

Larvae influence thermoregulatory fanning behavior in honeybees (*Apis mellifera* L.)

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Abstract For many animals, maintaining a specific range of temperatures during offspring development is critical for the survival of the young. While this is most studied in birds and mammals, some insects regulate nest temperatures to create an ideal environment for larval development. Here, we explore the thermoregulatory fanning behavior in honeybees performed to maintain colony temperatures in the presence of larvae. We found that honeybees are more likely to fan when larvae are present, but need direct contact with larvae to fan. We found no evidence that exposure to brood pheromone plays a role in stimulating fanning behavior. Finally, we saw a shift in the fanning response seasonally. These results show that the presence of developing offspring influences the fanning response in honeybees and help us to understand how honeybee colonies achieve the fine thermoregulation necessary for healthy larval development.

Keywords Division of labor · Task allocation · *Apis mellifera* L. · Thermoregulation · Care of offspring

Introduction

For many species, care of offspring is a critical component of offspring survival. Care of offspring, defined as parental investment that increases current reproduction at the expense of future reproduction (Wittenberger 1981; Zeh et al. 1985), is surprisingly widespread in insects, occurring in 10 orders (Zeh et al. 1985). In most orders, however, parents perform offspring care facultatively to increase survival of their offspring, but the young can survive without it (Mas and Kölliker 2008). Eusocial insects have obligate offspring care and larvae will not survive without adult investment (Mas et al. 2009). Larval begging by use of movements is known in some ants, such as *Novomessor* (Hölldobler et al. 1978) and *Myrmica* (Creemers et al. 2003), as well as *vespid* wasps (Ishay and Landau 1972; Ishay and Schwartz 1973; Hunt 1991). More often, though, chemical cues communicate larval status to adult caregivers. For example, adult bumblebees assess hunger status using larval cuticular hydrocarbons (Den Boer and Duchateau 2006). Honeybee larvae produce brood pheromone, which induces foragers to collect more pollen (Le Conte et al. 2001). Cues from offspring allow caregivers to adjust provisioning efforts in many insects (Mas and Kölliker 2008).

While most studies of offspring care focus on nutritional provisioning, many organisms also closely regulate the microclimate in which young are reared. Altricial offspring depend on caregivers for temperature regulation during critical developmental periods (Koteja 2000). This thermal regulation can occur passively, with a caregiver selecting a site for a nest, or actively, with the caregiver behaviorally and physiologically maintaining temperature (Warner and Shine 2008). For example, leaf-cutting and grass-cutting ants select appropriate depths in soil for brood chambers (Bollazzi and Roces 2002, 2007), fire ants move brood as

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nest temperatures vary throughout the day (Penick and Tschinkel 2008), while termites create elaborate architecture and nest orientation for regulation of air movement in their mounds (Korb 2003; Jacklyn 2010). Although caregivers rely on behavioral and chemical cues to assess satiation of offspring in many taxa, much less is known about how caregivers assess thermal status of offspring. In this paper we explore how cues from offspring coupled with cues from the environment shape thermoregulatory care of offspring in a eusocial honeybee.

Thermal information is one of the most critical environmental cues that caregivers consider when caring for young and the same principles apply to vertebrates as to social insects. Eusocial insect workers provide thermal control for development as well as extensive food provisioning for their larvae (Himmer 1927, 1932; Lindauer 1952). This type of care is often viewed as a colony or nest-level process in which the temperature, humidity, carbon dioxide and oxygen levels of the nest are manipulated to maintain optimal conditions for rearing young (Seeley 1974; Starks and Gilley 1999; Human et al. 2013). Honeybees actively cool their nest by fanning and using water evaporation, and warm their colonies by shivering, clustering, and pressing their bodies onto or entering brood comb (Heinrich and Esch 1994; Starks and Gilley 1999; Kleinhenz et al. 2003, Stabentheiner et al. 2010). Eusocial insects provide an interesting system by which to study how caregivers can effectively provide this extensive care.

