

## RESEARCH ARTICLE

# The cues of colony size: how honey bees sense that their colony is large enough to begin to invest in reproduction

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## ABSTRACT

As organisms develop, they first invest resources in survival and growth, but after reaching a certain condition they start to also invest in reproduction. Likewise, superorganisms, such as honey bee colonies, first invest in survival and growth, and later commit resources to reproduction once the number of workers in the colony surpasses a reproductive threshold. The first form of reproductive investment for a honey bee colony is the building of beeswax comb made of special large cells used for rearing males (drones). How do the workers sense that their colony is large enough to start building this ‘drone comb’? To address this question, we experimentally increased three possible cues of colony size – worker density, volatile pheromone concentration and nest temperature – and looked for effects on the bees’ comb construction. Only the colonies that experienced increased worker density were stimulated to build a higher proportion of drone comb. We then monitored and quantified potential cues in small and large colonies, to determine which cues change with colony size. We found that workers in large colonies, relative to small ones, have increased contact rates, spend more time active and experience less variable worker density. Whereas unicellular and multicellular organisms use mainly chemical cues to sense their sizes, our results suggest that at least one superorganism, a honey bee colony, uses physical cues to sense its size and thus its developmental state.

**KEY WORDS:** Superorganism, Development, Sociogenesis, Reproductive investment, *Apis mellifera*, Drone comb

## INTRODUCTION

In virtually all living systems, developmental changes are cued to increases in the system’s size. The bacteria *Vibrio fischeri*, which live in the light organ of the Hawaiian bobtail squid, *Euprymna scolopes*, begin emitting light only after reaching a high cell density (reviewed in Waters and Bassler, 2005). Similarly, the cells of humans undergo changes associated with puberty only when body size has reached a threshold level (reviewed in Grumbach, 2002). In both these examples, individual cells must detect group size, and then make appropriate changes. *Vibrio fischeri* use small hormone-like molecules to detect cell density, which act as transcription factors to alter gene expression (Waters and Bassler, 2005). In humans, adipose tissue produces the hormone leptin, which must reach a critical level before puberty begins (Grumbach, 2002). For

unicellular bacteria and multicellular humans, the cues that trigger these developmental changes are chemical. Colonies of social insects that work together to form a tightly integrated unit (a ‘superorganism’; Hölldobler and Wilson, 2009) also have developmental changes linked to increases in group size. One of the most striking is the switch to investing in reproduction, not just survival and growth, once the number of workers in the colony exceeds a threshold level (Smith et al., 2014).

A honey bee (*Apis mellifera* Linnaeus 1758) colony reproduces by producing drones and casting swarms. A colony’s first investment in reproduction, however, occurs when workers begin to build cells of beeswax comb with the large diameters needed for rearing drones, i.e. drone comb (reviewed in Boes, 2010). Building drone comb marks the onset of colony ‘puberty’; that is, the period during which a colony first begins to prepare for reproduction. Workers begin building drone comb once the number of workers in the colony has passed a reproductive threshold (Smith et al., 2014). In this study, we used both experimental and observational approaches to address a key life-history question: how do worker honey bees sense that their colony is large enough to begin to invest in reproduction?

Workers switch from building only worker comb to building both worker comb and drone comb when the colony has grown to have approximately 4000 workers (Smith et al., 2014). To detect this reproductive threshold, workers likely sense cues that are correlated with their colony’s size rather than count the number of colony members per se. We used two complementary approaches to understand how individual bees sense colony size. First, we independently increased three possible cues of colony size: worker density, volatile pheromone concentration and nest temperature. To assess whether bees respond to these cues by redirecting resources to reproduction, we measured the proportion of drone comb built after we experimentally increased each cue. Second, we quantified and compared potential cues in small and large colonies to determine which cues are reliable indicators of colony size.

## MATERIALS AND METHODS

This research was performed at the Liddell Field Station of Cornell University, in Ithaca, NY, USA (42°27.6’N, 76°26.7’W).

## Experimental study

In the experimental study, we manipulated three cues that workers might use to sense that their colony has enough workers to invest in drone comb: (1) worker density, (2) volatile pheromone level and (3) nest temperature. We chose these potential cues because of their relevance to honey bee life-history. Colonies living in the wild choose nest cavities of ca. 45 liters (Seeley and Morse, 1976); thus, as the number of workers increases, so too may their density. Chemical cues are pervasive throughout honey bee communication (e.g. colony defense, brood presence and queen status; reviewed in Slessor et al., 2005), so bees may use volatile pheromones to detect

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colony size. Finally, honey bees tightly regulate nest temperature (see p.170 of Seeley, 1985), so increasing the number of heat-emitting individuals may change temperature gradients.

In each treatment group, we increased one of the three cues, while keeping colony size the same for each group. The size of each colony was sufficient to stimulate comb construction, but close to the threshold for building drone comb. We then compared the proportion of drone comb built by colonies in each treatment group versus the control group. We reasoned that if the colonies in a treatment group built a higher proportion of drone comb than those in the control group, then this was evidence that workers in the treatment group use the manipulated cue as an indicator of colony size.

### Hive setup

The setup for each colony was as follows: a lower box, where the treatment was produced; a middle box, where the colony was kept; and an upper box, where the 2 liters 1:1 (v:v) sucrose feeder was placed to encourage comb building (Fig. 1). A modified floorboard between the middle and lower boxes provided an entrance for the bees in the middle box. This floorboard also had two 7 cm holes with screen on both sides. The screen kept the bees in the middle box from entering the lower box, and the holes allowed the treatment in the lower box to permeate the middle box. Two Thermochron iButton Devices (model number DS1923FS, Embedded Data Systems; hereafter referred to as iButtons) were used – one placed in the center of the middle frame and the other at the same height, but in the corner furthest from the hive entrance – to measure temperature every 30 min at the center and the edge of each colony's middle box.

