following spring and summer. These summer-flying *M. rotundata* have ample forage and foraging time during the summer flowering season. Such differences in the lives of these two species may result in differences in the mechanisms by which they learn and in their abilities to learn. So, although *M. rotundata* did not perform as well as *O. lignaria* in all of the current bioassays, *M. rotundata* may equal or outperform *O. lignaria* in other olfactory conditioning experiences.

Other conditioning studies have either tested females only, or tested females and males together, without differentiating by sex. No other conditioning study has compared the performance of female and male solitary bees. Perhaps this is because the importance of males is discounted because males only need to forage and mate, and therefore, would not have as great of a need for learning as females, who live longer and need to make repeated trips between nest sites and foraging sites. In the current study, males did just as

well as females, and in fact, comparison of females and males found seven instances in which males performed better than females, as compared to three instances in which the females outperformed the males. So, this study afforded no evidence that males are less able to learn than females. The similar learning ability between males and females should not be surprising, because male bees are genetically identical to their mothers, and therefore, should inherit similar learning abilities. Also, female and male *O. lignaria* have been shown to have similar patterns of development in their mushroom bodies, the brain region responsible for learning in insects (Withers et al., 2008).

Menzel (2001) did not believe that honey bees should differ from other bees in their cognitive capacities, because all bees have essentially the same goals, such as navigation between nesting and foraging sites. However, previous experiments comparing social and solitary bees' learning capabilities conclude that social bees demonstrate better learning than solitary bees as a result of differences in social systems or as a result of social bees being evolutionarily more advanced (*Bombus bimaculatus* and *Xylocopa virginica*: Dukas and Real, 1991; *Apis mellifera* and *M. rotundata*: Campan and Lehrer, 2002). Amaya-Márquez et al. (2008) compared the learning capability of *O. lignaria* to the results obtained by Dukas and Real (1991) for *A. mellifera* and *X. virginica*. Amaya-Márquez et al. determined that the solitary *O. lignaria* performed more similarly to the social *B. bimaculatus* than to the solitary *X. virginica*. They argued that life history plays a more important role than social system in determining species' cognitive capabilities.

In the current study, *O. lignaria* and *M. rotundata* were conditioned in a foraging context using very similar methods, but *O. lignaria*'s learning ability was better demonstrated than that of *M. rotundata.* It would be difficult to make the argument that *O. lignaria* has more derived learning ability, given that these two species are in the same family, are somewhat specialists on particular plant families, and have other biological and ecological similarities. However, the physiological, ecological, and other life history differences that exist between these two species may be responsible for the different outcomes in this bioassay (Gould and Marler, 1984; Heinrich, 1984; Amaya-Márquez et al., 2008).

The extent of adult experience in honey bees and *O. lignaria* is evidenced from changes in the mushroom bodies of the brain, although the changes are not exactly similar between the two species (Withers et al., 2008). Withers et al. (2008) posit that honey bees, like other social species, continue brain development after adult emergence from the natal cell. *Osmia lignaria* emerge from the natal nest with fully developed brains because they spend the entire winter as adults, during which time their brains may go through developmental changes that honey bees and *M. rotundata* do not experience until after emergence. In our study, bees were tested 3-5 days after emergence, but this represents a large difference in the time that the two species had been adults. It is possible that if *M. rotundata* were tested at a later time, their learning ability may have increased as brain development continued.

It may be, in fact, that *O. lignaria* and *M. rotundata* do not differ from each other or from social Hymenoptera because of decreased learning abilities in one species or

another, but rather they simply have different or selective learning abilities. Learning is context-dependent, and contexts may carry different levels of importance depending on a species' life history. It would not be adaptive to learn everything and then forget those things that are unimportant. Instead, it would make more sense to evolve the ability to learn only those things that are important (Gould and Marler, 1984). For example, a bee species that specializes on only a few flowers would not be likely to evolve the ability to learn an unlimited number of floral odors; instead it would be more efficient to have an innate ability to recognize the odors of the few flowers that are meaningful. On the other hand, a generalist bee species, which can utilize a wide variety of flowers, would be better served by having the ability to learn the floral odors that are relevant at a particular time and place, learning new odors as necessary.

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CHAPTER 6

CONCLUSIONS

Employing the use of *Osmia lignaria* and *Megachile rotundata* as additional and alternative pollinators may help to alleviate the current deficit of honey bees, which are in decline in the United States due to health problems. However, efficient management of these solitary bees requires that the problem of dispersal of commercial populations be resolved. In this dissertation, the study reported in Chapter 2 eliminates rough handling of pre-emergent bees as a possible cause of dispersal in *O. lignaria*. In Chapter 3, another approach to solving this problem was also considered: decreasing dispersal of *M. rotundata* by attracting nesting females. This approach shows promise, both for *M. rotundata* and for *O. lignaria* (Vorel and Pitts-Singer, unpublished), although more work is needed if the attractive compounds present in old nests are to be identified. Once identified, these compounds may be used to develop a method of luring females to provided nesting materials on a large commercial scale.

Attraction of females to provided nesting materials may be facilitated by the bees' ability to learn olfactory cues. Conditioning of solitary bees has been accomplished before (Dukas and Real, 1991; Campan and Lehrer, 2002; Amaya-Márquez et al., 2008). Chapters 4 and 5 demonstrate that, if the correct technique is utilized, *O. lignaria* and *M. rotundata* can learn to respond to olfactory cues in a foraging context. This is not surprising, but does provide a baseline measure of these species' learning abilities.

Dobson (1987) believed that the preferences exhibited by inexperienced *Colletes fulgidus longiplumosus* for certain flower, pollen, and pollenkitt odors were the result of

learning in the natal nest. It is possible that exposure to odors in the natal nest could impact developing bees in other ways. The often noted attraction of solitary bees to active nesting and to previously used nesting materials (Michener, 1960; Cardale, 1968; Stephen et al., 1969; Michener, 1974; Eickwort et al., 1977; Buttery et al., 1981; Parker et al., 1983; Fairey and Lieverse, 1986) may not be an entirely hard-wired response, but may be, in part, a learned response. *Megachile rotundata* have been known to nest in the same type of nesting materials as they emerged from, even if more suitable nest sites are available (Stephen, 1962). A straight-forward method of determining if learning within the natal nest influences bees' future nest selections would be to add a novel olfactory cue to the natal nest. If, upon emergence, females preferentially nest in materials treated with the novel cue, this would provide strong evidence for the influence of learning on nest selection. In addition, this learned attraction to novel cues could be used in the future to develop methods of attracting females to new or treated nesting materials, thus increasing retention of commercial populations while decreasing the incidence of disease.

Experiments in which developing *O. lignaria* and *M. rotundata* are exposed to novel cues in an effort to influence their nest choices have already been initiated (Vorel and Pitts-Singer, unpublished). In 2008-2009, *M. rotundata* cells were exposed to either PAA or citral throughout development, beginning at the first instar. In summer 2009, the odor-exposed bees were allowed to choose between odor-treated nested materials and untreated nest materials. However, additional replication is needed, and conclusions cannot be drawn from the data collected thus far.

In 2009, *O. lignaria* females were exposed to PAA during the two weeks prior to emergence from the cocoon. However, they did not preferentially nest in response to PAA. It appears that, if learning occurs within in the natal nest, it most likely occurs at an earlier point in time.

