COMPARING REVERSAL-LEARNING ABILITIES, SUCROSE RESPONSIVENESS, AND FORAGING EXPERIENCE IN SCOUT AND NON-SCOUT HONEY BEE (*APIS MELLIFERA*) FORAGERS

BY

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THESIS

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ABSTRACT

Honey bees (*Apis mellifera*) colonies divide foraging activities between scouts, who search for new sources of food, and non-scouts, who rely on information from waggle dances to find food sources. Molecular analyses of scouts and non-scouts have revealed differences in the expression of numerous genes, including several related to neurotransmitter signaling. Despite this progress, we know almost nothing about cognitive, sensory, or behavioral differences that underlie scouting. I tested three hypotheses related to differences between scouts and non-scouts. First, I hypothesized that scouts and non-scouts differ in their reversal-learning abilities. Scouts showed a significantly faster reversal in their response to an odor that was punished and then rewarded. The results also suggested an interaction between the effects of foraging role (scout or non-scout) and seasonal effects on reversal-learning abilities. Second, I hypothesized that variation in responsiveness to sucrose rewards is associated with scouting behavior. I found no significant difference in responsiveness between scouts and non-scouts. Third, I hypothesized that greater foraging experience increases the probability that a forager will engage in scouting behavior. I tested this by comparing wing damage between scouts and non-scouts and found that non-scouts showed greater wing damage in the early summer but not the late summer. Together, these three results contribute to our understanding of cognitive, sensory, and behavioral aspects associated with scouting behavior.

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INTRODUCTION

Cognitive and sensory abilities often vary with differences in ecological niche or role within a community (Shettleworth 2001; Healy et al. 2009; Mery 2013). Individuals within a population can show variation in their cognitive abilities (Dukas 2004) and consistent differences in their behavioral tendencies (Sih et al. 2004). Recent work has suggested that cognitive differences among individuals may create consistent differences in behavioral tendencies and vice versa (Sih & Del Giudice 2012; Niemela et al. 2013). This possibility evokes the question: do cognitive differences among individuals influence division of labor within animal societies? Honey bees (*Apis mellifera*) provide an excellent system to study this question because they have long served as models for understanding foraging-related division of labor (Robinson 1992; Seeley 1995) and the mechanisms underlying learning and memory (Menzel 1993).

Associations between division of labor and cognitive/sensory abilities suggest the possibility of a causal relationship between them. Honey bees use their sensory and cognitive abilities to collect nectar and pollen from a wide variety of flowers that change repeatedly over the course of the foraging season (Visscher & Seeley 1982; Menzel 1985; Menzel 1999). The performance of worker honey bees on cognitive tests depends on a wide variety of factors including environment early in life (Jones et al. 2005; Scheiner 2012), motivational state (Scheiner et al. 2005), and genetic differences (*olfactory acquisition*: Bhagavan 1994; *olfactory discrimination*: Benatar 1995; *latent inhibition*: Chandra et al. 2000, 2001; *reversal learning*: Ferguson et al. 2001). Experiments have revealed strong

relationships between the tasks that individual workers perform and their perceptual and cognitive abilities (*nursing*: Ben-Shahar & Robinson 2001, Ben-Shahar et al. 2000; *hygienic behavior*: Masterman et al. 2000, 2001; *pollen/nectar collection*: Scheiner et al. 2001a,b; Latshaw & Smith 2005; *resin collection*: Simone-Finstrom et al. 2010).

These previously cited experiments focus on the response of individual bees to information from the environment around them, but the honey bee foraging strategy also involves frequent exchange of information among workers (Seeley 1995). In temperate climates, honey bee colonies must gather food stores during the warmer months in order to support tens of thousands of bees and maintain high in-hive temperatures over the winter (Seeley & Visscher 1985). In environments with sparsely distributed resources, communication about food sources greatly enhances these gathering efforts (Sherman & Visscher 2002; Dornhaus & Chittka 2004). Workers accomplish this communication using a signal called a waggle dance, which conveys information about the distance, direction, and profitability of a resource (von Frisch 1967).

The honey bee social foraging strategy affects cognitive demands on workers. Colonies can divide labor between foragers, called scouts, who actively search for novel sources of food, and non-scouts, who focus on exploiting known sources (Seeley 1983). This division of labor increases efficiency by shifting the burden of exploring and collecting information to a subset of 5-36% of the foragers (Seeley 1983; Seeley & Visscher 1988). When food distributions change, most honey bee foragers are able to rely on the flexibility of a minority of their sister foragers (Townsend-Mehler & Dyer 2012).

Many studies have examined the question of what causes a forager to engage in scouting behavior. Resource quality (Beekman et al. 2007) and distribution (Seeley 1983) both influence the decision to scout. However, the strong relationship between the tendency to scout for nest sites and later to scout for food resources suggests a continuum with behavioral extremes in which some foragers never scout and other foragers scout repeatedly (Liang et al. 2012). In addition, both nest-site scouting (Robinson & Page 1989) and food scouting (Dreller 1998; Mattila & Seeley 2011) have a heritable component. Scouts and non-scouts do not differ in size (Seeley 1983) or age (Dreller 1998), but they may differ in cognitive or sensory abilities.

Recent results of brain gene expression comparisons between scout and non-scout foragers have implicated neurotransmitter systems in the regulation of scouting behavior (Liang et al. 2012). Among their many functions, these neurotransmitter systems play key roles in cognition and perception. Microarray analyses revealed differences between scouts and non-scouts in the expression of a number of genes related to neurotransmitter signaling (Liang et al. 2012). These included genes involved in dopamine, GABA, glutamate, and octopamine signaling (Liang et al. 2012), which play important roles in learning and memory across taxa (*dopamine*: Wise 2004; *GABA*: Paredes & Agmo 1992; and *glutamate*: Riedel et al. 2003), including honey bees (Gauthier & Grunewald 2012). Both octopamine and dopamine affect responsiveness to sucrose in honey bee foragers (Scheiner et al. 2002) and sensitivity to olfactory cues (Barron et al. 2002; Spivak et al. 2003). In addition, pharmacological experiments demonstrated a causal relationship between glutamate and

octopamine and the tendency to scout for food (Liang et al. 2012). Changes in the levels of these two neurotransmitters influenced foraging decisions, but whether this involves effects on learning, memory, and/or perception was not examined.

In the current study I tested predictions of three hypotheses about factors that could influence scouting behavior: reversal-learning abilities, sucrose responsiveness, and foraging experience. First, based on work by Ferguson et al. (2001), I hypothesized that the flexibility a forager needs to excel in olfactory reversal learning could increase her chances of finding and responding to a newly rewarding patch of flowers. If the ability to forget old associations and learn new associations influences the division of labor between scout and non-scout foragers, scouts and non-scouts should show differences in their reversal-learning abilities. To test this prediction, I used a well-established proboscis extension reflex (PER) assay in which bees first learn to associate one odor with a sugar reward and another odor with a mildly aversive stimulus and then must learn to switch their preferences (Ferguson et al. 2001).

Second, based on known differences in sucrose responsiveness between workers with different foraging specializations (*nectar/pollen/water foragers*: Page et al. 1998; Pankiw & Page 2000; Scheiner et al 1999, 2001a,b; *resin/pollen foragers*: Simone-Finstrom et al. 2010), I hypothesized that lower responsiveness to sucrose decreases the probability that a forager would search independently. As noted above, treatments with octopamine and dopamine influence sucrose responsiveness in honey bees (Scheiner et al. 2002), supporting the idea that scouts and non-scouts might also differ in their responsiveness to

sucrose. If a forager responds to only high sugar concentrations, it might make her more sensitive to the variation in nectar qualities encountered on exploratory foraging trips and decrease her likelihood of engaging in scouting behavior. Based on this hypothesis, scouts should show significantly higher responsiveness to sucrose than non-scouts. To test this prediction, I used another PER assay, which involves presenting bees with an ascending series of sucrose concentrations and recording the number of presentations that they reflexively respond to (Page et al. 1998; Pankiw & Page 1999).

Third, based on results from Lindauer (1952) and Seeley (1983), I hypothesized that greater foraging experience increases the probability that a forager will engage in scouting behavior. Seeley (1983) suggested that experienced foragers have a higher probability of scouting based on comparisons of the proportion of experienced (Seeley 1983) vs. novice foragers from a previous study (Lindauer 1952) that found a new food source without the aid of dance information. However, Dreller (1998) found no differences in the probability of scouting among marked cohorts of bees, indicating that scouts and non-scouts have similar age distributions. Increased experience with spatial cues could help foragers find new sources of food. In addition, the mushroom body neuropil, a structure in the honey bee brain involved in associative learning, both expands (Fahrbach & Dobrin 2009) and shows changes in gene expression pattern (Lutz et al. 2012) with increased foraging experience. Previous studies have used wing damage as an indication of foraging experience (Breed et al. 1990; Huang et al. 1994). Based on Seeley's (1983) results I expected scouts to show greater wing damage than non-scouts. To test this prediction, I recorded the presence or

absence of wing damage on scouts and non-scouts collected for both the reversal-learning and sucrose responsiveness experiments.