In the control treatment and density treatment, we left the lower box in each hive empty. In the density treatment, we increased the density of bees in the middle box by placing a wooden block in it, thus restricting the bees to three frames instead of five. Therefore, the bees in this treatment had less space in which to build comb, but having less space to build comb does not induce a colony to invest in drone comb (see reference versus comb area in figs 1–3 in Smith et al., 2014).

In the pheromone treatment, we installed a second colony in the lower box. This colony contained 10,000 workers, five frames of

brood with no drone comb and a naturally mated queen. We installed colonies in the lower boxes 2 days before the experimental colonies. To reduce mixing of foragers from the two colonies, we oriented the hive entrances of the colonies in opposite directions.

In the temperature treatment, we installed a 40 W incandescent lamp covered in aluminium foil in the lower box of each hive. A piece of wood placed above the lamp diffused the heated air in the lower box to prevent a hotspot from forming directly above the lamp. To verify that each lamp stayed lit, we checked the temperature of each hive's lower box every 24 h using a thermocouple thermometer (type K, Omega Engineering, Stamford, CT, USA).

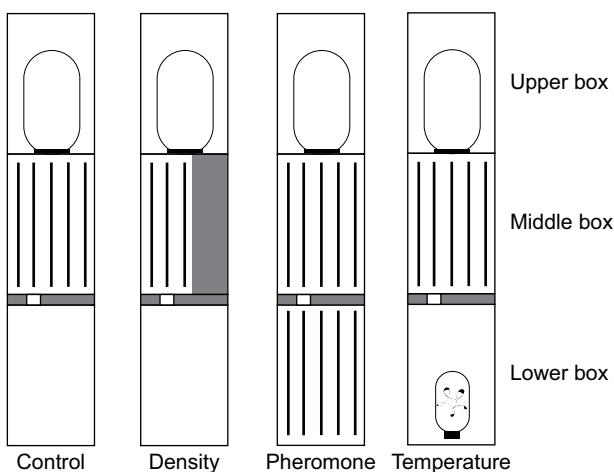
Honey bees regulate the brood nest temperature between 33 and 36°C to ensure healthy development of the brood (see p.170 of Seeley, 1985). To check that the lamp increased brood nest temperature but did not overheat the colony above, we monitored control hives with lit and unlit lamps, but without bees. The lit lamp increased the temperature of the middle box, but did not overheat it (heated control:  $31.1 \pm 7.2^\circ\text{C}$ , unheated control:  $24.4 \pm 8.1^\circ\text{C}$ ). When we compared the brood nest temperatures of the colonies in the different treatment groups, we found that only the colonies in the temperature treatment had elevated brood nest temperatures. Relative to the control treatment colonies, the temperature treatment colonies were warmer ( $P < 0.05$ ) at both the center and edge of the middle box (temperature treatment: center  $36.0 \pm 1.8^\circ\text{C}$ , edge  $30.7 \pm 5.8^\circ\text{C}$ ; control treatment: center  $34.8 \pm 1.3^\circ\text{C}$ , edge  $27.9 \pm 5.5^\circ\text{C}$ ; density treatment: center  $34.4 \pm 2.4^\circ\text{C}$ , edge  $27.6 \pm 4.7^\circ\text{C}$ ; pheromone treatment: center  $34.8 \pm 1.3^\circ\text{C}$ , edge  $28.8 \pm 5.5^\circ\text{C}$ ).

### Colony preparation and installation

The control treatment group had 10 colonies and the other treatment groups had 8 colonies each. To equalize the number of worker bees per colony, each one was started as an artificial swarm made with worker bees taken from one of the Liddell Field Station apiaries, and a queen purchased in May 2014 from C. F. Koehnen and Sons (Ord Bend, CA, USA). Each artificial swarm had  $1.07 \pm 0.03$  kg of worker bees  $-8200 \pm 200$  bees (Mitchell, 1970; Otis, 1982) – and was prepared following standard methodology (Seeley and Tautz, 2001). The bees were fed 1:1 (v:v) sucrose solution *ad libitum* for 96 h before being installed in their hive.

On day 0, 22 June 2014, the 34 artificial swarms were randomly assigned to a treatment group. Each colony was given one frame of fully drawn worker comb with at least 60% of the cells containing larvae and capped brood. This frame was placed in the middle of the box. The other frames given to each colony were empty, without comb or wax foundation, so they provided space for the workers to build whichever type of comb they wished. After being installed, the colonies were left undisturbed except on days 5 and 8 when we measured, using a  $2 \times 2$  cm grid, the areas of worker comb and drone comb built in the initially empty frames. Drone comb is easily identified because the cells are larger than those of worker comb (wall-to-wall distance: 6.2–6.9 mm versus 5.2–5.7 mm) (Martin and Lindauer, 1966; Taber and Owens, 1970). We filled the sucrose feeder in the upper box of each hive on days 0, 3, 5 and 8.

At the end of the experiment, 4 July 2014, we estimated the number of workers in each colony using the Liebfeld method (Imdorf et al., 1987). All colonies decreased in size, to  $5013 \pm 812$  bees, but none of the treatment groups were significantly different from the control (Tukey HSD,  $P > 0.05$ ). This also confirmed that colonies in the pheromone treatment did not lose workers to the colony in the lower box.