Megachile rotundata are already widely used as pollinators for the commercial production of alfalfa seed. *Osmia lignara* are being heavily developed as pollinators for a variety of fruit trees. The work contained in this dissertation, as well as the results of future experiments, can be applied to make commercial management of these pollinators more efficient and productive.

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APPENDIX

RESULTS OF STATISTICAL ANALYSES FOR CHAPTER 5

Table A-1. Results of two-tailed Fisher's exact tests comparing the likelihood of conditioned and unconditioned bees choosing either faux feeder in Experiment One.

Bee Tested	Odor Pairing	No. Tested (Cond. / Uncond.)	Percent that Chose (Cond. / Uncond.)	P
O. lignaria ♀	Geraniol vs. Blank	60 / 61	68% / 36%	<0.01
O. lignaria ♂	Geraniol vs. Blank	57 / 37	61% / 43%	0.09
O. lignaria ♀	PAA vs. Blank	56 / 72	84% / 58%	<0.01
O. lignaria ♂	PAA vs. Blank	49 / 35	65% / 54%	0.37
M. rotundata ♀	Geraniol vs. Blank	51 / 50	20% / 6%	0.07
M. rotundata ♂	Geraniol vs. Blank	57 / 51	25% / 18%	0.48
M. rotundata ♀	PAA vs. Blank	46 / 50	39% / 26%	0.19
M. rotundata ♂	PAA vs. Blank	47 / 50	49% / 32%	0.10

Table A-2. Results of two-tailed Fisher's exact tests comparing the likelihood of conditioned and unconditioned bees choosing the scented faux feeder in Experiment One.

Bee Tested	Odor Pairing	No. Chose (Cond. / Uncond.)	Percent that Chose Scented (Cond. / Uncond.)	P
O. lignaria ♀	Geraniol vs. Blank	41 / 22	93% / 32%	<0.01
O. lignaria ♂	Geraniol vs. Blank	35 / 16	83% / 63%	0.16
O. lignaria ♀	PAA vs. Blank	47 / 42	85% / 36%	<0.01
O. lignaria ♂	PAA vs. Blank	32 / 19	84% / 47%	<0.01
M. rotundata ♀	Geraniol vs. Blank	10 / 3	90% / 33%	0.11
M. rotundata ♂	Geraniol vs. Blank	14 / 9	50% / 0%	<0.02
M. rotundata ♀	PAA vs. Blank	18 / 13	89% / 38%	<0.01
M. rotundata ♂	PAA vs. Blank	23 / 16	74% / 50%	0.18

Table A-3. Results of two-tailed Fisher's exact tests comparing female and male bees' likelihood of choosing either faux feeder in Experiment One.

Species Tested	Conditioning Status	Odor Pairing	No. Tested (♀ / ♂)	Percent that Chose (♀ / ♂)	P
O. lignaria	Cond.	Geraniol vs. Blank	60 / 57	68% / 61%	0.45
O. lignaria	Uncond.	Geraniol vs. Blank	61 / 37	36% / 43%	0.53
O. lignaria	Cond.	PAA vs. Blank	56 / 49	84% / 65%	<0.05
O. lignaria	Uncond.	PAA vs. Blank	72 / 35	58% / 54%	0.84
M. rotundata	Cond.	Geraniol vs. Blank	51 / 57	20% / 25%	0.64
M. rotundata	Uncond.	Geraniol vs. Blank	50 / 51	6% / 18%	0.12
M. rotundata	Cond.	PAA vs. Blank	46 / 47	39% / 49%	0.41
M. rotundata	Uncond.	PAA vs. Blank	50 / 50	26% / 32%	0.66

Table A-4. Results of two-tailed Fisher's exact tests comparing female and male bees' likelihood of choosing the scented faux feeder in Experiment One.

Species Tested	Conditioning Status	Odor Pairing	No. Chose (♀ / ♂)	Percent that Chose Scented (♀ / ♂)	P
O. lignaria	Cond.	Geraniol vs. Blank	41 / 35	93% / 83%	0.29
O. lignaria	Uncond.	Geraniol vs. Blank	22 / 16	32% / 63%	0.10
O. lignaria	Cond.	PAA vs. Blank	47 / 32	85% / 84%	1.00
O. lignaria	Uncond.	PAA vs. Blank	42 / 19	36% / 47%	0.41
M. rotundata	Cond.	Geraniol vs. Blank	10 / 14	90% / 50%	0.08
M. rotundata	Uncond.	Geraniol vs. Blank	3 / 9	33% / 0%	0.25
M. rotundata	Cond.	PAA vs. Blank	18 / 23	89% / 74%	0.43
M. rotundata	Uncond.	PAA vs. Blank	13 / 16	38% / 50%	0.71

Table A-5. Results of binomial tests of bees' likelihood of choosing the left or right faux feeder during testing in Experiment One.

Bee Tested	Conditioning Status	Odor Pairing	No. that Chose	Percent that Chose Left / Right	Z	P
O. lignaria ♀	Cond.	Geraniol vs. Blank	41	51% / 49%	0.16	0.88
O. lignaria ♂	Cond.	Geraniol vs. Blank	35	57% / 43%	0.85	0.40
O. lignaria ♀	Uncond.	Geraniol vs. Blank	22	50% / 50%	0.00	1.00
O. lignaria ♂	Uncond.	Geraniol vs. Blank	16	37% / 63%	-1.00	0.32
O. lignaria ♀	Cond.	PAA vs. Blank	47	49% / 51%	-0.15	0.88
O. lignaria ♂	Cond.	PAA vs. Blank	32	53% / 47%	0.35	0.72
O. lignaria ♀	Uncond.	PAA vs. Blank	41	49% / 51%	-0.16	0.88
O. lignaria ♂	Uncond.	PAA vs. Blank	19	53% / 47%	0.23	0.82
M. rotundata ♀	Cond.	Geraniol vs. Blank	10	50% / 50%	0.00	1.00
M. rotundata ♂	Cond.	Geraniol vs. Blank	14	57% / 43%	0.53	0.59
M. rotundata ♀	Uncond.	Geraniol vs. Blank	3	67% / 33%	0.58	0.56
M. rotundata ♂	Uncond.	Geraniol vs. Blank	9	56% / 44%	0.33	0.74
M. rotundata ♀	Cond.	PAA vs. Blank	17	24% / 76%	-2.18	<0.03
M. rotundata ♂	Cond.	PAA vs. Blank	23	52% / 48%	0.21	0.83
M. rotundata ♀	Uncond.	PAA vs. Blank	13	46% / 54%	-0.28	0.78
M. rotundata ♂	Uncond.	PAA vs. Blank	16	56% / 44%	0.50	0.62

Table A-6. Results of binomial tests of unconditioned bees' likelihood of choosing the scented or unscented faux feeder during testing in Experiment One.