We use honeybees, *Apis mellifera*, as a model system with which to test hypotheses about direct feedback from brood to adults during thermoregulatory care for offspring. When larvae are present in the honeybee colony, temperature is tightly regulated around 35 °C (Himmer 1927; Lindauer 1955; Fahrenholz et al. 1989). If nest temperatures rise above 37 °C, the larvae can develop malformations and die (Himmer 1932). To cool their nest, honeybees engage in active thermoregulatory behaviors (Jones and Oldroyd 2007). This includes spreading water on comb to evaporatively cool it (Kühnholz and Seeley 1997), heat shielding, where bees use their bodies to absorb then dissipate excess heat (Starks and Gilley 1999; Bonoan et al. 2014), and fanning behavior, used to circulate air through the colony (Cook and Breed 2013; Egley and Breed 2013). Fanning behavior is of particular interest because of the group dynamics needed to be effective, and is the focus of our study. Overall, these behaviors effectively maintain the proper climate for larval development and have major implications for the overall success of the colony.

Here, we tested the previously unexplored question: how do larvae influence the thermoregulatory fanning behavior in adult honeybees? We hypothesized that the presence of larvae would affect fanning behavior. Specifically, we predicted that honeybees would fan more when they are in

the presence of a larva. We also predicted that adult-larva interactions are important in the transfer of thermal cues, so adult honeybees that physically touch larvae will fan more. Honeybee larvae communicate with brood pheromone to influence foraging behavior, so we also predicted that brood pheromone would increase fanning behavior. Finally, if we observe effects of the presence of larvae on adult behavior in the lab, we should also see variation in the thermoregulatory response of bees across the season, as honeybees diminish or stop larval production in the winter. We predicted adult honeybees sampled from hives with larvae would fan more than bees from winter hives. Our goal is to provide a deeper understanding of the role the presence of larvae plays in this critical thermoregulatory behavior.

Methods

General honeybee care

We collected workers and larvae from twelve *Apis mellifera* colonies on the University of Colorado campus for these experiments. Ten frame wooden Langstroth hives with plastic frames housed our colonies. We conducted experiments between 1 June 2012 and 1 August 2014. All hives were used and were randomly selected for each collection.

Collecting fanner bees and larvae

To parse out the direct effect of larvae on fanning behavior, we collected only a single bee and a single larva. While bees are most likely to fan in groups (Cook and Breed 2013), we controlled for social effect using single bees with a single larva to directly test the effect of presence of larvae. We collected fanners as discerned by location on the landing platform, distinct upright but curved abdomen position, and rapid wing movement for a period of at least 10 s without changing position and orientation relative to hive opening. Studies on bumblebees use 10 s of sustained fanning to define a fanner (Weidenmüller 2004). This posture and time distinguish them from other worker bees, such as foragers who may be departing the colony, guards who may fan their Nasanov gland (Free 1967), and other fanning-like behaviors that take place at the entrance of the colony (Yang et al. 2009). We chose to focus on porch fanners as they are easy to collect and are more likely to be fanning because of temperature rather than to regulate carbon dioxide (Seeley 1974) and humidity (Human et al. 2006). Furthermore, while these fanners are situated on the porch, they move throughout the colony and likely interact with larvae directly and with other honeybees that interact with larvae, and therefore can receive thermal information about them. We collected a single fanning adult honeybee by grabbing a

leg with forcep, and placed her into a mesh cage (cylindrical: height: 20 cm, radius: 6 cm). We then opened the hive to collect larvae as carefully as possible and with no smoke to ensure some continuing of normal behavior with such a large disturbance, however when we opened hives, we did not use them again for 2 h, until normal hive behavior returned. We carefully extracted worker larvae with forceps in the fourth or fifth instars from the same colony that workers were collected from. After hives were sealed back up, we transported the bee and larva in the cage to the lab, where we performed our behavioral assay.