**Fig. 1. Experimental setup for manipulating potential cues of colony size.** The middle box of each hive contained 8000 worker bees and a queen bee, one frame of brood, and two or four empty frames that provided space for comb building. Vertical lines in the boxes represent bee frames.

### Data analysis

Statistical analyses were performed using R software version 3.1.1 and the packages lme4, lmerTest and lsmeans (R Core Team, 2014; Bates et al., 2015; <https://CRAN.R-project.org/package=lmerTest>; Lenth, 2016). Colonies with a dead or non-laying queen were removed from the analyses, so our final sample sizes were 10, 7, 7 and 8 colonies in the control, density, pheromone and temperature treatments, respectively. The proportion of drone comb built was compared between treatments using a linear mixed-effects model, with colony ID as the random factor. The best-fit model was determined by comparing AIC values with a Chi-square test (Akaike, 1974). The best-fit model included the interaction between treatment and experimental day (day 5 and day 8), and was significantly different from a null model that did not include these predictor variables ( $P < 0.001$ ). We then performed pairwise comparisons with a Tukey adjustment to check for significant differences between the treatment groups.

### Observational study

The goal in the observational study was to monitor and compare cues in small and large colonies to see which cues reliably change with colony size, and how. Presumably, cues more tightly correlated with colony size are more likely to be used by workers to detect colony size. We set up four 4-frame observation hives: two with 5000 bees (small colony size: colony S1 and S2) and two with 10,000 bees (large colony size: colony L1 and L2). We monitored these colonies for 8 days.

### Observation hive setup

The observation hives were built as described in Seeley (1995; see p.73), but held four frames of comb instead of two. From top to bottom, the four frames contained: capped honey, capped brood, empty comb and nothing (empty frame). All the cells in the capped honey frame were filled with honey. Most cells (80%) in the capped brood frame were filled with capped brood. All cells in the empty comb frame were empty. The empty frame had no comb at first; it provided a place for workers to build comb. None of the frames contained drone comb, because drone comb inhibits drone comb construction (Pratt, 1998) and we wanted to confirm that the large colonies were large enough to build drone comb. On both sides of each observation hive, we drew on the glass a grid that divided each frame into eight equal regions. All four hives were oriented with their entrances facing north, and all were kept in the same climate-controlled building (room temperature ca. 25°C).

Four colonies from the Liddell Field Station apiary were selected as source colonies for stocking the observation hives. Each colony was headed by a queen purchased in May 2014 from C. F. Koehnen and Sons. To generate two cohorts of bees of known age in each observation hive colony, we removed from each source colony over 100 freshly emerged bees on 30 July and 7 August 2014, we paint-marked these bees (Posca Paint Pens, Japan), and then we returned them to their respective colonies. On 11 August 2014, we collected from each source colony the following materials: the queen, one frame of brood, as many marked bees as possible (ca. 90–150 bees) and additional workers (5000 for small colonies, 10,000 for large colonies, as determined by mass). After installing the queen, brood frame and workers in each observation hive, we left the colonies undisturbed for 1 day, and then we began monitoring them on 13 August 2014. The cues that we monitored fell into three categories: worker density, worker behavior and colony temperature.

### Worker density

#### Contact rate

To see whether bees could use contact rate to assess colony size, we quantified contact rates between individuals in small and large colonies. From 13 to 20 August 2014, we followed randomly chosen marked bees for 30 s, counting the number of times the focal bee contacted or was contacted by other bees. A contact was defined as any touching between the focal bee and another bee. We followed 20 individuals (10 from each age cohort) in each colony each day, for a total of 640 observations.

#### Antennation rate

To see whether bees could use rate of antennation with other bees (i.e. antennae-to-antennae contact, each lasting more than 1 s) to sense colony size, we assessed how the antennation rate a bee experiences differs between small and large colonies. From 13 to 20 August 2014, we followed randomly chosen marked bees for 30 s, counting the number of times the focal bee made antennal contact with other bees. We followed 20 workers (10 from each age cohort) in each colony each day, for a total of 640 observations.

#### Transect line

Workers move throughout their colony's nest. To see whether the density of bees surrounding a moving worker differs between small and large colonies, we quantified worker density in multiple locations in our study colonies. On 14 August 2014, from 08:00 h to 22:00 h, we photographed each observation hive every 2 h. We then digitally drew two 20 cm horizontal transect lines across each frame (capped honey, capped brood, empty comb, empty frame) and counted the number of bees 'touched' by this line. We pooled the number of bees along a transect line across all time points for each colony, after confirming that time of day did not significantly improve the statistical model (see below).

#### Worker velocity and turning angle

We digitally tracked individually marked bees to see whether a worker bee's velocity changes with colony size. Using DLTdv5 software (Hedrick, 2008), we digitized the paths of marked bees in videos taken on 13 August 2014. For each colony, we tracked as many marked bees as possible in a 1 min video (colony S1, 57 bees; colony S2, 46 bees; colony L1, 31 bees; colony L2, 37 bees; total, 171 bees). To quantify the velocity of bees while moving (i.e. not while engaged in a task), we set a minimum threshold velocity of  $0.75 \text{ mm s}^{-1}$ . This threshold was determined after assessing the digitized paths of a subset of non-moving bees.