METHODS AND MATERIALS

Bees

Honey bee colonies were maintained using standard beekeeping techniques at the University of Illinois in Urbana, Illinois. They were derived from naturally mated queens (a mix of mainly European subspecies of the western honey bee, *Apis mellifera*).

Scout collections with the hive-moving assay

The two main assays used previously to differentiate scouts and non-scouts are the hive-moving assay and the novel-feeder assay. The hive-moving assay identifies scouts based on their decision to forage in a novel environment without information from recruitment dances (Seeley 1983; Dreller 1998). The novel feeder assay identifies scouts based on their ability to find novel artificial food sources in the presence of a productive food source, which they are already familiar with (Liang et al. 2012). The scouts selected in both assays show similar brain gene expression patterns (Liang et al. 2012). Therefore, I chose to use the hive-moving assay to collect scouts and non-scouts for comparison because it identifies a large number of scouts more quickly than does the novel feeder assay.

To collect scouts and non-scouts, I used a hive-moving assay with similar methods to Liang et al. (2012). I selected hives containing ten frames, or roughly 20,000 worker bees, for this assay. I sealed each hive at night using an entrance reducer and duct tape and moved it to a

new location, outside the colony's foraging range (>4 km). The following morning, I attached an entrance, consisting of two mesh tubes joined to form a T-shape and sealed with corks, to the entrance reducer with one opening pointing upwards (Figure 1A). This setup allowed bees to easily walk out and up to exit the hive but made it difficult for them to re-enter, preventing any recruitment during the collection. Given that undertakers often leave the hive to remove dead bees soon after it is opened, I excluded bees exiting the hive and returning to it within the first ten minutes by collecting them with an insect vacuum (BioQuip Products, Rancho Dominguez, CA) and holding them until I had finished collecting scouts.

Previous dissections of honey stomach contents revealed that most bees collected after 10 minutes return with some nectar, indicating that they had foraged (Liang et al. 2012). Therefore, I assumed that bees returning after ten minutes had left the hive to search for novel food sources. I collected scouts returning to the hive with soft forceps (BioQuip Products) and placed them in modified insect vacuum collecting chambers (BioQuip Products, Figure 1B). I avoided collecting scouts with pollen on their legs due to known differences in sucrose responsiveness and learning ability between pollen and nectar foragers (Page et al. 1998; Scheiner et al 1999, 2001a,b). The collecting chambers had a hole drilled in the side so that a 1.5 ml feeding tube filled with sucrose solution could be inserted. I filled the feeding tubes with 30% (w/v) sucrose because previous comparisons (Pankiw et al. 2001) showed that feeding high- and low-pollen hoarding bees this concentration of sucrose did not diminish differences in sucrose responsiveness between the two groups. Each cage contained 6-7 scouts. After collecting the desired number of

scouts, I removed the entrance reducer from the hive to allow foragers to discover food sources and recruit their nestmates. I then transported the chambers of scouts from the collection site to the lab, where they remained under artificial lights at room temperature until harnessing (Figure 1C).

I collected non-scouts at least 1 hour after the scout collections, to give adequate time for recruitment. Given that only 5-36% of foragers engage in scouting behavior (Seeley 1983), I assumed that most of these foragers were not scouts. I again sealed the hive with an entrance reducer, and I collected foragers as described above. I then transported them to the same room as the scouts. I collected scouts and non-scouts in the morning on 7 days from different colonies between June 19th and July 4th, 2013 for sucrose responsiveness comparisons. I collected another set of scouts and non-scouts from 6 colonies on 12 days between August 6th and September 10th, 2013 for reversal-learning comparisons. For the reversal learning collections, I collected scouts and non-scouts from each colony twice with at least three days between collections to allow the colony to recover from the move.

Harnessing for reversal-learning and sucrose responsiveness comparisons

I harnessed scouts and non-scouts in brass tubes or waxed paper tubes (Custom Paper Tubes, Cleveland, OH) to prepare them for the reversal-learning or sucrose responsiveness tests, respectively. I buried the collecting chambers under ice for 8-10 minutes until all bees inside were incapacitated, and then I placed the bees on the surface of the ice. I secured each bee to a tube with a strip of gorilla tape (Gorilla Glue Co., Cincinnati, OH)

placed between her head and thorax so that she could freely move her antennae and proboscis. In the case of nurses, nectar foragers, and pollen foragers (see section below), I placed an additional piece of tape with a Kimwipe strip (Kimberly-Clark Professional, Roswell, GA), added to avoid sticking to the bee, around their thoraces and hind legs (Figure 1D). This second piece served to blind observers to each bee's role within the colony. This process took approximately 30 minutes from the time I placed the first chambers on ice until I harnessed the last bee (with no bee chilled for more than 16 minutes). I harnessed between 20-36 bees for each test. I wrote the group identity of the bee on the back of each tube. Once harnessed, I rearranged the bees to ensure that the person testing them was blind to which group they came from.

Reversal-learning comparisons

To compare the reversal-learning abilities of scouts and non-scouts, I used an established olfactory reversal-learning test (Bitterman et al. 1983; Ben-Shahar et al. 2000; Ferguson et al. 2001). Half an hour after harnessing, I fed them to satiation with 1.25 M sucrose. Then I placed them in a dark, humidified container at room temperature for a period of 22-26 hours before the beginning of the test. After that period, I placed them in the testing room and allowed them to acclimate for half an hour. Then, to select bees with an intact proboscis extension response, I touched their antennae with a drop of 1.5 M sucrose 3 times with a 1-minute intertrial interval. I selected only bees that responded by putting out their mouthparts immediately on all 3 presentations. For each test, 4-5 bees of each group

were selected. As in the sucrose responsiveness tests, I rearranged these bees to ensure that the tester was blind to which group they came from.

The test consisted of twelve discrimination trials, followed by a waiting period of 30 minutes (Ben-Shahar et al. 2000), and then twelve reversal trials. I used a predetermined sequence of rewarding and punishing trials (designed to prevent bees from learning the pattern of rewards and punishments rather than the odor cues; Bitterman et al. 1983) in both the discrimination and reversal phases (+--+-++-+--+) with an intertrial interval of 10 minutes. During each trial I placed a harnessed bee in a ventilated enclosure under constant airflow. I allowed her to acclimate for 20 seconds, used a Stimulus Air Controller CS-55 (Syntech Research and Equipment, Kirchzarten, Germany) to pump air through an odor cartridge and over her antennae for six seconds, and then allowed her to recover for 20 seconds before removing her from the enclosure and placing the next bee in it. Each odor cartridge consisted of a 5¾" Pasteur pipette containing a strip of filter paper soaked with 8 ul of either a 2 M solution of 1-hexanol or 1-nonanol dissolved in heavy mineral oil (Sigma Aldrich, St. Louis, MO). For each test, I paired one odor with rewarding trials in the discrimination phase and punishing trials in the reversal phase (odor A) and the other odor with punishing trials in the discrimination phase and rewarding trials in the reversal phase (odor B). I alternated these pairings across the tests. I repeated this process for 9 bees during each trial and used the remaining minute to exchange odor cartridges.

During the rewarding trials, after 2 seconds of exposure to the odor, I touched the bee's antennae with a toothpick soaked in 1.5 M sucrose and then allowed her to lick the

toothpick until the end of the odor pulse (approximately 4 seconds). If a bee extended her mouthparts during the first 2 seconds of the odor pulse, I recorded that as a response. If she did not extend her mouthparts during that time, I recorded it as a lack of response. If a bee failed to respond after I presented her with the 1.5 M sucrose, I excluded her from later analyses.

During the punishing trials, after 2 seconds of odor exposure, I touched the bee's antennae with a toothpick soaked in 3 M NaCl (Ferguson et al. 2001). However, I did not allow her to drink because harnessed honey bees will ingest concentrated salt solutions, and ingesting 3 M NaCl causes high levels of mortality (Ayestaran et al. 2010). Bees allowed to collect solutions from a feeder learn to avoid concentrated salt solutions (Bermant & Gary 1966), but this learning may depend on tasting the salt with the proboscis, which did not occur in this study. The fact that most bees respond by extending their proboscides after touching the salt solution with their antennae suggests that it provides only a mildly aversive stimulus. Therefore, the lack of a sucrose reward may contribute more than the presence of salt to learning not to respond to the conditioned stimulus.