Bee Tested	Odor Pairing	No. that Chose	Percent that Chose Scented / Unscented	Z	P
O. lignaria ♀	Geraniol vs. Blank	22	32% / 68%	1.71	0.09
O. lignaria ♂	Geraniol vs. Blank	16	63% / 37%	-1.00	0.32
O. lignaria ♀	PAA vs. Blank	42	36% / 64%	1.85	0.06
O. lignaria ♂	PAA vs. Blank	19	47% / 53%	0.23	0.82
M. rotundata ♀	Geraniol vs. Blank	3	33% / 66%	0.58	0.56
M. rotundata ♂	Geraniol vs. Blank	9	0% / 100%	3.00	<0.01
M. rotundata ♀	PAA vs. Blank	13	38% / 62%	0.83	0.41
M. rotundata ♂	PAA vs. Blank	16	50% / 50%	0.00	1.00

Table A-7. Results of two-tailed Fisher's exact tests comparing the likelihood of conditioned and unconditioned bees choosing either faux feeder in Experiment Two.

Bee Tested	Odor Pairing (Positive vs. Negative)	No. Tested (Cond. / Uncond.)	Percent that Chose (Cond. / Uncond.)	P
O. lignaria ♀	Geraniol vs. PAA	50 / 50	94% / 84%	0.20
O. lignaria ♂	Geraniol vs. PAA	19 / 20	89% / 75%	0.41
O. lignaria ♀	PAA vs. Geraniol	59 / 50	86% / 84%	0.79
O. lignaria ♂	PAA vs. Geraniol	5 / 20	80% / 75%	1.00
O. lignaria ♀	Geraniol vs. Citral	52 / 45	71% / 73%	0.82
O. lignaria ♂	Geraniol vs. Citral	31 / 7	65% / 57%	1.00
O. lignaria ♀	Citral vs. Geraniol	46 / 45	43% / 73%	<0.01
O. lignaria ♂	Citral vs. Geraniol	8 / 7	38% / 57%	0.62
O. lignaria ♀	PAA vs. Citral	21 / 25	57% / 44%	0.55
O. lignaria ♂	PAA vs. Citral	2 / 7	100% / 57%	0.50
O. lignaria ♀	Citral vs. PAA	32 / 25	72% / 44%	0.06
O. lignaria ♂	Citral vs. PAA	3 / 7	100% / 57%	0.48
M. rotundata ♀	Geraniol vs. PAA	39 / 40	56% / 43%	0.26
M. rotundata ♂	Geraniol vs. PAA	40 / 44	73% / 80%	0.61
M. rotundata ♀	PAA vs. Geraniol	42 / 40	57% / 43%	0.27
M. rotundata ♂	PAA vs. Geraniol	40 / 44	68% / 80%	0.23
M. rotundata ♀	Geraniol vs. Citral	40 / 42	50% / 33%	0.18
M. rotundata ♂	Geraniol vs. Citral	40 / 41	55% / 56%	1.00
M. rotundata ♀	Citral vs. Geraniol	41 / 42	20% / 33%	0.16
M. rotundata ♂	Citral vs. Geraniol	40 / 41	45% / 56%	0.38
M. rotundata ♀	PAA vs. Citral	41 / 40	56% / 68%	0.36
M. rotundata ♂	PAA vs. Citral	40 / 40	70% / 73%	1.00
M. rotundata ♀	Citral vs. PAA	40 / 40	35% / 68%	<0.01
M. rotundata ♂	Citral vs. PAA	40 / 40	48% / 73%	<0.04

Table A-8. Results of two-tailed Fisher's exact tests comparing the likelihood of conditioned and unconditioned bees choosing the positively-scented faux feeder in Experiment Two. Some groups, designated NC for "not computed," were too small for statistical analysis.

Bee Tested	Odor Pairing (Positive vs. Negative)	No. Chose (Cond. / Uncond.)	Percent that Chose Positive Odor (Cond. / Uncond.)	P
O. lignaria ♀	Geraniol vs. PAA	47 / 42	72% / 43%	<0.01
O. lignaria ♂	Geraniol vs. PAA	17 / 15	88% / 53%	<0.05
O. lignaria ♀	PAA vs. Geraniol	51 / 42	57% / 57%	1.00
O. lignaria ♂	PAA vs. Geraniol	4 / 15	75% / 47%	0.58
O. lignaria ♀	Geraniol vs. Citral	37 / 33	70% / 48%	0.09
O. lignaria ♂	Geraniol vs. Citral	20 / 4	85% / 75%	0.54
O. lignaria ♀	Citral vs. Geraniol	20 / 33	45% / 52%	0.78
O. lignaria ♂	Citral vs. Geraniol	3 / 4	33% / 25%	1.00
O. lignaria ♀	PAA vs Citral	12 / 11	67% / 55%	0.68
O. lignaria ♂	PAA vs Citral	2 / 4	100% / 100%	NC
O. lignaria ♀	Citral vs PAA	23 / 11	61% / 45%	0.47
O. lignaria ♂	Citral vs PAA	3 / 4	100% / 100%	NC
M. rotundata ♀	Geraniol vs. PAA	22 / 17	68% / 29%	<0.03
M. rotundata ♂	Geraniol vs. PAA	29 / 35	66% / 31%	<0.02
M. rotundata ♀	PAA vs. Geraniol	24 / 17	25% / 71%	<0.01
M. rotundata ♂	PAA vs. Geraniol	27 / 35	89% / 69%	0.07
M. rotundata ♀	Geraniol vs. Citral	20 / 14	70% / 57%	0.49
M. rotundata ♂	Geraniol vs. Citral	22 / 23	55% / 35%	0.24
M. rotundata ♀	Citral vs. Geraniol	8 / 14	50% / 43%	1.00
M. rotundata ♂	Citral vs. Geraniol	18 / 23	50% / 65%	0.36
M. rotundata ♀	PAA vs. Citral	23 / 27	61% / 56%	0.78
M. rotundata ♂	PAA vs. Citral	28 / 29	79% / 83%	0.75
M. rotundata ♀	Citral vs. PAA	14 / 27	50% / 44%	0.75
M. rotundata ♂	Citral vs. PAA	19 / 29	26% / 17%	0.49

Table A-9. Results of two-tailed Fisher's exact tests comparing female and male bees' likelihood of choosing either faux feeder in Experiment Two.

Species Tested	Conditioning Status	Odor Pairing	No. Tested (♀ / ♂)	Percent that Chose (♀ / ♂)	P
O. lignaria	Cond.	Geraniol vs. PAA	50 / 19	94% / 89%	0.61
O. lignaria	Uncond.	Geraniol vs. PAA	50 / 20	84% / 75%	0.50
O. lignaria	Cond.	PAA vs. Geraniol	59 / 5	86% / 80%	0.54
O. lignaria	Cond.	Geraniol vs. Citral	52 / 31	71% / 65%	0.63
O. lignaria	Uncond.	Geraniol vs. Citral	45 / 7	73% / 57%	0.40
O. lignaria	Cond.	Citral vs. Geraniol	46 / 8	43% / 38%	1.00
O. lignaria	Cond.	PAA vs. Citral	21 / 2	57% / 100%	0.50
O. lignaria	Uncond.	PAA vs. Citral	25 / 7	44% / 57%	0.68
O. lignaria	Cond.	Citral vs. PAA	32 / 3	72% / 100%	0.55
M. rotundata	Cond.	Geraniol vs. PAA	39 / 40	56% / 73%	0.16
M. rotundata	Uncond.	Geraniol vs. PAA	40 / 44	43% / 80%	<0.01
M. rotundata	Cond.	PAA vs. Geraniol	42 / 40	57% / 68%	0.37
M. rotundata	Cond.	Geraniol vs. Citral	40 / 40	50% / 55%	0.82
M. rotundata	Uncond.	Geraniol vs. Citral	42 / 41	33% / 56%	<0.05
M. rotundata	Cond.	Citral vs. Geraniol	41 / 40	20% / 45%	<0.02
M. rotundata	Cond.	PAA vs. Citral	41 / 40	56% / 70%	0.25
M. rotundata	Uncond.	PAA vs. Citral	40 / 50	68% / 78%	0.34
M. rotundata	Cond.	Citral vs. PAA	40 / 40	35% / 48%	0.36

Table A-10. Results of two-tailed Fisher's exact tests comparing female and male bees' likelihood of choosing the positively-scented faux feeder in Experiment Two.