A bee changes travel direction when her path is obstructed. To see whether a bee moves differently depending on colony size, we quantified the turning angles for workers in small and large colonies. Using the digitized path data, we calculated the turning angle at each time point when a bee moved, relative to the previous and subsequent time points. This calculation does not distinguish between bees turning left versus right, but rather compresses turning angles between 0 and 180 deg.

### Worker behavior

#### Tasks

Workers perform tasks throughout the nest. To see how workers adjust their efforts among tasks as a function of colony size, we monitored the locations and the behaviors of marked bees from two age cohorts. From 13 to 20 August 2014, we scan sampled both sides of each observation hive and recorded the location, task and age cohort of each marked bee that we spotted. On the first day of

data collection, the bees in the older age cohort were 15 days old and the bees in the younger age cohort were 7 days old. We identified tasks as in Kolmes (1984), with some modifications (Table S1).

To analyze these data, we sorted the 43 specific tasks into seven general tasks: walking, resting, nursing, hive maintenance, worker maintenance, in festoon and foraging. A festoon is a cluster of bees hanging attached to one another; it resembles a curtain and often surrounds an area where comb is under construction. We tested whether bees were engaged in each of the seven general tasks differently based on colony size (large or small) and age cohort (old and young) using a binomial generalized linear mixed effects model. We accounted for the age of the marked bees, because bees change tasks according to their age (Seeley, 1982). Experimental day and colony ID were set as random factors. To further test whether bees in small or large colonies began foraging at a younger age, we made experimental day a predictor variable, which did improve the model ( $P < 0.05$ ), but was not significantly different for marked bees in small versus large colonies ( $P > 0.05$ ).

#### Location

To see whether bees use their distribution throughout the nest to assess colony size, we determined whether bees in the small and large colonies used the space differently within their hives. For example, bees in large colonies might spend more time at the periphery of the nest than do bees in small colonies. Using the location data that were collected for examining the workers' task distributions, we summed the number of marked bees in every grid square each day, from 13 to 20 August 2014. We then tested whether the locations of marked bees were different for workers in small and large colonies, and whether bees were more likely to be observed at the periphery or the center of the nest. We defined the nest periphery as the grid squares that touch the edges of the observation hive (40 per observation hive) and the nest center as the inner grid squares (24 per observation hive). We then tested for differences in the spatial distributions (periphery or center) for the marked bees in small and large colonies.

#### Colony temperature

To see whether temperature differs with colony size, we placed iButtons on the center of both sides of each of the four frames in each observation hive. The iButtons logged temperature every 30 min from 08:00 h on 13 August 2014 to 12:00 h on 20 August 2014.

#### Data analysis

All statistical analyses were performed using R software version 3.1.1 and the packages lme4, lmerTest, pbkrTest and lsmeans (Bates et al., 2015; <https://CRAN.R-project.org/package=lmerTest>; Halekoh and Højsgaard, 2014; Lenth, 2016). For each cue that we monitored, we built a generalized linear mixed effects model to test for differences between small and large colonies, with colony ID as the random factor. We then added fixed effects, such as colony size,

and tested whether each fixed effect significantly improved the model versus a null model using AIC comparison and a Chi-square test (Akaike, 1974). We then used an  $F$ -test with a Kenward–Roger approximation to determine the significance of a given fixed effect. If there were multiple predictor variables, we performed pairwise comparisons with a Tukey adjustment. Values are reported as means  $\pm$  s.d.

## RESULTS

### Experimental study

Every colony built comb, but not every colony built drone comb. Table 1 shows for each treatment group the mean amount of comb built and the mean proportion of this comb that was drone comb. Fig. 2 shows the proportion of drone comb built in each treatment. Only in the density treatment was the proportion of drone comb significantly higher than in the control treatment ( $P = 0.047$ ).

### Observational study

Both large colonies built comb, including drone comb. Colony L1 built 24 cm<sup>2</sup> of worker comb and 594 cm<sup>2</sup> of drone comb; colony L2 built no worker comb and 10 cm<sup>2</sup> of drone comb. Neither small colony built any comb. Therefore, the two large colonies were above the threshold colony size needed to build drone comb, and the two small colonies were below the threshold colony size. The workers in the small colonies would likely have built worker comb had there not been empty comb available.

### Worker density

#### Contact rate

Bees in large colonies had significantly higher contact rates than bees in small colonies ( $P = 0.016$ ). In small colonies, bees received  $10.9 \pm 6.6$  contacts per 30 s, whereas in large colonies they received  $14.9 \pm 6.5$  contacts per 30 s. Adding the age cohort of the marked bees did not significantly improve the model over one that included only colony size ( $P > 0.05$ ).

#### Antennation rate

Antennation rates between bees did not significantly differ between small and large colonies ( $P > 0.05$ ). In small colonies, bees had  $0.43 \pm 0.76$  antennations per 30 s; in large colonies, they had  $0.49 \pm 0.78$  antennations per 30 s.

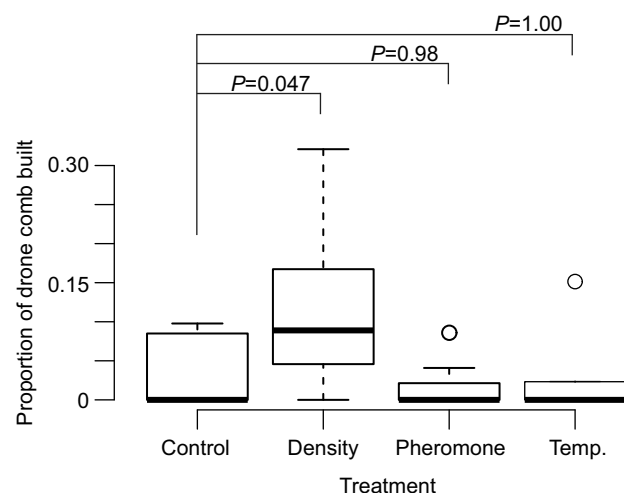


Fig. 2. The proportion of drone comb built by the colonies in each treatment group.

