STAY COOL: EXPLORING A GROUP PERFORMED THERMOREGULATORY FANNING BEHAVIOR IN HONEYBEES

by

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by Chelsea Nicole Cook has been approved for the Department of Ecology and Evolutionary Biology

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The final copy of this thesis has been examined by the signatories, and I find that both the content and the form meet acceptable presentation standards of scholarly work in the above mention discipline

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Stay Cool: Exploring A Group Performed Thermoregulatory Fanning Behavior In Honeybees
Thesis directed by Professor Michael D. Breed

ABSTRACT

A defining characteristic of societies is the ability of their members to perform tasks efficiently. Many proposed mechanisms by which these tasks are performed focus on jobs that can be accomplished by many individuals working independently. However most research neglects those jobs that require coordination among individuals to effectively execute the task. Thermoregulatory fanning behavior in worker honeybees (*Apis mellifera L.)* requires that coordination. Honeybees respond to increasing environmental temperatures by fanning to circulate the air in their hives and cool the colony down, as temperatures exceeding 36°C causes larval death. The goal of my dissertation is to explore the environmental, social, and physiological mechanisms that regulate this critical response. To explore fanning behavior, I developed an assay where I manipulate the environmental and social contexts to measure behavioral differences. My research shows that honeybees respond more readily to increasing temperatures when they are a part of a group, and are not likely to fan when alone. Additionally, when single honeybees are heated with a larva, they are more likely to fan than honeybees are heated alone. In both of these social contexts, I found that tactile cues are critical for the fanning response to occur; if honeybees are prevented from touching larvae or other adults, they are less likely to fan. I then manipulated the environmental context by altering the rates at which we heated groups of honeybees. I found that honeybees in groups cue in on rate of temperature change by responding faster to more quickly increasing temperatures. Finally, physiologically, I found that octopamine and tyramine play a role in regulating the fanning response. I found that fanners had lower brain concentrations of both octopamine and tyramine, and that honeybees treated with octopamine and tyramine together had a dampened fanning respond. The fanning response is critical for the survival of a colony. Furthermore, fanning is an excellent model system by which to explore the additive effects of individual and group responses to a changing environment. My research elucidates several mechanisms that control the fanning response. My dissertation increases our knowledge about honeybee behavior and expands upon our knowledge of division of labor in social insect societies.

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CHAPTER 1

INTRODUCTION

A fundamental goal of the study animal behavior research is to better understand the mechanisms by which animals interact and societies are organized. Social organization has allowed animals that live in groups to become some of the most widespread and ecologically successful organisms. While social insects make up only 2% of all insect species, they comprise over half of all insect biomass (Hölldobler & Wilson, 2009). The success of social insects is likely due to the efficiency created by partitioning of tasks among individuals such that those tasks are performed more effectively compared to solitary insects.

Task partitioning, or division of labor, is present in some form in most social species (Sherman, Lacey, Reeve, & Keller, 1995). Division of labor can be seen within small families, or among large groups of tens of thousands. On a broad scale, division of labor can be organized simply by spatial arrangement, with the individual who is closest to the unfinished task performing it, to fine control that may include accomplishing a task by coordinating as a group. While division of labor is widespread in social groups, the ways in which the labor is divided varies greatly from species to species, and can even vary from task to task within a species.

Many societies exhibit enhanced efficiency in accomplishing tasks when compared to solitary individuals. This increased efficiency is often the result of a division of labor, which occurs when tasks are partitioned among group members, and can lead to individuals becoming specialized and therefore more efficient at performing tasks

(Bourke, Franks, & Keller, 1995; Hölldobler & Wilson, 2009). Labor can be divided in small groups, as between two parents or parents and offspring (Sherman et al., 1995). Division of labor can also occur in large groups, between thousands of individuals, such as in ant colonies (Wilson, 1971). In both cases, tasks are completed more efficiently as compared to individuals who must perform all tasks alone (Hölldobler and Wilson 1990). This increased efficiency is a major advantage that had led to the success and persistence of these social groups.

Division of labor in social groups can be based on temporal polyethism, in which worker age determines task specialization (reviewed in Robinson, 1992; Camargo, Forti, Lopes, Andrade, & Ottati, 2007), dominance hierarchies, wherein rank determines task performance (Bonabeau, Sobkowski, Theraulaz, & Deneubourg, 1991; Donnell, 1998; Honk & Hoegweg, 1981; Powell & Tschinkel, 1999), and physical castes, in which worker size and/or shape specialization determines task type (Hölldobler & Wilson, 1990; Oster & Wilson, 1978). These simple mechanisms are species level characteristics that provide much of the basic framework for variation in task specialization among individuals in societies. However, because these mechanisms only focus on the larger scale of division of labor and neglect shifts that happen in response to environmental changes, they do not fully explain the variation in effort between or within colonies.