Use of screens to restrict physical contact between larvae and adults

To explore the potential cues adult honeybees could use while physically interacting with the larva, we designed a similar cage with an auxiliary chamber. This chamber was made of the same metal screen material as the rest of the cage. The chamber was the same size and shape for the bee (cylindrical: height: 20 cm, radius: 6 cm), with the additional chamber (cylindrical: height: 3 cm, radius: 6 cm) added on, so as to not change the volume to which the bee is confined. We performed the same protocol for collecting a fanner and a larva as previously stated, except we placed the larva into the auxiliary chamber, separated by 3 cm from the adult fanner bee. Again, we transported these cages into the lab to perform our behavioral assay. We performed 43 trials of bees separated from the larva.

Brood pheromone

Brood pheromone (BP) emerged as the next step in exploring the role of a larval chemical cue in the performance of adult fanning behavior. We acquired synthesized brood pheromone (Super Boost, Contech Enterprises Inc.). Super Boost is comprised of 10 fatty acid esters (Le Conte et al. 1990). Since we saw a response of increased fanning behavior in the presence 5 larvae, we wanted to use a 5 larvae equivalent dose to account for any evaporation or degradation. We kept Super Boost frozen ($-18\text{ }^{\circ}\text{C}$) until use, then let thaw for 5 min at room temperature. We vortexed the Super Boost for 1 min, then put 0.112 g into 10 mL of hexane. We then vortexed this mixture for 1 min. This stock solution was serially diluted 3 times until we had 200 doses of BP per 10 mL solution. When not in use, these solutions were kept frozen at $-18\text{ }^{\circ}\text{C}$.

We placed 250 micro liters of BP solution onto filter paper (Whatman #2 42.5 mm) in a fume hood, and the hexane was allowed to evaporate off for an hour. The filter paper was then collected and placed into zip top sealable plastic bags for transport to the field. When not immediately used, the filter papers were stored in the freezer.

We performed this study using a blind design, with the observer unaware of the treatment being observed. For this, we also prepared filter paper with only hexane, which was treated in the exact same manner as the BP solution and treated filter paper. We used completely separate forceps, bags, gloves, and other tools when handling the different samples to eliminate the possibility of contamination between treatments and controls.

We placed filter paper into color-coded cages (only CNC knew which color corresponded with treatment and control), and then brought the cages out into the field to collect fanners. One fanner was placed into the cage, then brought back into the lab and placed into a color-coded jar for acclimation and the heating trial. Either KJS or another lab assistant, CR, watched the trials and recorded data. Similar to our other experiments, bees were allowed 25 min to acclimate before beginning the heating regime and behavioral assay. Brood pheromone experiments were conducted from June to September 2013.

Seasonal presence of larvae at hive

Larvae present (spring/summer)

We identified fanners at the hive using the protocol described above and placed them into mesh cages in groups of ten. We collected spring data from 13 March 2013 to 8 April 2013, and summer data from May to September 2013 and 2014.

Larvae absent (winter)

During late fall and much of winter workers are likely generalists and perform any task that needs to be accomplished in the colony (Fluri et al. 1976; Huang and Robinson 1995; Pearce et al. 2001). Therefore, we collected groups of ten bees opportunistically at the entrance of the hive. We chose groups of ten because single bees are not likely to fan (Cook and Breed 2013). Bees were randomly collected using forceps and placed into individual wire mesh cages (cylindrical, 5 cm \times 2.5 cm). These cages were used to transport the bees back to the lab. Winter collections were performed from 23 October 2012 to 16 November 2012.