Species Tested	Conditioning Status	Odor Pairing	No. Chose (♀ / ♂)	Percent that Chose Positive (♀ / ♂)	P
O. lignaria	Cond.	Geraniol vs. PAA	49 / 15	69% / 87%	0.32
O. lignaria	Uncond.	Geraniol vs. PAA	42 / 15	43% / 53%	0.55
O. lignaria	Cond.	PAA vs. Geraniol	51 / 4	57% / 75%	0.63
O. lignaria	Cond.	Geraniol vs. Citral	37 / 20	70% / 85%	0.34
O. lignaria	Uncond.	Geraniol vs. Citral	33 / 4	48% / 75%	0.60
O. lignaria	Cond.	Citral vs. Geraniol	20 / 3	45% / 33%	1.00
O. lignaria	Cond.	PAA vs. Citral	12 / 2	67% / 100%	1.00
O. lignaria	Uncond.	PAA vs. Citral	11 / 4	55% / 100%	0.23
O. lignaria	Cond.	Citral vs. PAA	23 / 3	61% / 100%	0.53
M. rotundata	Cond.	Geraniol vs. PAA	22 / 29	68% / 66%	1.00
M. rotundata	Uncond.	Geraniol vs. PAA	17 / 35	29% / 31%	1.00
M. rotundata	Cond.	PAA vs. Geraniol	24 / 27	25% / 89%	<0.01
M. rotundata	Cond.	Geraniol vs. Citral	20 / 22	70% / 55%	0.35
M. rotundata	Uncond.	Geraniol vs. Citral	14 / 23	57% / 35%	0.31
M. rotundata	Cond.	Citral vs. Geraniol	8 / 18	50% / 50%	1.00
M. rotundata	Cond.	PAA vs. Citral	23 / 28	61% / 79%	0.22
M. rotundata	Uncond.	PAA vs. Citral	27 / 29	56% / 83%	<0.05
M. rotundata	Cond.	Citral vs. PAA	14 / 19	50% / 26%	0.27

Table A-11. Results of binomial tests of bees' likelihood of choosing the left or right faux feeder during testing in Experiment Two.

Bee Tested	Conditioning Status	Odor Pairing	No. that Chose	Percent that Chose Left / Right	Z	P
O. lignaria ♀	Cond.	Geraniol vs. PAA	47	47% / 53%	-0.44	0.66
O. lignaria ♂	Cond.	Geraniol vs. PAA	17	47% / 53%	-0.24	0.81
O. lignaria ♀	Uncond.	Geraniol vs. PAA	42	48% / 52%	-0.31	0.76
O. lignaria ♂	Uncond.	Geraniol vs. PAA	15	60% / 40%	0.77	0.44
O. lignaria ♀	Cond.	PAA vs. Geraniol	51	55% / 45%	0.70	0.48
O. lignaria ♂	Cond.	PAA vs. Geraniol	4	25% / 75%	-1.00	0.32
O. lignaria ♀	Cond.	Geraniol vs. Citral	37	35% / 65%	-1.81	0.07
O. lignaria ♂	Cond.	Geraniol vs. Citral	20	65% / 35%	1.34	0.18
O. lignaria ♀	Uncond.	Geraniol vs. Citral	33	55% / 45%	0.52	0.60
O. lignaria ♂	Uncond.	Geraniol vs. Citral	4	75% / 25%	1.00	0.32
O. lignaria ♀	Cond.	Citral vs. Geraniol	20	55% / 45%	0.45	0.65
O. lignaria ♂	Cond.	Citral vs. Geraniol	3	33% / 67%	-0.58	0.56
O. lignaria ♀	Cond.	PAA vs. Citral	12	50% / 50%	0.00	1.00
O. lignaria ♂	Cond.	PAA vs. Citral	2	50% / 50%	0.00	1.00
O. lignaria ♀	Uncond.	PAA vs. Citral	11	64% /36%	0.90	0.37
O. lignaria ♂	Uncond.	PAA vs. Citral	4	50% / 50%	0.00	1.00
O. lignaria ♀	Cond.	Citral vs. PAA	23	65% / 35%	1.46	0.14
O. lignaria ♂	Cond.	Citral vs. PAA	3	33% / 67%	-0.58	0.56
M. rotundata ♀	Cond.	Geraniol vs. PAA	22	36% / 64%	-1.28	0.20
M. rotundata ♂	Cond.	Geraniol vs. PAA	29	45% / 55%	-0.56	0.58
M. rotundata ♀	Uncond.	Geraniol vs. PAA	17	53% / 47%	0.24	0.81
M. rotundata ♂	Uncond.	Geraniol vs. PAA	35	60% / 40%	1.18	0.24
M. rotundata ♀	Cond.	PAA vs. Geraniol	24	63% / 37%	1.22	0.22
M. rotundata ♂	Cond.	PAA vs. Geraniol	27	56% / 44%	0.58	0.56
M. rotundata ♀	Cond.	Geraniol vs. Citral	20	45% / 55%	-0.45	0.65
M. rotundata ♂	Cond.	Geraniol vs. Citral	22	50% / 50%	0.00	1.00
M. rotundata ♀	Uncond.	Geraniol vs. Citral	14	57% / 43%	0.53	0.59
M. rotundata ♂	Uncond.	Geraniol vs. Citral	23	61% / 39%	1.04	0.30
M. rotundata ♀	Cond.	Citral vs. Geraniol	8	37% / 63%	-0.71	0.48
M. rotundata ♂	Cond.	Citral vs. Geraniol	18	61% / 39%	0.94	0.35
M. rotundata ♀	Cond.	PAA vs. Citral	23	57% / 43%	0.63	0.53
M. rotundata ♂	Cond.	PAA vs. Citral	28	39% / 61%	-1.13	0.26
M. rotundata ♀	Uncond.	PAA vs. Citral	27	52% / 48%	0.19	0.85
M. rotundata ♂	Uncond.	PAA vs. Citral	29	52% / 48%	0.19	0.85
M. rotundata ♀	Cond.	Citral vs. PAA	14	50% / 50%	0.00	1.00
M. rotundata ♂	Cond.	Citral vs. PAA	19	47% / 53%	-0.23	0.82

Table A-12. Results of binomial tests of unconditioned bees' likelihood of choosing either the positively-scented or the negatively-scented faux feeder during testing in Experiment Two.