Table 1. Comb building by each treatment group

Treatment	No. of colonies	Total comb built (cm <sup>2</sup> )	Drone comb built (cm <sup>2</sup> )	Proportion drone comb
Control	10	1822 $\pm$ 359	50 $\pm$ 83	0.03 $\pm$ 0.04
Density	7	1572 $\pm$ 116	190 $\pm$ 178	0.12 $\pm$ 0.11
Pheromone	7	1969 $\pm$ 107	37 $\pm$ 68	0.02 $\pm$ 0.03
Temperature	8	1702 $\pm$ 397	42 $\pm$ 86	0.02 $\pm$ 0.05

Data are means  $\pm$  s.d.

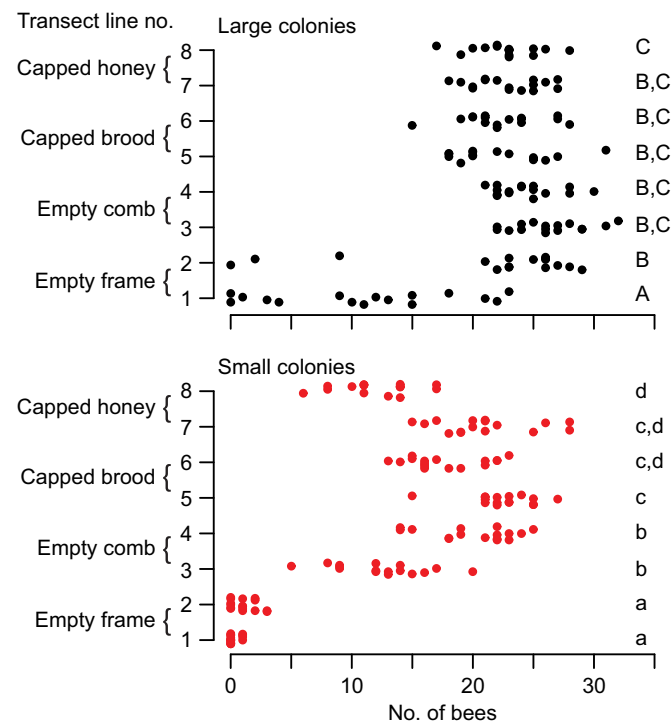


### Transect line

When we compared the number of bees along eight transect lines (Fig. 3), we found that the location of the transect line was significant ( $P<0.001$ ), with the highest number of bees in the nest center, atop the capped brood and on empty comb frames. The interaction between transect line location and colony size was also significant ( $P<0.001$ ). Large colonies had more bees along their transect lines than did small colonies ( $P<0.005$ ). Comparing the number of bees at each transect line between the small and large colonies (Fig. 3), we found fewer bees in the small colonies than in the large colonies (pairwise comparisons,  $P<0.01$ ), except for lines 5 and 7, where there was no difference. When we examined how the number of bees along a transect line differed within small and large colonies, we found more variation in worker density in the nests of small colonies than in those of large colonies (see letters in Fig. 3). In large colonies, worker density was comparatively uniform throughout the nest, except for transect line 1, where there were fewer bees than elsewhere (Fig. 3).

### Worker velocity and turning angle

The mean velocities of bees in small and large colonies were not statistically different: small colonies,  $0.316\pm0.149\text{ cm s}^{-1}$ ; large colonies,  $0.361\pm0.140\text{ cm s}^{-1}$  ( $P>0.05$ ). There was also no difference in the maximum velocities: small colonies,  $0.905\pm0.510\text{ cm s}^{-1}$ ; large colonies,  $1.071\pm0.438\text{ cm s}^{-1}$  ( $P>0.05$ ). Furthermore, the turning angles did not significantly differ between workers in small and large colonies: small colonies,  $77.3\pm55.7\text{ deg}$ ; large colonies,  $78.3\pm56.9\text{ deg}$  ( $P>0.05$ ) (Fig. 4).



**Fig. 3. Number of bees observed along each transect line in large and small colonies.** Transect lines are numbered from the bottom of the observation hive (1) to the top (8). Letters on the right denote statistical differences between the transect lines within large colonies or small colonies ( $P<0.05$ ). Along all transect lines, except 5 and 7, large colonies had more bees than did small colonies.