Handling control for reversal-learning comparisons

I handled scouts and non-scouts differently prior to testing due to constraints imposed by the hive-moving assay. This assay requires collecting scouts first, allowing foragers to recruit, and then collecting non-scouts for comparison. This situation necessitated caging scouts prior to harnessing for at least 1 hour longer than non-scouts. To determine

whether possible differences in reversal-learning performance between scouts and non-scouts could result from these differences in handling, I collected two groups of non-scouts, which I refer to as early and late non-scouts. I collected these workers from the same 6 colonies as the scouts and non-scouts. At least 3 days following a collection of scouts and non-scouts, I moved the colony back to its original yard. I collected early non-scouts in the same manner as scouts (described above) except that the hole in the entrance reducer was left open overnight, and collections began only after I observed foragers returning to the colony with large pollen loads and/or distended abdomens, signs indicating successful recruitment. As in the scout/non-scout collections, I returned to collect late non-scouts at least 1 hour after the early non-scout collection, which meant that early non-scouts had at least 1 more hour in cages with access to 30% sucrose than late non-scouts. These collections occurred between August 18th and September 13th, 2013.

Positive control for comparing sucrose responsiveness

In order to ensure that the sucrose responsiveness assay I used was capable of detecting possible differences between scouts and non-scouts, I compared three additional groups: nurses, nectar foragers, and pollen foragers. Pollen foragers are known to show higher sucrose responsiveness than nectar foragers (Page et al. 1998; Scheiner et al 1999, 2001) and foragers show higher responsiveness than nurses (Pankiw & Page 1999; Behrends et al. 2007; Behrends & Scheiner 2010). I identified nurses by finding a frame of wax honeycomb in a beehive that contained many mature larvae and then collecting workers that repeatedly put their heads into these cells. I identified pollen and nectar foragers by

blocking the entrance to the colony and observing whether returning workers had large pollen loads on their hind legs or noticeably distended abdomens and no pollen on their legs, respectively. I collected all three groups with soft forceps into collecting chambers as described above. I made these collections from 8 different colonies between June 16th and July 5th, 2013.

Sucrose responsiveness comparisons

These tests involved presenting an ascending series of sucrose concentrations. When a bee detects sucrose she reflexively extends her proboscis so the number of times that a worker extends her proboscis in response to the series provides a measurement of her responsiveness (Page et al. 1998). Given evidence that recovery time after harnessing can affect sucrose responsiveness (Pankiw and Page 2003), I allowed the bees to recover following harnessing for either 30 or 60 minutes. For each colony, I divided scouts and non-scouts equally between these two recovery time treatments and randomized the order of the treatments to minimize any effects of caging time. In contrast, I allowed all nurses, nectar foragers, and pollen foragers collected as positive controls to recover for 30 minutes (Pankiw et al. 1999). After the recovery period, I presented each bee with water by touching a 2 µl drop to both of her antennae using a 10 µl pipetman. If a bee responded by extending her proboscis, I allowed her to drink to satiation. Then I presented each bee with 0.1, 0.3, 1, 3, 10, and 30% sucrose w/v, with a presentation of water in between each pair of concentrations to minimize sensitization (Page et al. 1998; Pankiw & Page 1999; Pankiw et al. 2001). These solutions were made with 99.8% pure sucrose (Sigma Aldrich, St. Louis,

MO). The interval between presentations for a given bee was between 2 and 3 minutes. I did not allow bees to drink during this time.

I measured the sucrose responsiveness of each bee based on her response to the sucrose concentrations. I recorded all proboscis extension responses. If a bee did not respond to any of the concentrations offered, I presented her with honey. If she responded to the honey, I assumed that she had a response threshold above 30% sucrose. If she failed to respond, I excluded her from later analyses.

Comparison of wing damage between scouts and non-scouts

To compare the amount of foraging experience of scout and non-scout foragers, I recorded whether or not the scouts and non-scouts I had collected showed signs of wing damage. Wing damage accumulates exponentially over the foraging careers of bees (Cartar 1992; Mueller & Wolf-Mueller 1993; Higginson & Barnard 2004) and probably results from wing collisions with vegetation during foraging trips (Foster & Cartar 2011). In this study, I defined wing damage as any tear or missing piece of either of the two forewings (Huang et al. 1994). Hind wings rarely show any damage (Higginson & Barnard 2004). I used records from all scouts and non-scouts collected for the sucrose responsiveness and reversal-learning comparisons, as well as 3 additional collections on May 29th, June 3rd, and June 4th, 2013.

Statistical analyses

To compare reversal-learning performance, using the methods of Ferguson et al. (2001), I translated responses into four scores: the number of times a worker responded to the rewarded odor in the discrimination phase (A+), the number of responses to that odor when it was punished during the reversal phase (A-), the number of responses to the punished odor in the discrimination phase (B-), and the number of responses to that odor when it was rewarded in the reversal phase (B+). I chose the two odors, 1-hexanol and 1-nonanol, because previous work had found that honey bee workers in a y-maze do not prefer one odor over the other (Carcaud et al. 2009). However, analysis of my reversal-learning data showed a significant preference for 1-nonanol over 1-hexanol. Therefore, when analyzing the data, I included the effect of odor pairing, meaning which odor I paired with a reward and then a punishment (A) and which odor I paired with a punishment and then a reward (B). I used generalized linear mixed models (GLIMMIX procedure in SAS) with a Poisson distribution and a log link function to analyze the effects of role (scout or non-scout), day of collection, collection (first or second for each colony), odor pairing (A=1-hexanol/B=1-nonanol or A=1-nonanol/B=1-hexanol), and the interactions of these variables with role on the four reversal-learning scores.

Given that some bees died during the starvation period before conditioning, I used probability of survival as an estimate of the stressfulness of this treatment for scouts and non-scouts. I analyzed the effect of role, day of collection, and the interaction between these two variables on the probability of survival using a logistic regression (GLIMMIX

procedure in SAS) with a binomial distribution and a logit link function. I included colony of origin as a random effect in both the analyses of reversal-learning and of mortality. I repeated this analysis using data on the proportion of foragers that had an intact PER following the starvation period, which means the proportion that responded quickly to all three presentations of sucrose in the test prior to conditioning.

To compare levels of sucrose responsiveness, I translated responses to sucrose solutions into PER scores in which I considered a response to all concentrations a "6" and no response to any of the concentrations a "0" (Page et al. 1998). Just as with the reversal-learning scores, I used generalized linear mixed models (GLIMMIX) to analyze the effects of role within the colony, recovery time, and their interaction on workers' PER scores. I included colony of origin/date of collection (because colony and date of collection were confounded) as a random effect. For the positive controls, I also performed a second analysis to better understand the relative variation in PER scores of nurses, pollen foragers, and nectar foragers across colonies in this experiment. In this analysis, I included role within the colony, colony of origin/date of collection, and the interaction between them as fixed effects in generalized linear mixed models (GLIMMIX).

To estimate the probability of a type II error in the comparison of scouts and non-scouts, I used data from the positive controls for a power analysis with the twosamplewilcoxon Power procedure in SAS. I used the wilcoxon rank sum test for the power analysis because SAS has a well-established procedure for this analysis, and, given the lower power of non-parametric tests, it should provide a conservative power estimate.

To compare wing damage, I again used a logistic regression (GLIMMIX) with a binomial distribution and a logit link function to model the effects of role on the probability of showing wing damage (1 for wing damage present and 0 for wing damage absent; Huang et al. 1994). I included colony/day of collection as a random effect. I analyzed the data from foragers collected early in the summer (June-July) separately from those of foragers collected late in the summer (August-September) in order to test for seasonal effects. I also used records of weight gain from a hive used to monitor foraging conditions around the University of Illinois Bee Research Facility (N. Lawrence & C. Nye 2013, Unpublished data) to examine seasonal changes in the availability of food resources that could affect foraging rates.

RESULTS

Reversal-learning comparisons

Of the foragers collected, 52.5±3.5% of scouts and 45.9±3.5% of non-scouts passed the test for an intact proboscis extension reflex (PER) by responding quickly to all three presentations of sucrose prior to conditioning. I randomly chose approximately half of these foragers for conditioning in the reversal-learning assay. The acquisition and extinction curves of the conditioned scouts and non-scouts are shown in Figure 2.

I found a significant effect of role (scout or non-scout) on responses to the rewarded odor in the reversal phase, B+ (Table 1; Figure 3). In addition, odor pairing had a significant effect on responses to both the punished odor in the discrimination phase (B-) and the rewarded odor in the reversal phase (B+) but no significant interaction between role and odor pairing (Table 1; Figure 3). There was also a trend toward a significant interaction between day of collection and role (Table 1). Each day of conditioning occurred one day after collecting, harnessing, and feeding the bees and two days after moving the colony for the hive-moving assay. I moved each of the six colonies in this experiment twice, and I found a significant effect of collection (first or second for each colony) and the interaction between day of collection and role on response to the punished odor during the initial discrimination phase, B- (Table 1). No variable or interaction between variables had a

significant effect on responses to the first rewarded (A+) and then punished (A-) odor (Table 1; Figure 2).