In addition to these fundamental factors affecting the division of labor, there are a number of mechanisms that facilitate adjustments in work allocation in response to factors like colony ontogeny, seasonality, and environmental stressors such as low food

availability, drought, or pressure from predators and parasites. Key regulatory mechanisms include individual variation in response thresholds to tasks (Page , Erber, Fondrk, & Page, 1998), information feedback loops (Seeley, 1982), “foraging for work” (Pinter-Wollman, Wollman, Guetz, Holmes, & Gordon, 2011; Tofts & Franks, 1992; Tofts, 1993), genetic variation among workers (Jones, Myerscough, Graham, & Oldroyd, 2004), and nutritional status of workers (Toth, Kantarovich, Meisel, & Robinson, 2005; Toth & Robinson, 2005). Depending on the species in question, several of these factors may interact to predict the behavior of workers.

Honeybees are a model system with which to study division of labor. Honeybees have distinct reproductive castes where workers are essentially sterile, and the reproductive queen is the only fertile female in the colony (Winston, 1991). Among the worker caste of honeybees exists a complex division of labor (Ratnieks & Anderson, 1999; Seeley, 1982). This division of labor is temporal, where workers change tasks over their lifetime, which is about 30 days. The youngest honeybees perform tasks inside the colony, like larval care. The oldest bees are foragers (Winston, 1991). Between these two main task groups lies a group loosely defined as “middle aged bees”. These bees perform several tasks, including guarding (Breed, Robinson, & Page, 1990), undertaking (Moore, Breed, & Moor, 1987), and fanning (Egley & Breed, 2013). Variation among workers in response threshold, genetics, nutritional experience, and hormonal status may play particularly key roles in driving task specialization in worker honeybees (Beshers & Fewell, 2001; Johnson, 2010; Pankiw & Page, 2003).

Thermoregulation is a critical task that must be performed to ensure colony success. Honeybees thermoregulate in both cold and hot ambient temperatures. If hive temperatures exceed 36°C, the larvae inside will develop malformations (Himmer, 1932). Several behaviors contribute to thermal cooling, including heat shielding (Siegel, Hui, Johnson, & Starks, 2005; Starks & Gilley, 1999), foraging for water that is then used for evaporative cooling (Kühnholz & Seeley, 1997), and fanning to circulate air and remove excess heat (Egley & Breed, 2013). To perform fanning, honeybees will form a group at the entrance and fan their wings. This behavior results in the influx of cooler air. To effectively circulate air to cool the hive, a group of several bees is often needed. If ambient temperatures drop, bees will shiver to generate metabolic heat (Heinrich & Esch, 1994; Starks, Johnson, Siegel, & Decelle, 2005) and will press their abdomens onto or enter brood comb to more effectively spread the heat generated (Kleinhenz, Bujok, Fuchs, & Tautz, 2003). Precise thermoregulation of the colony is necessary for functioning and reproduction of the colony. While some work has been done on this critical behavior (Jones et al. 2004) the social context or the exact mechanisms that influence fanning behavior has yet to be explored.

Using a response threshold model, my second chapter defines the social context of the fanning response. I test the hypothesis that if honeybees are using response thresholds, they will fan when by themselves. To test this hypothesis, I collected a single fanning honeybee and heated her in a behavioral assay that my advisor and I designed. I show that the fanning response is not simply a response to a thermal threshold, as a single honeybee rarely fans. I then repeated this assay with groups of three bees and

groups of ten bees. I found that honeybees are significantly more likely to begin fanning, and that they will fan at lower thermal response thresholds when being heated in groups of ten bees, compared to bees heated in groups of three, or bees heated by themselves. I also collected honeybees in different task groups to evaluate the fanning response in honeybees other than fanners. I collected nurses, guards, and foragers, in addition to fanners. I also show that across behavioral task groups, honeybees exhibit different probabilities of fanning. Fanner honeybees are most likely to fan, as are guard bees. Nurses are next likely to fan. Pollen foragers are the least likely to begin fanning. This chapter, which was published in *Animal Behaviour* in 2013, sets the stage to allow me to more deeply explore the fanning response in other contexts.

Thermoregulation is critical for the development of larvae in a honeybee colony. If temperatures exceed 36°C the developing larvae inside can die (Himmer,1932; Martin Lindauer, 1952). However, the fanning response has been unexplored in the context of larvae. In my third chapter, I test the hypothesis that a honeybee will be more likely to fan in the presence of a larva than when she is by herself. I do this by employing a similar technique of collecting a single fanner honeybee, and placing her with a larva from the same colony. I found that a single honeybee is significantly more likely to fan with a larva than when she is alone. Furthermore, I found that a single bee was more likely to fan if she had direct contact with the larva than when she was prevented from touching it. This led us to hypothesize that perhaps a tactile pheromone could be inducing the fanning response. I then used one of the most well studied honeybee larval pheromones, brood pheromone (Le Conte, Mohammedi, & Robinson, 2001; Pankiw, Page, & Fondrk, 1998;

Sagili, Pankiw, & Metz, 2011a), to test this hypothesis. I found that brood pheromone did not increase the fanning response. While brood pheromone didn't seem to be playing a role in the worker honeybee fanning response, there could be many other cues from larvae that workers can be using to know when to fan. This paper is currently in review at *Insectes Sociaux*.