Temperature regime and behavioral assay

Once bees and larvae were collected at the hives, we brought them into the lab. We placed the cage into a two-liter glass container (9 cm \times 24 cm), and then loosely sealed the top. We then set the container on a heating unit (Proctor Simplex). We placed a piece of aluminum sheeting between the coil of the heating unit and the container, which reduced how quickly the jar increased in temperature. Once

inside the container, we left the bee alone for 25 min to acclimate. This amount of time allowed for the bee's activity level to steady after being caught and transported. We then began the heating regime, starting at room temperature (an average of 25.24 ± 0.110 °C), by heating the air inside of the jar 1 °C per minute. We measured the temperature using a high accuracy (± 0.3 °C Cole Parmer) digital thermometer that was placed through a fitted hole through the top of the jar. Behavior of the bees during the heating trials was observed constantly during the entire assay. We recorded bees as fanning if they began fanning their wings while standing still for at least 10 s, which is the same criteria we used when we collected fanners from the hives. Additional data we recorded consisted of the temperature at which bees began to fan (herein, thermal threshold) and which hive they came from, which we treated as a random effect in our models. We also recorded any interactions the adult bee had with the larva and whether the hive was in the sun or the shade at collection, but later excluded them from the model as they were not significant predictors of fanning behavior.

We recorded the occurrence of fanning behavior, worker and larvae interaction, and corresponding temperatures were recorded until the bees reached their thermal maximum and ceased all activity. We recorded fanning behavior by our previously mentioned characteristics for fanners as characterized by a worker displaying distinct upright body position and rapid wing movement for a period of at least 10 s without changing position (Egley and Breed 2013). We also noted date and time of collection, as well as whether the hive was in the sun at the time of collection, and the total time the trial took, but they are not included in our results as they did not explain variation in the fanning response.

Statistical analysis

To test all hypotheses we used a generalized linear mixed model and treated hive as a random effect. We used both probability of fanning and thermal response threshold as our response variables. This gave us a more comprehensive perspective of how fanning behavior could be modulated by larvae. To look at probability of fanning, we performed a logistic regression with a binomial error distribution (link = logit). To explore thermal response threshold, we performed a linear regression with a Gaussian distribution. To evaluate the magnitude of a significant effect, we used a post hoc (Tukey) test. In all models, we treated presence of larvae in the hive, presence of larva in a trial, separation, and pheromone as categorical predictor variables. With all of our models, we started with the most inclusive model, including two additional factors: whether the hive was in the sun (binomial) and total trial time (continuous). If predictor

variables were insignificant ($\alpha = 0.05$), we dropped them from the model. We used R and R Studio, version 0.98.1103 and the package LME4 or generalized linear mixed model analysis (Bates et al. 2014).

Results

Effect of presence of larvae on fanning behavior

Honeybees were significantly more likely to fan when being heated in the presence of a larva compared to being heated without a larva present ($N = 318$: $Z = 3.258$, $p = 0.00112$; Fig. 1I). The presence of larva had no significant effect on the thermal response threshold of fanner honeybees ($N = 103$, $F = 1.125_{2,100}$, $p = 0.3288$; Fig. 2I). When performing these behavioral assays we observed workers interacting extensively with larvae. Workers often antennated larvae, probed with them with their proboscis, and even picked larvae up and carried them around the experimental cage. These observations led to the following tests of the hypothesis that physical contact by adult bees with larvae could be critical in triggering fanning behavior.

Use of screens to restrict physical contact between larvae and adults

Adult worker bees separated from the larva but with olfactory contact were significantly less likely to fan than workers with direct contact with larvae ($N = 89$: $Z = 3.326$, $p = 0.00088$; Fig. 1I). There was no significant difference between probability of fanning when there was no larva compared to larva present but divided from the worker by a screen ($N = 156$: $Z = 1.385$, $p = 0.166$; Fig. 2I).

Brood pheromone

Honeybees were not more likely to fan when exposed to brood pheromone as compared to controls ($N = 98$: $Z = -0.492$, $p = 0.6226$; Fig. 1II). Brood pheromone did not significantly affect the threshold temperature at which bees began to fan. Bees that were treated with brood pheromone fanned at 26.6 ± 2.9 °C, and bees that were not exposed to brood pheromone fanned at 24.68 ± 1.61 °C ($N = 46$: $F = 3.457$, $df = 1, 29$, $p = 0.0731$; Fig. 2II).