In a similar analysis, the handling controls showed no significant effect of handling, day of collection, odor pairing, or the interaction between these variables on foragers' responses to the rewarded odor in the discrimination phase (A+), the rewarded odor in the reversal phase (B+) or the punished odor in the reversal phase (A-) (Table 2; Figure 4). However, day of collection and odor pairing had significant effects on the responses to the punished odor in the discrimination phase (B-) (Table 2; Figure 4). This analysis differed from the analysis of scouts and non-scouts because I collected handling control foragers only once from each colony so I could not include the effect of collection (first or second for each colony).

Unfortunately, 20.2±2.9% of scouts and 22.7±2.9% (mean±s.e.m) of non-scouts harnessed in this experiment died before conditioning. However, role showed no significant effect on the probability that a bee would die during this period ($F_{1,15}$=2.86, p=0.1115). In addition, day of collection had a small but significant effect on mortality ($F_{1,15}$=4.81, p=0.0445). There was no significant interaction between day of collection and role ($F_{1,15}$=2.99, p=0.1041). In addition, role ($F_{1,15}$=2.18, p=0.1602), day ($F_{1,15}$=0.02, p=0.8821), and the interaction between the two variables ($F_{1,15}$=0.55, p=0.4707) had no significant effect on the proportion of scouts and non-scouts with an intact proboscis extension reflex following the starvation period.

Comparison of the sucrose responsiveness of scouts and non-scouts

Analysis of the PER scores of scouts (n=165) and non-scouts (n=161) showed no significant effect of role ($F_{1,12}$=0.20, p=0.6626; Figure 5A). However, I found a slight but significant improvement in sucrose responsiveness after a longer period of holding prior to testing ($F_{1,310}$=7.29, p=0.0073; Figure 5A), suggesting that a longer recovery time after collection/harnessing changes the alertness or hunger state of the bee. I found no significant interaction between role and recovery time ($F_{1,310}$=0.31, p=0.5802). Mean PER scores and standard errors for each colony/day of testing are shown in Figure 6.

Comparison of the sucrose responsiveness of nurses, nectar foragers, and pollen foragers

Analysis of the PER scores of nurses (n=98), nectar foragers (n=62), and pollen foragers (n=72) showed a significant effect of role ($F_{2,21}$=5.93, p=0.0091; Figure 7A). Pairwise comparisons showed a significant difference between pollen foragers and nurses ($F_{1,14}$=8.67, p=0.0107) and a significant difference between pollen foragers and nectar foragers ($F_{1,14}$=9.62, p=0.0078) but no significant difference between nurses and nectar foragers ($F_{1,14}$<0.01, p=0.9574). In addition, to examine the relative variation of nurses compared to other groups, I used generalized linear mixed models that included colony/day of collection as a fixed effect. In this analysis, only the comparisons between nurses and nectar foragers ($F_{7,144}$=2.57, p=0.0159) and nurses and pollen foragers ($F_{7,154}$=2.98, p=0.0059) showed significant interactions between role and colony/date of

testing, indicating that the sucrose responsiveness of nurses varied considerably relative to the other groups (Figure 8).

I used these data to estimate the statistical power of the scout and non-scout comparisons, given the sample size of at least 76 bees in each group for both the 30-minute and 60-minute recovery times. The power analysis indicated a 7.7% chance of failing to detect a difference equal to that between nurses and pollen foragers and a 2.6% chance of failing to detect a difference equal to that between nectar foragers and pollen foragers.

Comparison of wing damage between scouts and non-scouts

In the early summer, I found a significant effect of role on wing damage ($F_{1,11}$=5.12, p=0.0449). During these collections, non-scouts showed a higher probability of wing damage than scouts did (Figure 9). In the late summer I found no significant effect of role ($F_{1,17}$=0.19, p=0.6722; Figure 9). I also found a significant effect of season on wing damage ($F_{1,28}$=14.22, p<0.0008; Figure 9), suggesting that foragers collected in the late summer were more likely to have foraging experience than foragers collected during the early summer. Based on measurements of the rate of weight gain of a monitoring hive (N. Lawrence & C. Nye 2013, Unpublished data), both the early and late summer included periods of high and low foraging success, but the late summer included the period of lowest foraging success (Figure 10).

DISCUSSION

Many studies have examined factors affecting the division of labor between scout and non-scout honey bee foragers (Lindauer 1952; Seeley 1983; Seeley & Visscher 1988; Dreller 1998; Beismeijer & Seeley 2005; Beekman et al. 2007; Mattila & Seeley 2011; Liang et al. 2012). In the current study, I tested predictions of three hypotheses about the effects of cognition, perception, and foraging experience on scouting behavior. It is important to note that confirming differences between scouts and non-scouts does not, in itself, prove that these factors play a causal role in the likelihood of scouting. It remains possible that differences in the behavior of scouts and non-scouts may cause differences in one or more of the factors instead.

First, based on the work of Ferguson et al. (2001), I reasoned that reversal-learning abilities could aid foragers in discovering newly rewarding patches of flowers. I tested the prediction that scouts show a faster response in a reversal-learning assay to a previously punished odor once it became rewarding. I found that scouts did show a significantly faster response than recruits. Second, I also reasoned that lower responsiveness to sucrose could decrease the probability that a forager would search independently. I tested the prediction that scouts show greater responsiveness than non-scouts in a sucrose responsiveness assay. I found no significant difference in responsiveness between the two groups. Third, based on previous results (Lindauer 1952; Seeley 1983), I hypothesized that greater foraging experience increases the probability that a forager will engage in scouting behavior. I tested the prediction that scouts have more wing damage than non-scouts. In

contrast, I found that non-scouts had more wing damage than scouts in the early part of the foraging season but no difference in the later part of the foraging season.

The reversal-learning results conformed to my prediction that scouts would show a faster response to an odor when it suddenly predicted a reward. When I compared the number of responses to the previously punished odor, B+, I found a significant effect of role on this score. In addition, collection (first or second for each colony) had an unexpected effect on responses to the punished odor during the discrimination phase, B-, which may indicate effects of seasonal changes or moving-related stress on the ability to discriminate between the two odors. There was also a significant interaction effect between role and day of collection on the B- score. Trials in early August indicated that scouts respond to a punished odor more frequently than non-scouts do, but trials in late August and September showed the reverse pattern.

These changes over the course of the experiment could stem from seasonal effects on honey bee behavior. A larger proportion of foragers engage in scouting during periods when flower abundance and distribution makes finding rewarding patches difficult (Seeley 1983). In addition, the novel-feeder assay identifies a larger proportion of foragers as scouts during the late summer and fall than during the early summer (SZ Liang, pers. comm.), which seems reasonable given changes in flower distribution and abundance between summer and fall (Visscher & Seeley 1982; Seeley & Visscher 1988; Beismeijer & Seeley 2005). Data collected from a monitoring hive (N. Lawrence & C. Nye 2013, Unpublished data) showed that foraging conditions changed repeatedly over the summer,

but the least productive period occurred in early August. Therefore, I would expect scouts collected in the late summer and fall to represent a somewhat different subpopulation of foragers than those collected in the early summer.

In contrast to my prediction that scouts would show greater responsiveness to sucrose than non-scouts, I found no differences in sucrose responsiveness between them. My methods differed from previous studies, including adding a period of caging with access to sucrose and chilling bees for a longer period before harnessing them. Despite these differences, I replicated previous findings (Page et al. 1998; Pankiw & Page 1999; Scheiner et al 1999, 2001a,b) of differences in responsiveness between pollen foragers and nurses and between pollen foragers and nectar foragers. In contrast to previous work (Behrends et al. 2007; Behrends & Scheiner 2010), I did not replicate previous results indicating differences between nurses and nectar foragers, but I found a significant amount of variation among nurses from different colonies. Unlike the comparison between nurses and nectar foragers, the comparisons between pollen and nectar foragers revealed no significant interaction with colony. Therefore, it seems likely that the factors contributing to variation in responsiveness among nurses did not influence the responsiveness of foragers. Power tests using data from the comparisons of nurses, pollen foragers, and nectar foragers indicate that the lack of differences between scouts and non-scouts is not likely to be due to an underpowered analysis. In addition, I also tested whether doubling the recovery time might lessen any effects of chilling the bees and reveal significant differences between scouts and non-scouts. I still found no difference.