Bee Tested	Odor Pairing	No. that Chose	Percent that Chose Positive / Negative	Z	P
O. lignaria ♀	Geraniol vs. PAA	42	43% / 57%	-0.93	0.35
O. lignaria ♂	Geraniol vs. PAA	15	53% / 47%	0.26	0.80
O. lignaria ♀	Citral vs. Geraniol	33	52% / 48%	0.17	0.86
O. lignaria ♂	Citral vs. Geraniol	4	25% / 75%	-1.00	0.32
O. lignaria ♀	Citral vs. PAA	11	45% / 55%	-3.00	0.76
O. lignaria ♂	Citral vs. PAA	4	0% / 100%	2.00	<0.05
M. rotundata ♀	Geraniol vs. PAA	17	29% / 71%	-1.70	0.09
M. rotundata ♂	Geraniol vs. PAA	35	31% / 69%	-2.20	<0.03
M. rotundata ♀	Citral vs. Geraniol	14	43% / 57%	-0.53	0.59
M. rotundata ♂	Citral vs. Geraniol	23	65% / 35%	1.46	0.14
M. rotundata ♀	Citral vs. PAA	27	44% / 56%	-0.58	0.56
M. rotundata ♂	Citral vs. PAA	29	17% / 83%	-3.53	<0.01

Table A-13. Results of two-tailed Fisher's exact tests comparing the likelihood of conditioned and unconditioned bees choosing either faux feeder in Experiment Three.

Bee Tested	Odor Pairing (Positive vs. Negative)	No. Tested (Cond. / Uncond.)	Percent that Chose (Cond. / Uncond.)	P
O. lignaria ♀	Geraniol vs. PAA	38 / 56	84% / 54%	<0.01
O. lignaria ♂	Geraniol vs. PAA	39 / 63	82% / 52%	<0.01
O. lignaria ♀	PAA vs. Geraniol	36 / 56	92% / 54%	<0.01
O. lignaria ♂	PAA vs. Geraniol	38 / 63	79% / 52%	<0.02
O. lignaria ♀	Geraniol vs. Citral	38 / 84	84% / 37%	<0.01
O. lignaria ♂	Geraniol vs. Citral	42 / 56	79% / 55%	<0.02
O. lignaria ♀	Citral vs. Geraniol	41 / 84	80% / 37%	<0.01
O. lignaria ♂	Citral vs. Geraniol	42 / 56	71% / 55%	0.14
O. lignaria ♀	PAA vs. Citral	39 / 50	82% / 70%	0.22
O. lignaria ♂	PAA vs. Citral	34 / 47	94% / 64%	<0.01
O. lignaria ♀	Citral vs. PAA	37 / 50	89% / 70%	<0.04
O. lignaria ♂	Citral vs. PAA	34 / 47	88% / 64%	<0.02
M. rotundata ♀	Geraniol vs. PAA	51 / 50	24% / 10%	0.11
M. rotundata ♂	Geraniol vs. PAA	50 / 55	26% / 15%	0.15
M. rotundata ♀	PAA vs. Geraniol	41 / 50	32% / 10%	<0.02
M. rotundata ♂	PAA vs. Geraniol	51 / 55	27% / 15%	0.15
M. rotundata ♀	Geraniol vs. Citral	43 / 51	14% / 6%	0.29
M. rotundata ♂	Geraniol vs. Citral	42 / 51	79% / 8%	<0.01
M. rotundata ♀	Citral vs. Geraniol	46 / 51	39% / 6%	<0.01
M. rotundata ♂	Citral vs. Geraniol	49 / 51	45% / 8%	<0.01
M. rotundata ♀	PAA vs. Citral	44 / 55	11% / 9%	0.75
M. rotundata ♂	PAA vs. Citral	54 / 52	57% / 13%	<0.01
M. rotundata ♀	Citral vs. PAA	44 / 55	30% / 9%	<0.02
M. rotundata ♂	Citral vs. PAA	44 / 52	30% / 13%	0.08

Table A-14. Results of two-tailed Fisher's exact tests comparing the likelihood of conditioned and unconditioned bees choosing the positively-scented faux feeder in Experiment Three.

Bee Tested	Odor Pairing (Positive vs. Negative)	No. Chose (Cond. / Uncond.)	Percent that Chose Positive Odor (Cond. / Uncond.)	P
O. lignaria ♀	Geraniol vs. PAA	32 / 30	94% / 43%	<0.01
O. lignaria ♂	Geraniol vs. PAA	32 / 33	72% / 24%	<0.01
O. lignaria ♀	PAA vs. Geraniol	33 / 30	82% / 57%	0.05
O. lignaria ♂	PAA vs. Geraniol	30 / 33	93% / 76%	<0.01
O. lignaria ♀	Geraniol vs. Citral	32 / 31	75% / 45%	<0.03
O. lignaria ♂	Geraniol vs. Citral	33 / 31	73% / 52%	0.12
O. lignaria ♀	Citral vs. Geraniol	33 / 31	88% / 45%	<0.01
O. lignaria ♂	Citral vs. Geraniol	30 / 31	100% / 48%	<0.01
O. lignaria ♀	PAA vs Citral	32 / 35	88% / 57%	<0.01
O. lignaria ♂	PAA vs Citral	32 / 30	91% / 60%	<0.01
O. lignaria ♀	Citral vs PAA	33 / 35	94% / 43%	<0.01
O. lignaria ♂	Citral vs PAA	30 / 30	73% / 40%	<0.02
M. rotundata ♀	Geraniol vs. PAA	12 / 5	67% / 20%	0.13
M. rotundata ♂	Geraniol vs. PAA	13 / 8	62% / 63%	1.00
M. rotundata ♀	PAA vs. Geraniol	13 / 5	85% / 80%	1.00
M. rotundata ♂	PAA vs. Geraniol	14 / 8	79% / 38%	0.08
M. rotundata ♀	Geraniol vs. Citral	6 / 3	67% / 33%	0.52
M. rotundata ♂	Geraniol vs. Citral	33 / 4	73% / 25%	0.09
M. rotundata ♀	Citral vs. Geraniol	18 / 3	56% / 67%	1.00
M. rotundata ♂	Citral vs. Geraniol	22 / 4	68% / 75%	1.00
M. rotundata ♀	PAA vs. Citral	5 / 5	80% / 60%	1.00
M. rotundata ♂	PAA vs. Citral	31 / 7	100% / 29%	<0.01
M. rotundata ♀	Citral vs. PAA	13 / 28	92% / 89%	1.00
M. rotundata ♂	Citral vs. PAA	11 / 7	91% / 71%	0.53

Table A-15. Results of two-tailed Fisher's exact tests comparing female and male bees' likelihood of choosing either faux feeder in Experiment Three.