### Worker behavior

#### Tasks

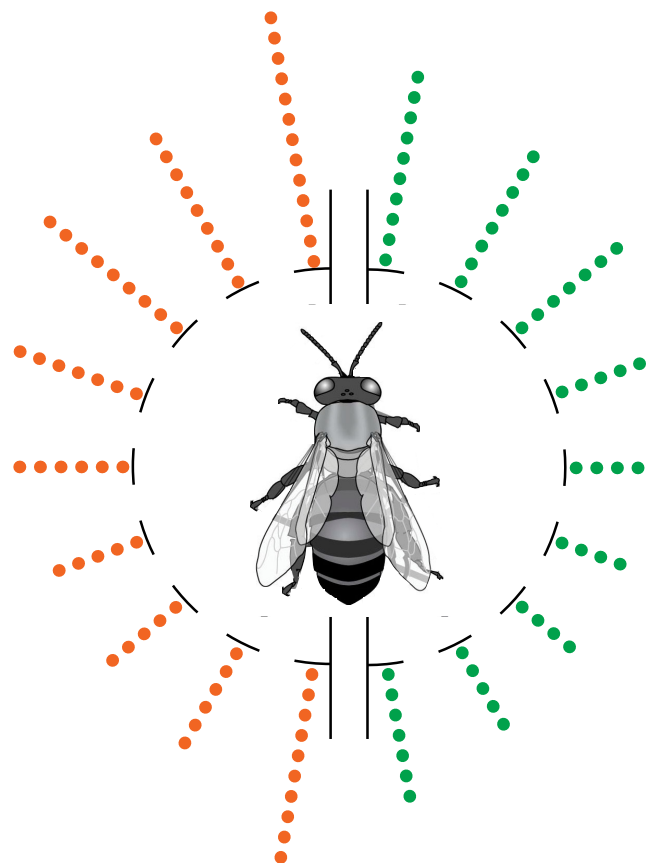
We observed 3504 tasks being performed by the marked bees (Table 2). Walking, resting, hive maintenance, worker maintenance and foraging behavior were performed with significantly different frequencies between the age cohorts ( $P<0.05$ ). The difference in frequency of nursing was marginally non-significant ( $P=0.056$ ). Frequency of being in a festoon was not significantly different between the age cohorts ( $P>0.05$ ). Comparing the tasks performed by bees in small and large colonies, only resting and being in the festoon were significantly different ( $P<0.05$ ). Bees in small colonies were observed resting more often than bees in large colonies. Bees in large colonies were observed in the festoon more often than bees in small colonies.

#### Location

The marked bees were observed in 3504 locations. The locations of the marked bees did not significantly differ between small and large colonies ( $P>0.05$ ). When we categorized the bees' location as either at the periphery or at the center of the observation hive, we still found no significant difference between small and large colonies ( $P>0.05$ ).

#### Colony temperature

The overall nest temperature did not significantly differ between small and large colonies ( $P>0.05$ ) (Fig. 5 and Table 3). Comparing



**Fig. 4. Worker turning angle for bees in small (left, orange dots) and large (right, green dots) colonies.** Each dot represents 50 turns by a marked bee of the angle shown. Calculation of the turning angle did not distinguish between bees turning left and turning right, but rather compressed angles between 0 and 180 deg.

**Table 2.** The percentage of time that marked bees were observed engaged in different tasks in relation to colony size and age cohort

Task	Small colonies		Large colonies		P-value	
	Old	Young	Old	Young	Colony size	Age cohort
Walking	30.9 (332)	24.1 (248)	31.4 (187)	27.6 (222)	0.68	0.0011*
Resting	12.1 (130)	15.5 (159)	5.9 (35)	7.8 (63)	<0.0001*	0.037*
Nursing	2.6 (28)	3.8 (39)	3.5 (21)	5.7 (46)	0.12	0.056
Hive maintenance	35.5 (382)	45.6 (469)	39.4 (235)	40.9 (329)	0.97	0.0003*
Worker maintenance	14.7 (158)	9.1 (93)	10.9 (65)	11.7 (94)	0.99	0.019*
In festoon	0.9 (10)	0.9 (9)	7.4 (44)	5.1 (41)	<0.0001*	0.58
Foraging	3.4 (36)	1.1 (11)	1.5 (9)	1.1 (9)	0.42	0.0054*
Total counts	(1076)	(1028)	(596)	(804)		

Workers from the small and large colonies are divided into old and young cohorts. Counts are given in parentheses. Asterisks indicate significance.

the temperatures of each frame separately for the small and large colonies, we found that only for the lowest frame (empty frame) was there a significant difference ( $P<0.05$ ). In small colonies, the empty frame was 6.4°C cooler than in large colonies.

**DISCUSSION**

We used both experimental and observational approaches to investigate the cue(s) worker bees use to sense colony size. The experimental study increased three cues independently in an attempt to ‘trick’ the bees into overestimating their colony’s size as being above the reproductive threshold. The observational study monitored cues in small and large colonies to identify reliable indicators of increased colony size, and hence candidates for the cue (s) that the bees use to sense their colony’s size.

**Experimental study**

Three potential cues of colony size were increased in the experimental study: density of workers, quantity of volatile

pheromones and temperature in the nest. Only an increase in worker density resulted in a higher proportion of drone comb built relative to the control colonies. These results suggest that workers somehow sense worker density and use this sensation to assess their colony’s size. However, increased worker density may not be the critical cue per se; it seems likely that the critical cue varies with colony density and increases as worker density increases (e.g. contact rates, antennation frequencies, difficulty in movement). To explore which specific stimuli reliably change with colony size, and so might be used by the bees as cues of colony size, we conducted an observational study.