The similar sucrose responsiveness of scouts and non-scouts contrasts with comparisons of other foraging specializations, including comparisons of nectar, pollen, water (Pankiw & Page 2000), and resin foragers (Simone-Finstrom et al. 2010). This contrast could arise from differences in the nature of the foraging specializations. I focused on nectar scouts and non-scouts, which differ in their foraging strategy, but not the resource they collect. In contrast, the foraging specializations mentioned above all involve collecting different resources, which differ in their gustatory properties (de Brito Sanchez et al. 2007).

Although nectar scouts and non-scouts interact with the same type of resource, they likely experience different levels of variation in the quality of the resources they encounter on foraging trips. Non-scouts exploit resources advertised by other foragers and often wait in the hive for dances to reactivate or recruit them when a patch becomes less profitable (Biesmeijer & Seeley 2005). In contrast, scouts often investigate multiple sites before finding a site worth advertising to their sister foragers (Seeley 1983). The results of the current study suggest that scouts and non-scouts would both perceive such differences in the variation of nectar quality. Scouts must choose to search for new resources despite perceiving this difference. Therefore, choosing to scout may reflect a lower level of risk aversion or a greater expectation of reward when faced with uncertainty. To test these hypotheses, future studies could compare the choices of scouts and non-scouts in assays designed to measure differences in risk aversion (Shafir et al. 1999) and expectations of reward (Bateson et al. 2011).

Contrary to the prediction that scouts would show greater wing damage than non-scouts, comparisons of wing damage across the two experiments indicated that non-scouts had a slightly larger amount of foraging experience than scouts did in the early summer but not the late summer. Wing damage does not provide a precise estimate of the number of trips made by foragers and cannot differentiate between a high rate of foraging over a short period or a low rate of foraging over a long period. The difference I found could derive from foragers with no previous experience having a higher tendency to search for new food sources. It could also result from differences in the number of foraging trips made per day by scouts and non-scouts.

While greater experience with spatial cues could help foragers to find novel sources of food, wing damage also has direct effects on foraging behavior. It decreases the flight performance of honey bees, which limits the distance that they fly on each trip (Higginson et al. 2011) and their choosiness when moving among flowers in a patch (Higginson & Barnard 2004). The flight difficulties associated with wing damage appear to affect a forager's perception of food patch profitability so that when she advertises a patch using the waggle dance, she conveys less excitement through the speed of her dance (Seeley et al. 2000) than a forager with undamaged wings would (Higginson et al. 2011). Such effects suggest that foragers with wing damage tend to make decisions that minimize unnecessary energy expenditure (Higginson et al. 2011), which could make them less likely to engage in exploratory behavior. Confirming this hypothesis would require experimental manipulations.

The increase in the probability of wing damage in both groups between early and late summer corresponds to known seasonal changes in the age distribution of foragers (Fukuda 1983). The fact that scouts and non-scouts differed in the early summer supports the effectiveness of the hive-moving assay in identifying scouts and non-scouts and, therefore indirectly strengthens the sucrose responsiveness results. The fact that scouts and non-scouts did not differ in late summer indicates that the declines in learning performance associated with very long foraging careers (Behrends et al. 2007) probably did not bias the results of the reversal-learning comparisons. Perhaps more importantly, these results support the assumption that seasonal changes in the distribution of rewarding flower patches play a role in determining the subpopulation of foragers that will scout in the hive-moving assay.

The results of these three analyses contribute to our understanding of differences between scouts and non-scouts that could influence the tendency toward scouting behavior. The comparison of reversal-learning abilities showed an interesting difference between the two groups. The comparison of sucrose responsiveness revealed scouting as the only honey bee foraging specialization known to have no association with sucrose responsiveness. Finally, the comparison of wing damage between scouts and non-scouts showed an unexpected relationship, and highlighted the importance of seasonal changes on the likelihood of scouting. These differences in reversal-learning performance and wing damage leave open the possibility that cognitive differences and differences in foraging experience may play a causal role in the division of labor between scouts and non-scouts.

Fortunately, recent work on the molecular determinants of scouting behavior has provided tools for manipulating the propensity of foragers to engage in scouting (Liang et al. 2012). Thus, future studies could examine the effect of these treatments on reversal-learning abilities and variables related to foraging experience such as the propensity of novice foragers to use dance information or the rate of trips made by experienced foragers. Using known differences between scouts and non-scouts on the behavioral and molecular levels, future experiments could examine the specific roles of these neurotransmitter systems in regulating scouting behavior.

TABLES AND FIGURES

Table 1 Results from generalized linear mix model analyses of reversal-learning scores of scouts and non-scouts. Cells contain F-values and p-values from analyses of the effect of role within the colony (scout or non-scout), day of collection, first or second collection from each colony, which odors were paired with the reward/punishment (1-hexanol or 1-nonaol), and interactions with role on four scores. These scores represent the sum of responses to the two odors in the discrimination phase and the reversal phase. A indicates the rewarded and then punished odor and B indicates the punished and then rewarded odor. "+" indicates rewarded and "-" indicates punished. For each analysis, scores from 48 scouts and 47 non-scouts were used

	Discrimination Phase		Reversal Phase	
	A+	B-	B+	A-
Role	$F_{1,82}$=0.82 p=0.3686	$F_{1,82}$=1.71 p=0.1945	**$F_{1,82}$=5.28** **p=0.0242***	$F_{1,82}$=1.80 p=0.1830
Day	$F_{1,82}$=0.02 p=0.8905	$F_{1,82}$<0.01 p=0.9972	$F_{1,82}$=3.00 p=0.0870	$F_{1,82}$=0.68 p=0.4130
Collection ($1^{st}/2^{nd}$)	$F_{1,82}$=0.04 p=0.8338	**$F_{1,82}$=4.87** **p=0.0301***	$F_{1,82}$=2.01 p=0.1598	$F_{1,82}$=0.17 p=0.6838
Odor pairing	$F_{1,82}$=0.62 p=0.4347	**$F_{1,82}$=6.69** **p=0.0115***	**$F_{1,82}$=6.12** **p=0.0154***	$F_{1,82}$=3.72 p=0.0571
Day*Role	$F_{1,82}$=1.37 p=0.2456	**$F_{1,82}$=6.72** **p=0.0113***	$F_{1,82}$=3.81 p=0.0544	$F_{1,82}$=1.86 p=0.1761
Collection*Role	$F_{1,84}$<0.01 p=0.9517	$F_{1,82}$=3.02 p=0.0860	$F_{1,82}$=0.02 p=0.8840	$F_{1,82}$=0.24 p=0.6270
Odor pairing*Role	$F_{1,82}$=0.50 p=0.4807	$F_{1,82}$=0.25 p=0.6185	$F_{1,82}$=0.09 p=0.7667	$F_{1,82}$=0.60 p=0.4416

Table 2 Results from generalized linear mix model analyses of reversal-learning scores of handling control foragers. Cells contain F-values and p-values from analyses of the effect of role within the colony (scout or non-scout), day of collection, first or second collection from each colony, which odors were paired with the reward/punishment (1-hexanol or 1-nonaol), and interactions with role on four scores. These scores represent the sum of responses to the two odors in the discrimination phase and the reversal phase. A indicates the rewarded and then punished odor and B indicates the punished and then rewarded odor. "+" indicates rewarded and "-" indicates punished. For each analysis, scores from 22 early non-scouts and 22 late non-scouts were used

	Discrimination Phase		Reversal Phase	
	A+	B-	B+	A-
Role	$F_{1,35}$=1.26 p=0.2693	$F_{1,35}$=0.01 p=0.9147	$F_{1,35}$=0.27 p=0.6070	$F_{1,35}$=0.29 p=0.5929
Day	$F_{1,35}$<0.01 p=0.9444	**$F_{1,35}$=5.09** **p=0.0304***	$F_{1,35}$=0.46 p=0.5031	$F_{1,35}$=0.85 p=0.3642
Odor pairing	$F_{1,35}$=0.02 p=0.8900	**$F_{1,35}$=9.61** **p=0.0038****	$F_{1,35}$=2.64 p=0.1135	$F_{1,35}$=0.93 p=0.3418
Day*Role	$F_{1,35}$=1.03 p=0.3161	$F_{1,35}$=0.15 p=0.7043	$F_{1,35}$=0.19 p=0.6620	$F_{1,35}$=0.11 p=0.7403
Odor pairing*Role	$F_{1,35}$=0.50 p=0.4859	$F_{1,35}$=0.43 p=0.5169	$F_{1,35}$=0.02 p=0.8902	$F_{1,35}$=0.80 p=0.3772