Species Tested	Conditioning Status	Odor Pairing	No. Tested (♀ / ♂)	Percent that Chose (♀ / ♂)	P
O. lignaria	Cond.	Geraniol vs. PAA	38 / 39	84% / 82%	1.00
O. lignaria	Uncond.	Geraniol vs. PAA	56 / 63	54% / 52%	1.00
O. lignaria	Cond.	PAA vs. Geraniol	36 / 38	92% / 79%	0.19
O. lignaria	Cond.	Geraniol vs. Citral	38 / 42	84% / 79%	0.58
O. lignaria	Uncond.	Geraniol vs. Citral	84 / 56	37% / 55%	<0.04
O. lignaria	Cond.	Citral vs. Geraniol	41 / 42	80% / 71%	0.44
O. lignaria	Cond.	PAA vs. Citral	39 / 34	82% / 94%	0.16
O. lignaria	Uncond.	PAA vs. Citral	50 / 47	70% / 64%	0.67
O. lignaria	Cond.	Citral vs. PAA	37 / 34	89% / 88%	1.00
M. rotundata	Cond.	Geraniol vs. PAA	51 / 50	24% / 26%	0.82
M. rotundata	Uncond.	Geraniol vs. PAA	50 / 55	10% / 15%	0.56
M. rotundata	Cond.	PAA vs. Geraniol	41 / 51	32% / 27%	0.82
M. rotundata	Cond.	Geraniol vs. Citral	43 / 49	14% / 29%	0.13
M. rotundata	Uncond.	Geraniol vs. Citral	51 / 51	6% / 8%	1.00
M. rotundata	Cond.	Citral vs. Geraniol	46 / 49	39% / 45%	0.68
M. rotundata	Cond.	PAA vs. Citral	44 / 54	11% / 57%	<0.01
M. rotundata	Uncond.	PAA vs. Citral	55 / 52	9% / 13%	0.55
M. rotundata	Cond.	Citral vs. PAA	44 / 52	30% / 21%	0.36

Table A-16. Results of two-tailed Fisher's exact tests comparing female and male bees' likelihood of choosing the positively-scented faux feeder in Experiment Three.

Species Tested	Conditioning Status	Odor Pairing	No. Chose (♀ / ♂)	Percent that Chose Positive (♀ / ♂)	P
O. lignaria	Cond.	Geraniol vs. PAA	32 / 32	94% / 72%	<0.05
O. lignaria	Uncond.	Geraniol vs. PAA	30 / 33	43% / 24%	0.12
O. lignaria	Cond.	PAA vs. Geraniol	33 / 30	82% / 93%	0.26
O. lignaria	Cond.	Geraniol vs. Citral	32 / 33	75% / 73%	1.00
O. lignaria	Uncond.	Geraniol vs. Citral	31 / 31	45% / 52%	0.80
O. lignaria	Cond.	Citral vs. Geraniol	33 / 30	89% / 100%	0.11
O. lignaria	Cond.	PAA vs. Citral	32 / 32	88% / 91%	1.00
O. lignaria	Uncond.	PAA vs. Citral	35 / 30	57% / 60%	1.00
O. lignaria	Cond.	Citral vs. PAA	33 / 30	94% / 73%	<0.04
M. rotundata	Cond.	Geraniol vs. PAA	12 / 13	67% / 62%	1.00
M. rotundata	Uncond.	Geraniol vs. PAA	5 / 8	20% / 63%	0.27
M. rotundata	Cond.	PAA vs. Geraniol	13 / 14	85% / 79%	1.00
M. rotundata	Cond.	Geraniol vs. Citral	6 / 14	67% / 64%	1.00
M. rotundata	Uncond.	Geraniol vs. Citral	3 / 4	33% / 25%	1.00
M. rotundata	Cond.	Citral vs. Geraniol	18 / 22	56% / 68%	0.52
M. rotundata	Cond.	PAA vs. Citral	5 / 31	80% / 100%	0.14
M. rotundata	Uncond.	PAA vs. Citral	5 / 7	60% / 29%	0.56
M. rotundata	Cond.	Citral vs. PAA	13 / 11	92% / 91%	1.00

Table A-17. Results of binomial tests of bees' likelihood of choosing the left or right faux feeder during testing in Experiment Three.

Bee Tested	Conditioning Status	Odor Pairing	No. that Chose	Percent that Chose Left / Right	Z	P
O. lignaria ♀	Cond.	Geraniol vs. PAA	32	47% / 53%	-0.35	0.72
O. lignaria ♂	Cond.	Geraniol vs. PAA	32	38% / 62%	-1.41	0.16
O. lignaria ♀	Uncond.	Geraniol vs. PAA	30	57% / 43%	0.73	0.47
O. lignaria ♂	Uncond.	Geraniol vs. PAA	33	55% / 45%	0.52	0.60
O. lignaria ♀	Cond.	PAA vs. Geraniol	33	58% / 42%	0.87	0.38
O. lignaria ♂	Cond.	PAA vs. Geraniol	30	37% / 63%	-1.46	0.14
O. lignaria ♀	Cond.	Geraniol vs. Citral	32	66% / 34%	1.77	0.08
O. lignaria ♂	Cond.	Geraniol vs. Citral	33	58% / 42%	0.87	0.38
O. lignaria ♀	Uncond.	Geraniol vs. Citral	31	42% / 58%	-0.90	0.37
O. lignaria ♂	Uncond.	Geraniol vs. Citral	31	58% / 42%	0.90	0.37
O. lignaria ♀	Cond.	Citral vs. Geraniol	33	42% / 58%	-0.87	0.38
O. lignaria ♂	Cond.	Citral vs. Geraniol	30	43% / 57%	-0.73	0.47
O. lignaria ♀	Cond.	PAA vs. Citral	32	60% / 40%	1.06	0.29
O. lignaria ♂	Cond.	PAA vs. Citral	32	50% / 50%	0.00	1.00
O. lignaria ♀	Uncond.	PAA vs. Citral	35	54% / 46%	0.51	0.61
O. lignaria ♂	Uncond.	PAA vs. Citral	30	47% / 53%	-0.37	0.72
O. lignaria ♀	Cond.	Citral vs. PAA	33	48% / 52%	-0.17	0.86
O. lignaria ♂	Cond.	Citral vs. PAA	30	50% / 50%	0.00	1.00
M. rotundata ♀	Cond.	Geraniol vs. PAA	12	42% / 58%	-0.58	0.56
M. rotundata ♂	Cond.	Geraniol vs. PAA	13	54% / 46%	0.28	0.78
M. rotundata ♀	Uncond.	Geraniol vs. PAA	5	40% / 60%	-0.45	0.65
M. rotundata ♂	Uncond.	Geraniol vs. PAA	8	63% / 37%	0.71	0.48
M. rotundata ♀	Cond.	PAA vs. Geraniol	13	54% / 46%	0.28	0.78
M. rotundata ♂	Cond.	PAA vs. Geraniol	14	71% / 29%	1.60	0.11
M. rotundata ♀	Cond.	Geraniol vs. Citral	6	33% / 67%	-0.82	0.41
M. rotundata ♂	Cond.	Geraniol vs. Citral	33	58% / 42%	0.87	0.38
M. rotundata ♀	Uncond.	Geraniol vs. Citral	3	0% / 100%	1.73	0.08
M. rotundata ♂	Uncond.	Geraniol vs. Citral	4	75% / 25%	1.00	0.32
M. rotundata ♀	Cond.	Citral vs. Geraniol	18	67% / 33%	1.41	0.16
M. rotundata ♂	Cond.	Citral vs. Geraniol	22	36% / 64%	-1.28	0.20
M. rotundata ♀	Cond.	PAA vs. Citral	5	40% / 60%	-0.45	0.65
M. rotundata ♂	Cond.	PAA vs. Citral	31	58% / 42%	0.90	0.37
M. rotundata ♀	Uncond.	PAA vs. Citral	5	40% / 60%	-0.45	0.65
M. rotundata ♂	Uncond.	PAA vs. Citral	7	14% / 86%	-1.89	0.06
M. rotundata ♀	Cond.	Citral vs. PAA	13	54% / 46%	0.28	0.78
M. rotundata ♂	Cond.	Citral vs. PAA	11	55% / 45%	0.30	0.76

Table A-18. Results of binomial tests of unconditioned bees' likelihood of choosing either the positively-scented or the negatively-scented faux feeder during testing in Experiment Three.