Seasonality

Season significantly influenced whether bees fanned. Bees were most likely to fan in the summer (brood present) or in the early spring (brood present) ($N = 60$ trials, spring-summer: $Z = -0.784$, $p = 0.433$; Fig. 1III) and least likely

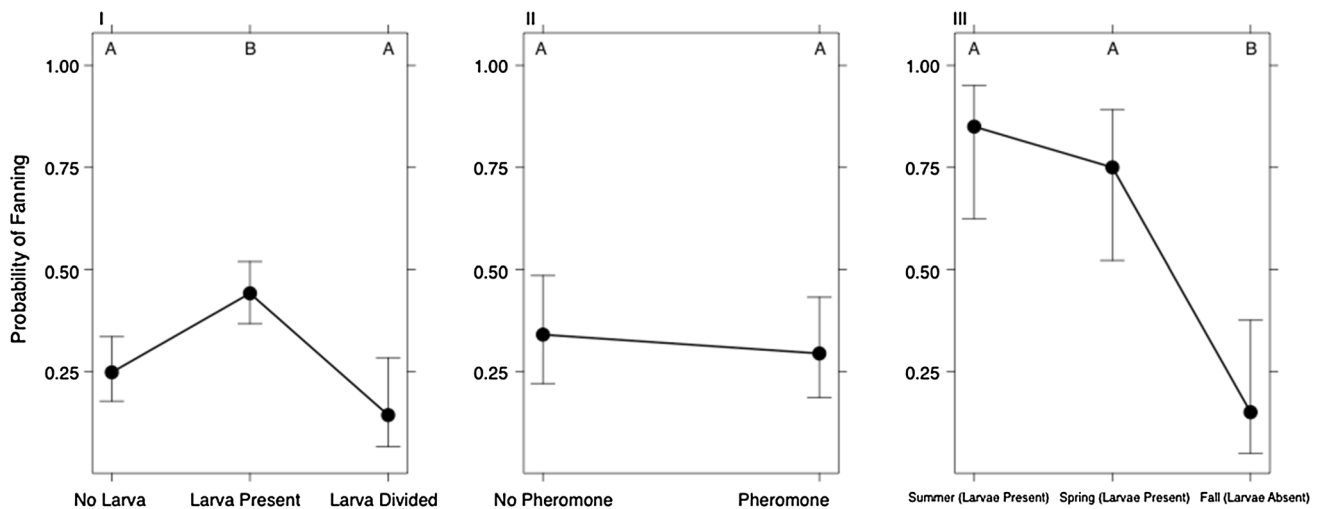


Fig. 1 Probability of fanning in honeybees. *I* shows the mean probability of fanning \pm the 95 % confidence intervals of fanning with no larva, presence with direct contact of larva, and presence but no contact with larva (divided). $N = 308$. *II* shows the mean probability of fanning \pm the 95 % confidence intervals of fanning when exposed to brood pheromone or not. There was no significant difference in probability of fanning when honeybees were exposed to brood pheromone. $N = 97$. *III* shows the mean probability of fanning

in honeybees sampled from hives with larvae present are significantly more likely to fan compared to honeybees sampled from hives without larvae present. Mean probability of fanning \pm 95 % confidence intervals across larvae presence at the hive. Probabilities are generally lower in these trials as there were 10 honeybees in the group, and honeybees are more likely to fan when they are in groups of ten compared to single bees. $N = 60$. Letters indicate statistically significant differences between groups