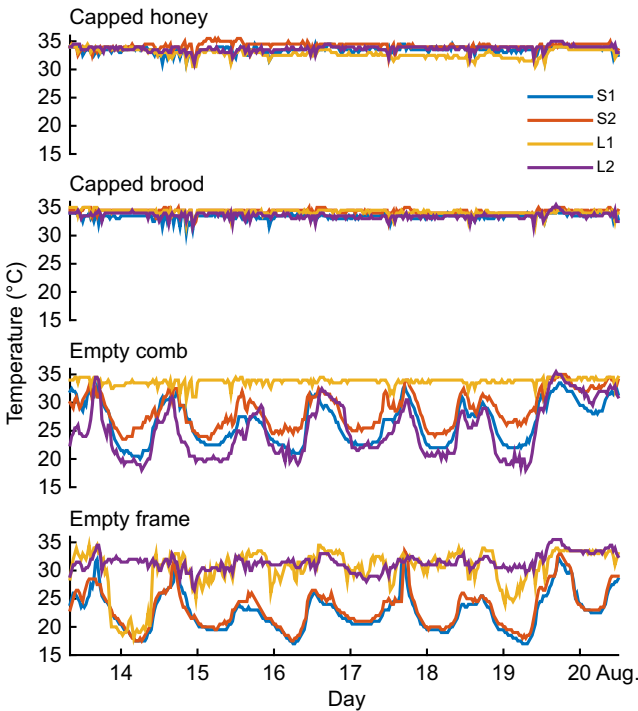
**Observational study**

We monitored potential cues in small and large colonies to determine which stimuli reliably change with colony size (Table 4). We grouped these potential cues into three categories: worker density, worker behavior and colony temperature.

**Worker density**

Experimentally increasing worker density was the only treatment that increased the proportion of drone comb built relative to the control (Fig. 2). Therefore, for the observational study, we looked closely at stimuli that we expected to co-vary with worker density and that can be sensed by individual bees.

An individual bee experiences a higher frequency of contacts with other bees in a large colony than in a small colony. Given a set nest cavity size, a larger colony has more bees packed together, so we expected contact rates to increase, but workers in a colony with twice as many bees did not receive twice as many contacts (small colonies with 5000 bees:  $10.9\pm6.6$  contacts per 30 s; large colonies with 10,000 bees:  $14.9\pm6.5$  contacts per 30 s). Also, bees did not distribute themselves uniformly in the nest, so contact rates will vary with worker density throughout the nest. At the colony level, we monitored worker density by recording the number of bees along eight transect lines. We found that in the nest center the number of bees along a transect line was independent of colony size. At the nest



**Fig. 5.** The temperature of the four frames in the observation hives. Each line denotes data for a single colony, plotted against time of day (where each tick mark indicates 00:00 h on the indicated day in August 2014). The temperatures were significantly different between the small (S) and large (L) colonies only at the lower periphery of the nest, i.e. on the empty frame.

**Table 3.** Mean temperature of frames in small and large colonies

Frame	Temperature (°C)		P-value
	Small colonies	Large colonies	
Capped honey	33.7±0.7	33.1±0.7	0.24
Capped brood	34.0±0.7	34.1±0.6	0.87
Empty comb	27.3±3.7	31.0±4.4	0.35
Empty frame	22.9±3.4	29.3±4.3	0.023*
Mean nest temperature	29.5±5.3	31.9±3.6	0.15

Frames are ordered as in the observation hive, with capped honey at the top, and the initially empty frame (combless) at the bottom. Asterisks indicate significance.

Table 4. Summary of results found in the experimental and observational studies

Cue tested	Difference?	Notes
<b>Experimental study</b>		
Increased worker density	Yes	Increasing worker density increases the proportion of drone comb built.
Increased volatile pheromones	No	No effect.
Increased nest temperature	No	No effect.
<b>Observational/correlational study</b>		
Contact frequency	Yes	Contact rate higher in larger colonies.
Antennation frequency	No	No difference.
Worker density throughout nest	Yes	Number of workers on transect lines is higher and less variable in larger colonies.
Worker velocity	No	No effect of colony size on the mean velocity or maximum velocity of moving workers.
Worker turning angle	No	No difference. Turning angles are nearly identical for individuals in small and large colonies.
Worker task distribution	Yes/no	Workers in large colonies spend significantly less time resting and more time in the festoon. All other behaviors are no different for workers in small and large colonies.
Worker location	No	No difference.
Colony temperature	Yes/no	Higher temperature in large colonies, but only on the lowest frame.

periphery, however, there was a higher density of bees in larger colonies, suggesting that workers spread themselves uniformly over some comb surfaces, such as the brood nest in the center of the colony, and this pushes additional bees to the periphery. Therefore, if a worker were to walk from the nest center to periphery, she would experience greater variation in worker density (and presumably contact rate) in a small colony relative to a large colony. Bees patrol their nest widely (Johnson, 2008), so variation in worker density could be used to assess colony size.

We found no difference in bee velocity as a function of colony size. Neither mean nor maximum velocity of tracked bees differed between small and large colonies. This was surprising, given that larger colonies contain more individuals and so have more potential obstacles to a bee moving between two points. We therefore tested whether bees in large colonies are forced to turn more often than bees in small colonies, given that their velocity does not change. This also turned out not to be the case: bees in small and large colonies had the same mean turning angles (Fig. 4). It is unlikely, therefore, that workers use velocity or turning angle to sense colony size.

How likely is it that workers use contact rates to sense their colony's size? Simpson et al. (2001) showed that locusts sense the density of conspecifics in their immediate environment by the frequency of contacts they receive on the hind femur. Honey bee workers may do the same. Our results show that the density of workers throughout the nest changes more dramatically in a small colony than in a large colony, so if a worker receives a constant rate of contact as she walks through her colony, then she is likely in a large colony. If a worker receives a variable rate of contact as she moves through her colony, then she is likely in a small colony. This variation in contact rate might serve as a cue of colony size.

**Worker behavior**

We tested whether worker tasks change with colony size. For example, do workers in small colonies spend more time nursing young larvae than workers in large colonies? If the tasks that workers are engaged in change with colony size, then workers might use this information to sense the size of their colony.