Bee Tested	Odor Pairing	No. that Chose	Percent that Chose Positive / Negative	Z	P
O. lignaria ♀	Geraniol vs. PAA	22	36% / 64%	-1.28	0.20
O. lignaria ♂	Geraniol vs. PAA	28	18% / 82%	-3.40	<0.01
O. lignaria ♀	Citral vs. Geraniol	20	60% / 40%	0.89	0.37
O. lignaria ♂	Citral vs. Geraniol	31	48% / 52%	-0.18	0.86
O. lignaria ♀	Citral vs. PAA	28	36% / 64%	-1.51	0.13
O. lignaria ♂	Citral vs. PAA	25	40% / 60%	-1.00	0.32
M. rotundata ♀	Geraniol vs. PAA	5	40% / 60%	-0.45	0.65
M. rotundata ♂	Geraniol vs. PAA	8	63% / 37%	0.71	0.48
M. rotundata ♀	Citral vs. Geraniol	3	0% / 100%	1.73	0.08
M. rotundata ♂	Citral vs. Geraniol	4	25% / 75%	-1.00	0.32
M. rotundata ♀	Citral vs. PAA	5	60% / 40%	0.45	0.65
M. rotundata ♂	Citral vs. PAA	7	86% / 14%	1.89	0.06

CURRICULUM VITAE

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PROFESSIONAL EXPERIENCE SUMMARY

- Program management
- Outreach
- Revamping and maintaining a webpage
- Effective communication skills
- Leadership and teamwork
- Working closely with growers

EDUCATION

Utah State University, Logan, Utah	Exp. completion
Ph.D. Candidate, Department of Biology	Dec. 2009
Academic Advisor: Dr. M. E. Pfrender	4.00 GPA
Research Advisor: Dr. T. L. Pitts-Singer	
Weber State University, Ogden, Utah	
B.S., Zoology, Summa cum Laude	Aug. 2004
Advisor: Dr. J. F. Mull	3.94 GPA
A.S., Biotechnology, High Honors	May 2003
Advisor: Dr. J. Clark	3.94 GPA

EMPLOYMENT

Utah State University, Logan, Utah	Oct. 2009 –
Cooperative Agricultural Pests Survey Coordinator	present
Cooperative Extension Service, Department of Biology	

RESEARCH EXPERIENCE

Graduate Student Research with Dr. T. L. Pitts-Singer 2004 - present
USDA-ARS Bee Biology and Systematics Laboratory,
Logan, Utah

- Examining chemically-mediated behavior of solitary bees used as commercial pollinators.
- Completed two experiments exploring the learning capabilities of blue orchard bees (*Osmia lignaria*) and alfalfa leafcutting bees (*Megachile rotundata*).
- Nearing completion of two other learning experiments.
- Completed two experiments assessing the role of olfactory cues for these two bee species during nest selection.
- Completed an experiment addressing whether handling procedures affect nest establishment of blue orchard bees in agricultural settings.

Graduate Research Assistantship with Dr. T. L. Pitts-Singer May 2005 – Aug. 2007, Summers 2008 & 2009
USDA-ARS Bee Biology and Systematics Laboratory,
Logan, Utah

- Performed scientific research in Bee Behavior Lab, working on dissertation projects, advisor's projects, and collaborators' projects.
    - Successfully designed experiments.
    - Collected and analyzed data.
    - Prepared manuscripts and presentations.

Undergraduate Research with Dr. John Mull 2002 - 2004
Weber State University, Ogden, Utah

- Explored possible functions for metapleural gland secretions of western harvester ants, *Pogonomyrmex occidentalis*.

Undergraduate Research with Dr. Michelle Arnold Summer 2003
Weber State University, Ogden, Utah

- Worked with a team of students on the use of low-level radiation for detecting long-term lead accumulation in bone.

TEACHING EXPERIENCE

Graduate Teaching Assistant
Utah State University, Department of Biology, Logan, Utah

- Biol 4000 Human Dissection — Fall 2009
  Assisted students with in-class activities. Assisted with preparing and administering quizzes and tests.
- Biol 2320 Human Anatomy Lab — Spring 2009
  Presented material to students and assisted with preparing and administering quizzes and tests.
- Biol 1610 General Biology Lab — Fall 2008
  Lectured weekly, facilitated weekly hands-on activities, administered quizzes, and was responsible for all grading.
- Biol 1060 Biological Discoveries — Fall 2007 & Spring 2008
  Prepared and gave weekly lectures, prepared and facilitated weekly hands-on activities, prepared and administered tests, and was responsible for all grading.

Tutor
Weber State University Student Support Services, Ogden, Utah — Aug. 2001 – Aug. 2004

- Helped students succeed in math, English, and science classes.
- 'Tutor of the Month' for January 2002
- Served on the hiring committee during the search for a new director.

Supplemental Instructor
Weber State University Academic Support Services, Ogden, Utah — Aug. 2001 – May 2003
Prepared for and facilitated study groups for Math 1010 and Zoology 1110 classes.

INVITED PRESENTATIONS (presenter underlined)

Vorel, C. A., and T. L. Pitts-Singer. 2009. Attraction to old nests during nest selection in a solitary bee, *Megachile rotundata*. Weber State University, Ogden, UT.

SUBMITTED PRESENTATIONS (presenter underlined)

Oral Presentations

Vorel, C. A., and T. L. Pitts-Singer. 2008. Odor discrimination in two conditioned solitary bees, *Osmia lignaria* and *Megachile rotundata* (Apidae: Megachilidae). Annual Meeting of the Entomological Society of America, Reno, NV.

Vorel, C. A., and T. L. Pitts-Singer. 2008. Olfactory conditioning of solitary bees. Utah State University Graduate Student Research Symposium, Logan, UT.

Vorel, C. A., and T. L. Pitts-Singer. 2007. Olfactory conditioning of solitary bees. Annual Meeting of the Animal Behavior Society, Burlington, VT.

Vorel, C. A., and T. L. Pitts-Singer. 2007. The proboscis extension reflex in hymenopterans of different social levels. Utah State University Graduate Student Research Symposium, Logan, UT.

Vorel, C. A. 2007. Use of olfactory cues from old nests for nest selection in the alfalfa leafcutting bee (*Megachile rotundata*). Annual Meeting of the Idaho Alfalfa and Clover Seedgrowers Commission, Boise, ID.

Poster Presentations

Vorel, C. A., and T. L. Pitts-Singer. 2009. Does rough handling decrease retention of blue orchard bees in commercial populations? Annual Meeting of the Entomological Society of America, Indianapolis, IN.

Vorel, C. A., and T. L. Pitts-Singer. 2008. Does rough handling decrease retention of blue orchard bees in commercial populations? Annual Meeting of the Animal Behavior Society, Snowbird, UT.