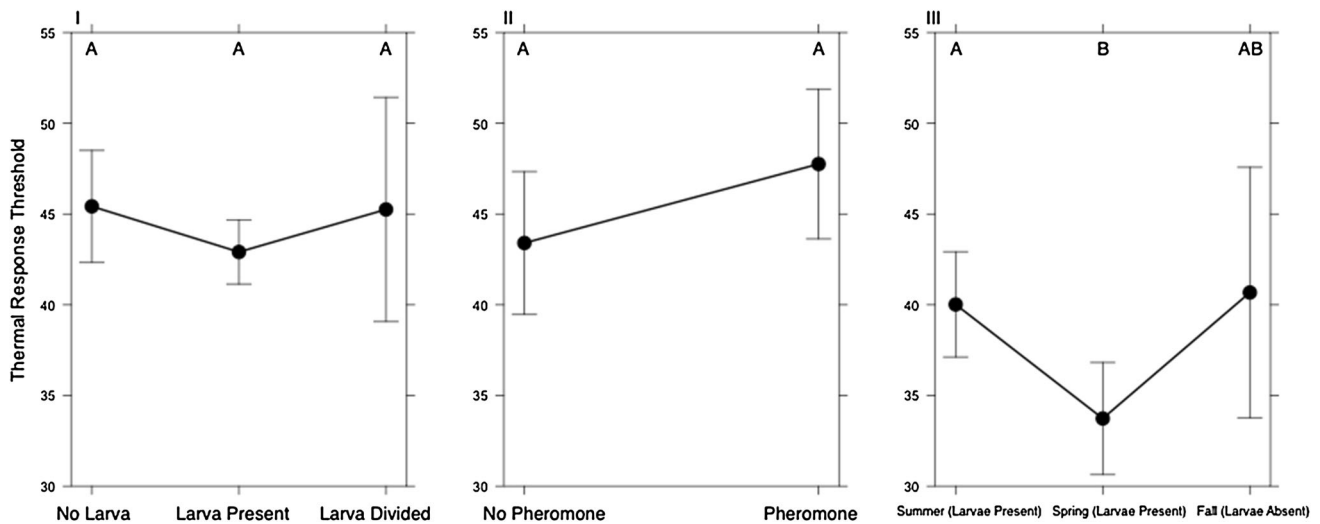


Fig. 2 Thermal response threshold of fanning. *I* shows the mean thermal response threshold \pm the 95 % confidence intervals of fanning with no larva, presence with direct contact of larva, and presence but no contact with larva (divided). $N = 308$. *II* shows the mean thermal response threshold \pm the 95 % confidence intervals when exposed to brood pheromone or not. There was no significant

difference in thermal response threshold when bees were exposed to brood pheromone. $N = 97$. *III* shows the mean thermal response thresholds \pm 95 % confidence intervals across larvae presence at the hive. Honeybees in the spring fanned at significantly lower thermal thresholds than bees in the summer and bees in the fall. $N = 60$. Letters indicate statistically significant differences between groups

to fan during the fall (brood absent) (Summer-Late Fall: $Z = -3.917$, $p < 0.0001$; Spring-Late Fall: $Z = -3.491$, $p = 0.0005$; Fig. 1III). During the fall only 15 % ($N = 20$ total trials during the fall) instances of fanning were observed, whereas 80 % ($N = 20$ total trials during the

spring) during the spring and 85 % ($N = 20$ total trials during the summer) in the summer. Bees in early spring began fanning at significantly lower thermal response thresholds than bees in the summer ($N = 40$ trials, $t = -3.021$, $p = 0.00492$; Fig. 2III), whereas there was no

significant difference in fanners between spring and late fall ($N = 40$ trials, $t = 0.179$, $p = 0.85$; Fig. 2III), and summer and late fall ($N = 40$ trials, $t = 1.87$, $p = 0.07$; Fig. 2III). This was likely because we saw so few fanners in the fall, and therefore the variance across thermal thresholds is higher.