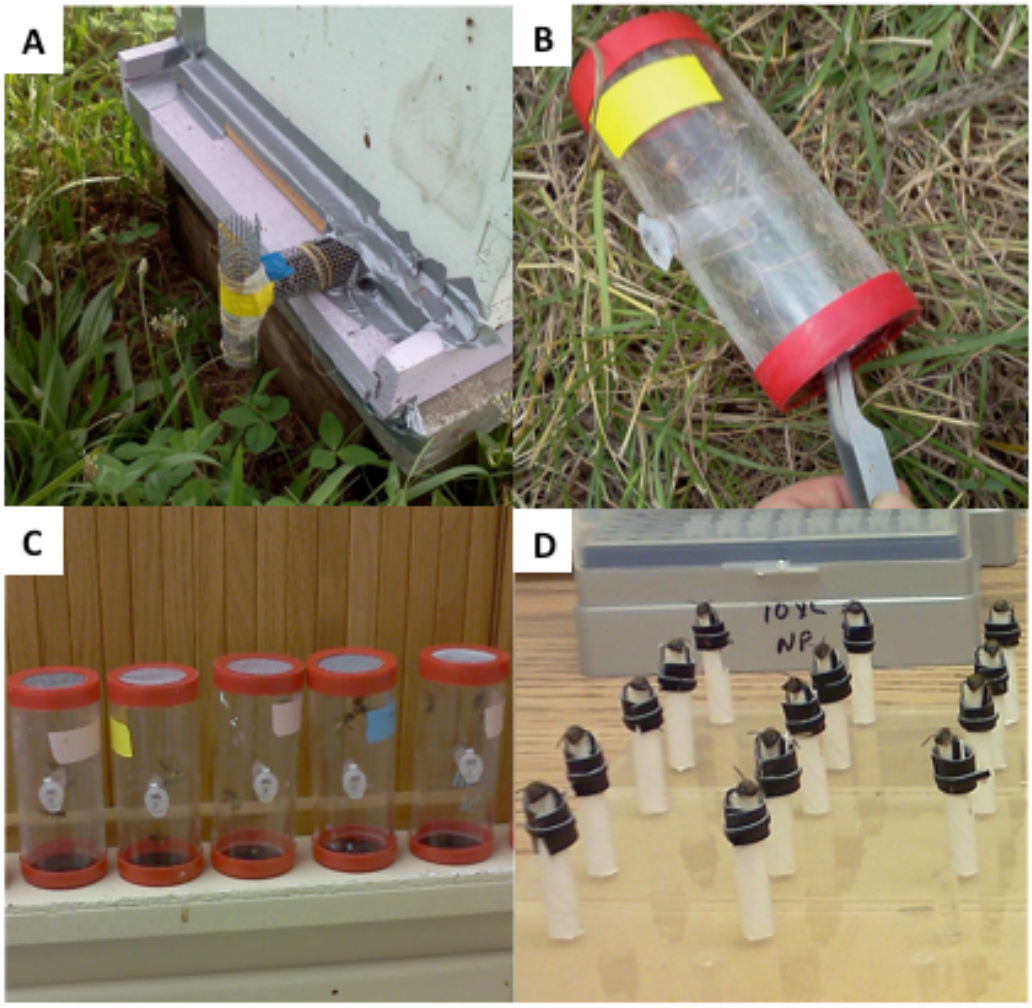

Figure 1 Photos of experimental procedure for sucrose responsiveness and reversal-learning comparisons. **A)** Mesh tube entrance added to colonies on the morning following a hive move to make it easy for scouts to exit the hive but difficult for them to re-enter. **B)** Use of soft forceps to capture scouts and place them in modified insect vacuum collecting chambers. **C)** Bees in collecting chambers in the lab with access to 30% sucrose feeders. **D)** Nurses, nectar foragers, and pollen foragers harnessed in waxed paper tubes

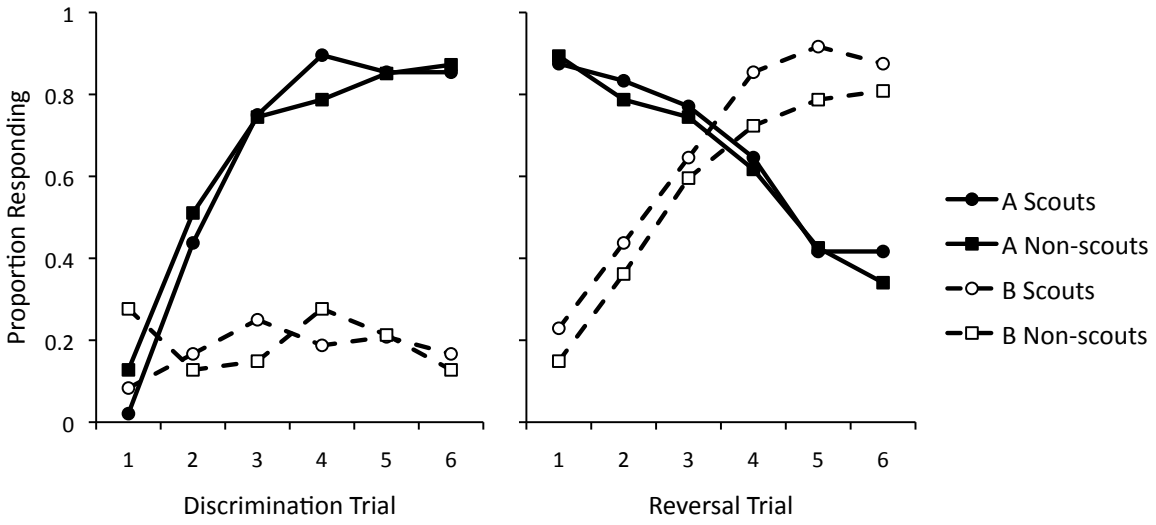

Figure 2 Acquisition and extinction curves of scouts (n= 48) and non-scouts (n= 47) in the reversal-learning tests. A refers to the first rewarded and then punished odor. B refers to the first punished and then rewarded odor

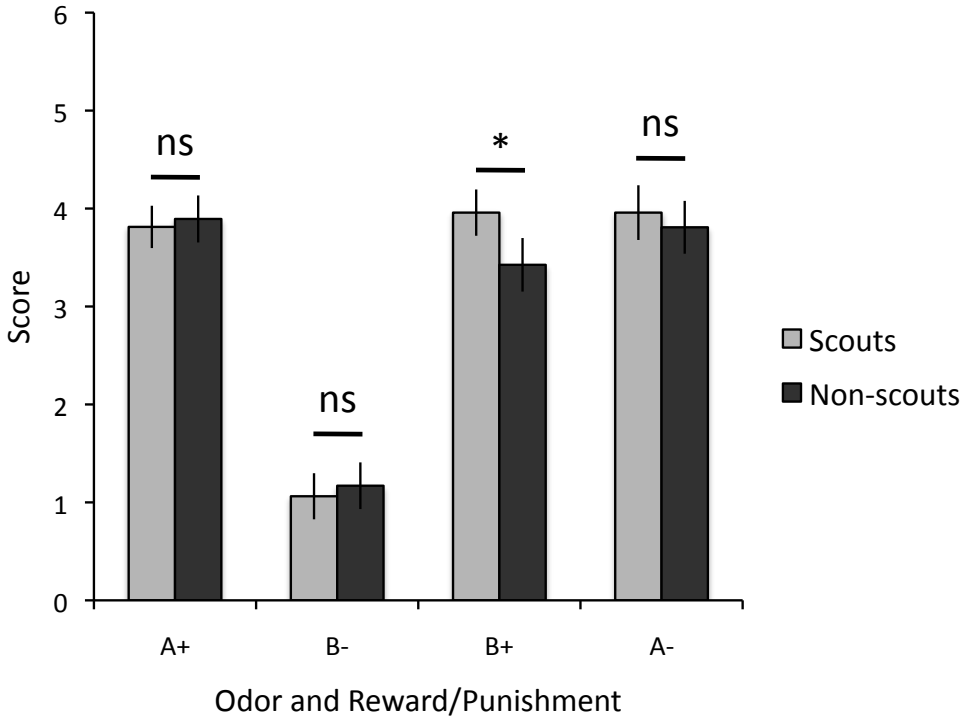

Figure 3 Comparison of responses across the discrimination and reversal phases between scouts (n=48) and non-scouts (n=47). Score indicates the number of responses out of six possible responses. A+ indicates the rewarded odor in the discrimination phase and A- indicates the same odor when it was subsequently punished in the reversal phase. B- indicates the punished odor in the discrimination phase and B+ indicates that same odor when it was subsequently rewarded in the reversal phase. Error bars represent 1 standard error of the mean.