Vorel, C. A., T. L. Pitts-Singer, and N. Boehme. 2007. Does sociality in Hymenoptera influence elicitation of the proboscis extension reflex in the laboratory? Annual Meeting of the Entomological Society of America, San Diego, CA.

Vorel, C. A., and T. L. Pitts-Singer. 2007. Nest lures for wild blue orchard bees in commercial orchards. Annual Almond Industry Conference, Modesto, CA.

Vorel, C. A., T. L. Pitts-Singer, and N. Frank. 2006. The proboscis extension reflex in Hymenopterans of different social levels. Annual Meeting of the Animal Behavior Society, Snowbird, UT.

Pitts-Singer, T. L., C. Guédot, and C. A. Vorel. 2006. Relating behavioral attributes of aggregating, solitary bees to those of social bees. XI Congress of the International Union for the Study of Social Insects, Washington, DC.

Vorel, C. A., and J. F. Mull. 2003. Composition and function of metapleural gland secretions in the western harvester ant, *Pogonomyrmex Occidentalis*. National Conference on Undergraduate Research, Salt Lake City, UT.

PUBLICATIONS

Vorel, C. A., and T. L. Pitts-Singer. The proboscis extension reflex not elicited in megachilid bees. J. Kansas. Ent. Soc. *Accepted*.

Vorel, C. A., T. L. Pitts-Singer, and J. Bosch. Influence of rough handling on blue orchard bee (*Osmia lignaria*) nest establishment. *In pre-submission review.*

Vorel, C. A., and T. L. Pitts-Singer. Influence of olfactory cues from old nests on nest selection by the solitary bees *Osmia lignaria* and *Megachile rotundata* (Hymenoptera: Megachilidae). *Submitted*.

GRANT FUNDED

Almond Board of California, *$4,400* 2007
"Nest Lures for Wild Blue Orchard Bees in Commercial Orchards"

- Verified attraction of blue orchard bees to old bee nests.
- Examined comparative attractiveness of individual components of old bee nests.

FELLOWSHIPS

Utah State University Vice President of Research Fellowship, *$15,000* 2004 - 2005
National Science Foundation Research Experience for Undergraduates Fellowship, *$2,500 stipend plus $500 research funds* Summer 2002

SCHOLARSHIPS

Claude E. Zobell Scholarship, *$1,500* 2007 - 2008
Seely-Hinckley Scholarship, *one year tuition* 2007 - 2008
James A. and Patty MacMahon Ecology Scholarship, *$1,000* 2007 - 2008
Weber State University College of Science Private Foundation Scholarship, *full year tuition* 2003 – 2004
Jerry B. Jensen Activity Scholarship, *one semester tuition* Spring 2003
Aspire-Utah Trio Scholarship, *one year tuition* 2003 – 2004
Aspire-Utah Trio Scholarship, *$500* 2002 - 2003
Services for Women Students Academic Scholarship, *one year tuition* 2002 – 2003

AWARDS

Weber State University, Dept. of Zoology, Outstanding Senior Award	2004
Weber State University, Dept. of Zoology, Evolutionary Ecology Award	2004
Utah Campus Compact/SSUN Civic Engagement Award	2004
Weber State University Academic Honor Award	Multiple Semesters

PROFESSIONAL SOCIETY MEMBERSHIPS

Entomological Society of America	2007 - present
• Rules and Ethics Committee	2008 - 2009
Kansas Entomological Society	2007 - present
Animal Behavior Society	2005 - present
Sigma Xi Honor Society for Scientific Research	2003 - present
Honor Society of Phi Kappa Phi	2002 - present
Golden Key Honor Society	2002 - present

PROFESSIONAL MEETING AND CONFERENCE INVOLVEMENT

Annual Meeting of the Entomological Society of America — Dec. 2008
- Served as Volunteer Audio/Visual Aide.

Annual Meeting of the Animal Behavior Society — Aug. 2006
- Along with Dr. T. Pitts-Singer and Dr. Sylvia Halkin, organized and led a group of 25 meeting attendees on a tour of Northern Utah points of interest.

Annual Meeting of the Animal Behavior Society, Attendee — Aug. 2005

Weber State University Academy of Leadership Participant — Spring 2002, Fall 2002
- Steering Committee member — Fall 2003
  - Food and Transportation Subcommittee Chair

Utah Academy of Leadership Participant — 2002, 2003

PROFESSIONAL AND ACADEMIC ACTIVITIES

Utah State University

Department of Biology Seminar Speaker Committee Member — 2008 - present

Graduate Student Representative at Department of Biology's successful presentation for USU Departmental Teaching Excellence Award — Feb. 2009

Reviewed manuscript for Journal of Plant Breeding and Crop Science — Feb. 2009

Biology Graduate Student Association Member — 2004 - present
- President — 2007 - 2008
- Vice President of Fundraising and Finances — 2006-2007
- Officer — 2005-2006

Entomology Club Member — 2005 - present
- Lead tours of USU Insect Collection and participate in numerous public outreach activities, such as Aggie Family Day and American West Heritage Center's Baby Animal Days.

USDA-ARS Bee Biology and Systematics Laboratory

Lead tours for university classes and community groups. — 2005 - present
Manage research greenhouses. — 2008 - present
Supervise undergraduate employees and volunteers. — 2006 - present

Weber State University

Zoology Club Member — 2003 - 2004
Math Club Member — 2001 - 2003
- Vice-president — 2002 - 2003

Taste for Diversity Committee Member — 2002 - 2003
Student Support Services Participant — 2001 - 2003
Services for Women Students "Elegant Evening" Committee Member — 2002
Campus Leaders Alliance Member — 2002 - 2004

VOLUNTEER EXPERIENCE

Utah Friends of Basset Hounds — Dec. 2008 – present
Dog Foster
- Evaluate, care for, and help find homes for rescued dogs.
- Assist with fundraising.

Aggie Cat Services — Sep. 2008 – present
Feeding Station Leader
- Coordinate volunteers feeding a feral cat colony on Utah State University campus.
- Feed and monitor health of feral cat colony on campus.
- Serve as communication liaison between volunteers and group officers.

IRS Volunteer Income Tax Assistance (VITA) Program — Feb. 2007 – Apr. 2008
Tax Preparer

- Prepared and filed income tax forms for low income individuals and families.

Ogden Nature Center — Jan. 2005 – Oct. 2005
Bird Rehabilitation Program

- Fed and cared for resident birds.
- Assisted in caring for infirm birds.

Corporation for National & Community Service — 2002 - 2004
Americorps Volunteer

- Served with other members of a national service network on a variety of education-related service projects.

Weber State University Volunteer Involvement Program
Head Start Chair — Aug. 2003 – May 2004

- Designed program and supervised group of volunteers that worked with preschool children.

Christmas Tree Express Chair — Aug. 2002 – May 2003

- Coordinated decoration and display of Christmas trees by campus organizations; trees were subsequently donated to needy families.
- Revamped and maintained website for Volunteer Involvement Program.

Utah Campus Compact's Students Serving Utah Network (SSUN) — June 2003 – May 2004
Representative for Weber State University

- Attended all SSUN functions as WSU's representative.
- Successfully planned and coordinated a campus-wide drive to increase student involvement in mentoring programs.