Discussion

The thermal status of young is a critical parameter of parental care in many eusocial insects. When exposed to high temperatures, single adult honeybees in the presence of larvae are significantly more likely to fan than single honeybees with no larvae present. This shows that a cue or cues from larvae increases the probability of fanning behavior of adult honeybees. This increase in response, however, is only seen when the bee has physical contact with the larva. When a bee is near, but unable to physically contact the larva, she exhibits similar fanning behavior to that of the control solitary bee. Fanning behavior significantly decreased when physical contact between adults and larvae was eliminated under high ambient temperature conditions. However, removing physical contact between adults and larvae had no significant impact on the thermal threshold of workers. This indicates that worker's ability to make physical contact with larvae plays a role in determining the probability, but not thermal onset, of fanning behavior.

Honeybee workers communicate with each other, queens, drones, and larvae in a myriad of ways. These include pheromones (Pankiw et al. 1998), vibrations (Donahoe et al. 2003; Cao et al. 2007), and direct contact (Gordon 1989). Direct physical contact can convey several types of information, including physical condition and chemical cues. In paired honeybees, lack of worker-worker contact inhibited ovary development, which was likely due to a volatile pheromone, as one bee developed ovaries while the other did not (Dor et al. 2005).

In honeybee colonies, larvae can communicate with adults chemically. One of those chemicals is brood pheromone, which increases pollen collection in foragers (Le Conte et al. 2001). We found, however, that brood pheromone had no effect on fanning probability or thermal threshold. While brood pheromone is an important cue in communication between the developing larvae and adults (Le Conte et al. 2001), honeybees must be receiving some other cue from larvae when making thermoregulatory choices. This could be different from or in addition to the brood pheromone, and appears to be cues from the larvae that allow adults to recognize their presence, and primes workers to fan. We allowed bees to come into direct contact

with brood pheromone, as it is unknown how it is distributed throughout the hive (Pankiw et al. 1998). Brood pheromone also plays an important role in the division of labor in honeybees (Sagili et al. 2011). Sagili et al. (2011) found that even relatively low concentrations of brood pheromone decreased the age that bees began foraging. Larval cues play a distinct role in orchestrating the action of workers, even in bees that are performing jobs other than brood care.

We found no effect of the presence of larvae on thermal response thresholds with single bees in the lab. This surprised us, as other environmental changes, like the number of bees present in a treatment group (Cook and Breed 2013) and season do affect the response threshold. Probability of performing a task and response thresholds together influence division of labor in social insects (Beshers and Fewell 2001). While much work on division of labor has focused on response thresholds (Robinson 1992; Beshers and Fewell 2001), probability of performing a behavior is also important, yet often overlooked (Jeanson and Weidenmüller 2014). Cook and Breed (2013) found that worker group size affected probability of fanning as well as thermal response thresholds. Presence of larvae can have a significant effect on division of labor by altering both whether a worker bee performs fanning or not, and at the hive, at what temperature they begin to fan. This study provides further evidence that emphasizes the influence that the presence of young can have in the division of labor in social insects.

Given our lab results, we decided to evaluate whether we would see similar patterns in the field. We found that the thermal response threshold varied across seasons, which correlates with presence of larvae. This makes sense, as the larvae are the more thermally sensitive individuals in the colony (Himmer 1927; Lindauer 1955; Fahrenholz et al. 1989). Having a lower, less variable thermal threshold during the summer, when temperatures can exceed optimal hive temperatures, could help enhance thermoregulatory responsiveness. This is especially critical when larvae are developing. While these are interesting results, these are strictly correlative with presence of larvae, and suggest an interesting direction to explore a more direct influence of larvae on fanning behavior in colonies.

Eusocial insect colonies offer useful systems to study care of offspring. Individuals other than parents provide offspring care, care is performed by a group (Wilson 1971), and often times, genetic diversity of a colony can be analyzed (Simone-Finstrom et al. 2014). Our study shows that larvae directly influence honeybee thermoregulatory behavior. Further, we show that adults are assessing thermal status by tactilely disseminated cues. These results provide direction for studying the mechanisms of assessing young in thermal offspring care that can be applicable across many taxa.

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