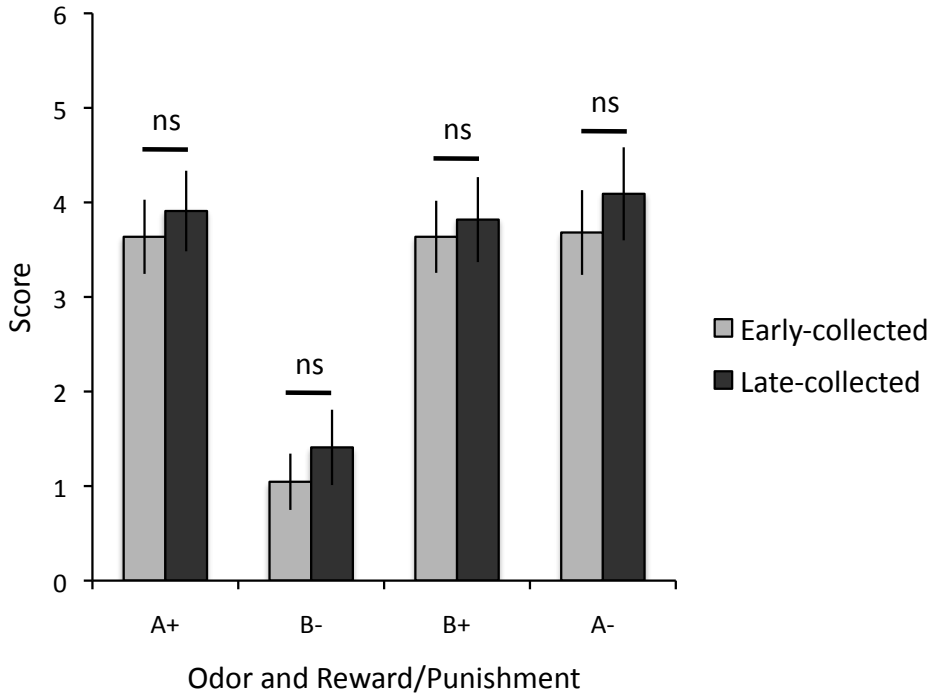

Figure 4 Comparison of reversal-learning scores between handling control groups. See Figure 2 caption for a description of each score. Depicted here are comparisons of scores of early-collected non-scouts (n=22) and late-collected non-scouts (n=22) collected from the same six colonies as the scouts and non-scouts shown in Figure 2. Early-collected = foragers collected earlier in the morning and held in cages for a longer period (≥1 hour longer), Late-collected = foragers collected later in the morning and held in cages for a shorter period. Error bars represent 1 standard error of the mean

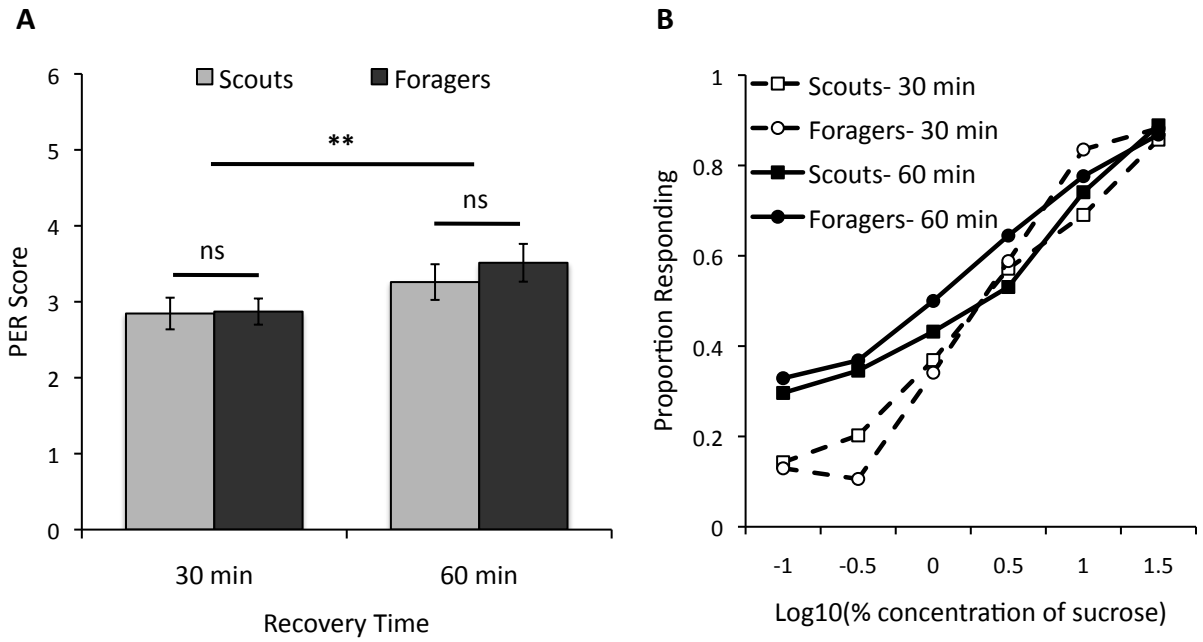

Figure 5 Comparison of sucrose responsiveness between scouts and non-scouts. **A)** Mean PER scores of scouts and non-scouts allowed to recover between harnessing and testing for 30 minutes (84 scouts and 85 non-scouts) or 60 minutes (81 scouts and 76 non-scouts). Error bars represent 1 standard error, ** indicates $p<0.01$, ns indicates nonsignificant. **B)** Proportion of each group responding to each sucrose concentration presented. The x-axis shows the log of the % sucrose (m/v)

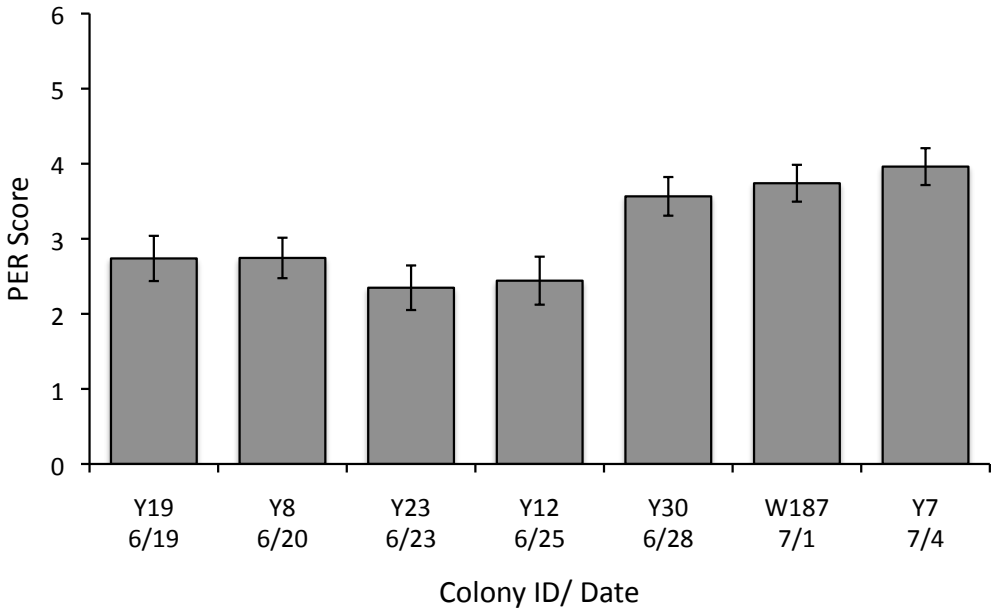

Figure 6 Comparison of sucrose responsiveness between colonies and across days. Mean PER scores are shown with error bars representing 1 standard error