Of the seven tasks we observed (condensed from 43 specific tasks into seven general tasks: walking, resting, nursing, hive maintenance, worker maintenance, in festoon, foraging), only in festoon and resting were significantly different between small and large colonies. Workers in large colonies were found in the festoon significantly more often than workers in small colonies. This is because large colonies have more bees with which to make a festoon than do small colonies. In the observation hives, the large colonies had large festoons and the small colonies had small festoons or none

at all. Could workers use the size of their festoon to determine whether they should begin building drone comb? Probably not. When a colony first inhabits a nest cavity, there are no combs yet, and so all the bees cluster together in a large festoon. Despite the large festoon, the workers first build worker comb, not drone comb (Smith et al., 2016). Workers presumably need to assess their colony's size in other situations as well, so we do not expect the size of a festoon to be a general indicator of colony size.

Workers in large colonies, relative to those in small colonies, also spend significantly less time resting, perhaps because of the higher contact rates in large colonies. Time spent inactive could be a way for a worker to sense her colony's size: the more time spent active, the larger the colony. While the mechanism is plausible, the trend seems unexpected. Michener's paradox states that per capita productivity decreases as colony size increases (Michener, 1964), and so predicts that workers in large colonies spend more time inactive than workers in small colonies, the opposite of what we found (Table 2). Michener's study, however, measured the productivity of a specific task (number of capped brood cells). Other studies show increased productivity with colony size, and thus workers spend less time inactive, such as during nest building, in contrast to Michener's paradox (e.g. Jeanne, 1986; Jeanne and Nordheim, 1996; see also Karsai and Wenzel, 1998; Strohm and Bordon-Hauser, 2003; Bouwma et al., 2006). To the best of our knowledge, our study is the first to look broadly at worker's task performance in relation to colony size, so it is the first to show that workers in large colonies spend less time resting, at least in *Apis mellifera*. Our study, however, only compared two small colonies with two large ones, and our definition of inactive bees (resting) includes all bees that were immobile. It is possible that some of these bees appeared inactive, but actually were heating the colony by contracting their flight muscles (Esch, 1960). Even so, by our definition, there were twice as many bees resting in small colonies as in large colonies, so workers could use the amount of time spent resting as a measure of colony size: the less time you rest, the larger your colony.

The worker task data also gave us information about the marked bees' locations in the nest. We tested whether workers in small and large colonies used the space within their nests differently, but found no differences. This indicates that although worker density varies in the nest, individuals are still moving throughout the entire nest.

**Colony temperature**

We monitored the temperature of colonies to quantify temperature variation with colony size. This was done to determine whether

workers in small and large colonies experience different temperature gradients while moving through their nests. We found no difference in temperature at the center of the observation hive in the small and large colonies, which corroborates existing studies that show honey bees tightly regulate the temperature of the brood nest (Seeley, 1985). However, small colonies were significantly cooler than large colonies on the lowest periphery of the observation hive. This is because large colonies have more bees than do small colonies, and each bee has a resting metabolism that keeps her a few degrees above ambient temperature (Kovac et al., 2007). Therefore, we expect large colonies to be warmer than small colonies simply as a result of a larger number of heat-emitting individuals. The experimental study, however, showed that increased temperature does not induce workers to build drone comb, so we do not expect bees to use nest temperature to sense colony size.

## Conclusions

The experimental study found that worker bees responded to an increase in worker density by producing more drone comb in preparation for reproduction. The observational study measured potential cues of colony size in small and large colonies living in nests of the same size, and hence with relatively low and high densities. We found that worker contact rate increases with colony size, and that worker density is less variable in large colonies. Workers in large colonies also spend less time resting than workers in small colonies, which may contribute to the increase in contact rate. Therefore, the observational study found contact rate to be the most likely indicator of colony density for honey bees.

The ability to sense developmental state is critically important for all organisms, including organisms whose development depends on group size. For a honey bee colony, workers evidently use density to sense group size. The underlying cue of worker density remains uncertain, but presumably it is a physical cue related to worker movement, although we did not test for non-volatile chemical cues. Whereas groups of unicellular bacteria and multicellular animals use chemical cues to sense their size, the one superorganism where it has been studied, a honey bee colony, evidently relies on physical cues to sense group size.

## Acknowledgements

The authors thank Tom Seeley for equipment, Erika Mudrak and Lynn Johnson from Cornell Statistical Consulting Unit and Collin Edwards for statistical advice, Sara Kaiser for the loan of iButtons, Paul Shamble for advice on video analysis, and Joyce Gao and Madeleine Ostwald for help in wrangling bees. We are also grateful to Cissy Ballen, Patricia Jones and Tom Seeley for providing helpful critiques of the manuscript. Michael Smith gives special thanks to Bill Wcislo for providing him with a space to quietly write at the Smithsonian Tropical Research Institute.

## Competing interests

The authors declare no competing or financial interests.

## Author contributions

P.A.K. participated in study design, collected field data and helped edit the manuscript. J.M.P. participated in study design, collected field data, helped with figures and helped edit the manuscript. M.L.S. conceived, designed and coordinated the study, collected and analyzed the field data, carried out the statistical analyses, drafted the figures and wrote the manuscript. All authors gave final approval for publication.

## Funding

This research was supported by two Graduate Research Fellowships from the National Science Foundation (DGE-1144153 to M.L.S. and DGE-1144152 to J.M.P.), and a Sigma Xi Grant-in-Aid of Research (to M.L.S.). The funders had no

role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.150342.supplemental>

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