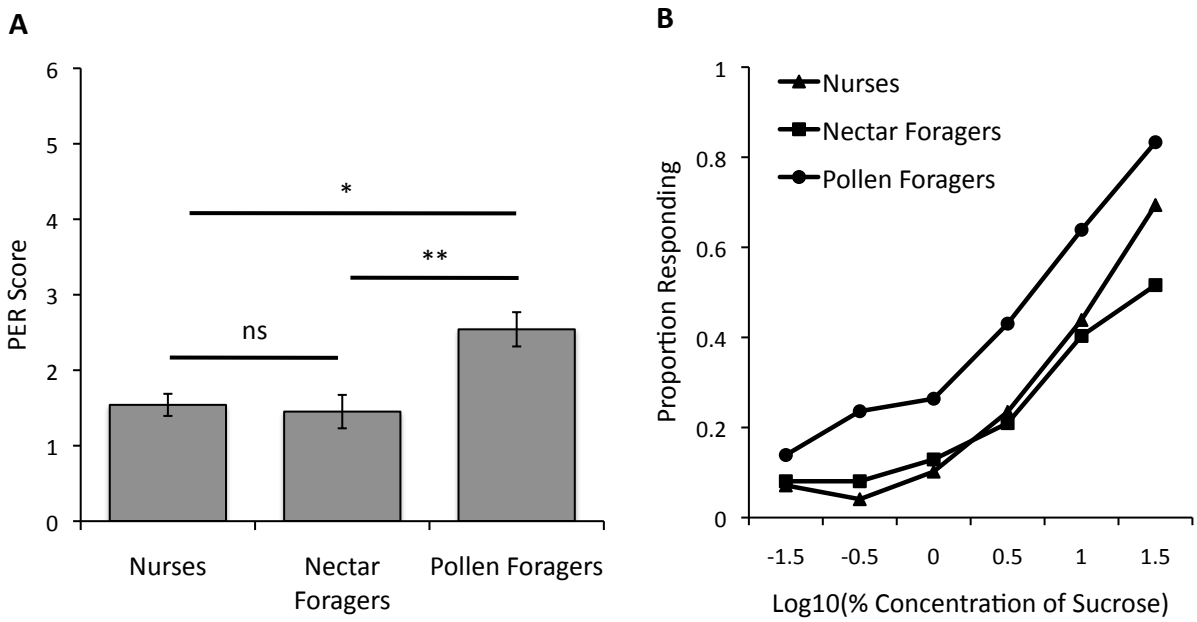

Figure 7 Comparison of sucrose responsiveness among nurses (n=98), nectar foragers (n=62), and pollen foragers (n=72). **A)** Mean PER scores of each group. Error bars represent 1 standard error. * indicates $p<0.05$, ** indicates $p<0.01$, ns indicates nonsignificant **B)** Proportion of each group responding to each sucrose concentration presented. The X-axis is the same as in Figure 5

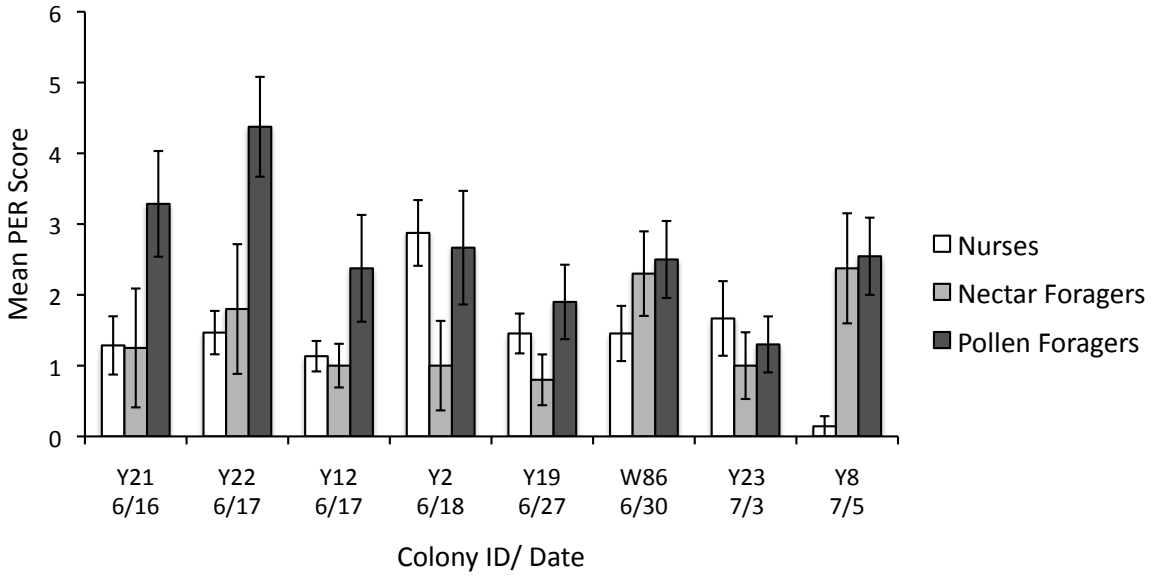

Figure 8 Comparison of sucrose responsiveness among nurses, nectar foragers, and pollen foragers across eight colonies. The mean PER scores of all groups are shown. Error bars represent 1 standard error

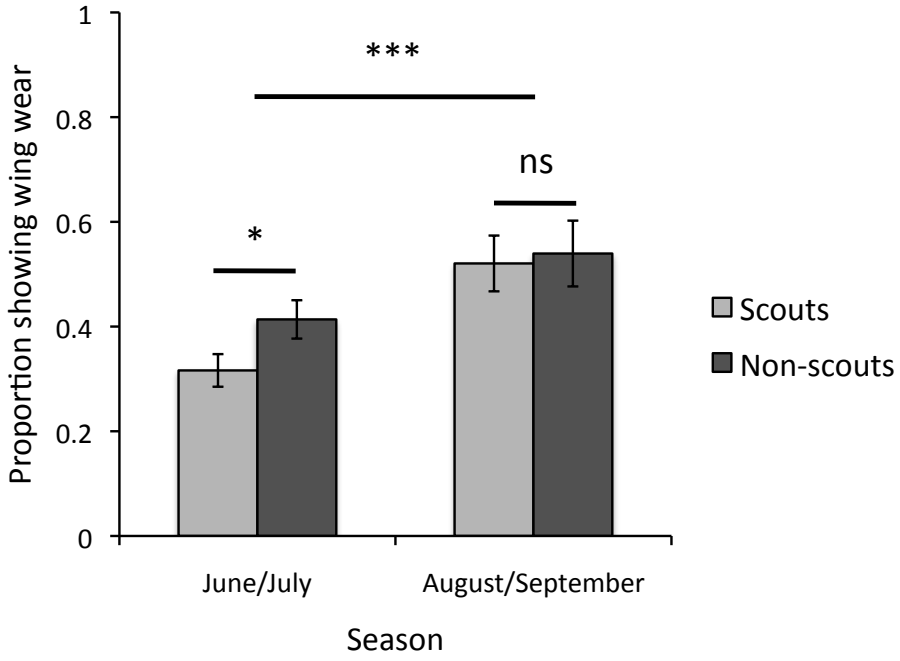

Figure 9 Comparison of the proportion of foragers showing wing damage among scouts and non-scouts. There were 10 collections from 8 colonies in June and July and 12 collections from 6 colonies in August and September. Error bars represent 1 standard error of the mean proportion across colonies/days of collection. * indicates $p<0.05$, *** indicates $p<0.0001$, and ns indicates nonsignificant

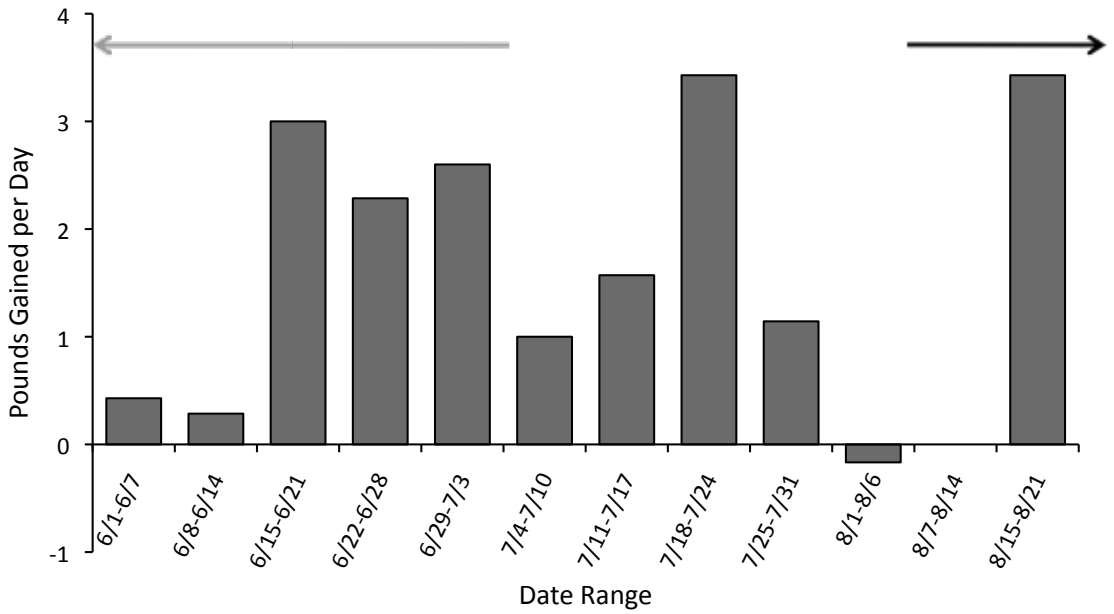

Figure 10 Changes in rate of weight gain by a hive used to monitor foraging conditions. Date ranges span 7 days, except 6/29-7/3 and 8/1-8/6, which were shortened due to missing data. The gray arrow indicates the time span during which wing damage was recorded in the early summer, and the black arrow indicates the time span during which wing damage was recorded in the late summer. Weight gain records for this hive began 2 days after the first wing damage records and ended 3 weeks before the final wing damage records. (N. Lawrence & C. Nye 2013, Unpublished data).

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