Honeybee thermoregulation is crucial for the survival of the colony. While honeybees are incredibly effective at maintaining nest temperatures between 33°C-35°C, I wanted to know if the rate of temperature change influenced the fanning response. In chapter 4, I hypothesize that honeybees would be more likely to begin to fan when the temperature is increasing quickly, compared to if the temperature was increasing slowly. To test this, I heated groups of ten, three, and single bees at slow (0.5°C/minute), medium (1°C/minute), and fast (2°C/minute) rates. I found that indeed, honeybees are more likely to begin fanning and fan at lower thermal response thresholds when they are heated rapidly, as compared to more slowly. Surprisingly, I only saw this response in groups of ten, but not in groups of three or solitary bees. Furthermore, I found that bees that were collected from hotter ambient temperatures at their hive were more likely to fan than bees that were collected at cooler ambient temperatures, but again, this was only seen in groups of ten bees. I show that honeybees in larger groups are cuing in on rate of temperature change and behaving to buffer that rapid increase. This paper is currently in review at *Animal Behaviour*.

In group-performed tasks, individuals must share information, either directly or indirectly, to effectively perform the behavior. Honeybees are more likely to fan in a

group (Cook and Breed 2013), which indicates that the interactions between individuals are passing important information, which may influence the fanning response. In chapter 5, I test the hypothesis that if honeybees are prevented from interacting in certain ways, the fanning response will be affected. I used a technique (Mann & Breed 1997; Katzav-Gozansky et al. 2004; Dor et al. 2005) to separate honeybees with single mesh, where they could slightly touch, see, perceive pheromones, & feel vibrations, and double mesh, where they could no longer touch but still see, perceive pheromones and feel vibrations. I found that honeybees that are separated, regardless of whether they can touch slightly or not at all, are less likely to fan and begin fanning at lower temperatures, compared to non-divided cages. These divided cages also disrupt the airflow that occurs when honeybees begin to fan. I then hypothesized that airflow could be a potential cue in the fanning response. To test this, I created cages that were divided with different sized mesh, so that different amounts of air would flow through the holes. Again, I found that no matter how much airflow could pass through, honeybees were still less likely to begin fanning compared to non-divided cages. Finally, I also show that honeybees will fan in both groups of nestmates and groups of non-nestmates, supporting previous research (Couvillion et al. 2013) that nest defense is less important away from the hive. Overall, this chapter begins to unravel the complex communication that honeybees seem to be utilizing to know when to fan.

Finally, exploring the proximate mechanisms that cause the fanning response can help us understand the physiological underpinnings of this behavior. Physiological mechanisms of behavior have been well explored in individual honeybees (Fussnecker,

Smith, & Mustard, 2006b; Lehman et al., 2006; Pankiw & Page, 2003; Sagili et al., 2011a; Schulz & Robinson, 1999; Schulz & Robinson, 2001b), but the implications of how these individual changes may be influencing group behavior have yet to be fully explored. In chapter 6, I test the hypothesis that fanner honeybees have different concentrations of biogenic amines than bees performing other tasks. To explore this, I use high-pressure liquid chromatography to explore the differences in 4 biogenic amines: octopamine, dopamine, tyramine, and serotonin. I compared these biogenic amines induced fanners in the lab to non-induced honeybees that were collected as fanners at the hive, brought into the lab, while one group was heated and the other was not. I also compared the biogenic amines of guards and fanners, as they are in similar environments and are of similar age. I did not find a significant difference in any amines between guards and fanners, but I did measure a significant drop in octopamine and tyramine in induced fanners compared to non-induced honeybees. I then wanted to verify that these biogenic amines were actually causing a difference in behavior. If they were, I hypothesized that fanners treated with these amines together would be less likely to fan, as higher amounts may be inhibiting the behavior. I treated groups of honeybees by feeding them with octopamine, tyramine, and octopamine with tyramine, along with two controls. In this blind experiment, I found that indeed, the octopamine with tyramine treated bees were significantly less likely to fan than any of the controls. Finally, I measured differences across the lifetime of the honeybees, and found expected differences in some biogenic amines, as based on previous work (Bateson, Desire, Gartside, & Wright, 2011; Farooqui, 2007; Giray et al., 2015). This chapter helps us to

understand how physiological changes in individuals can have implications in group responses. This also adds to the literature on the proximate mechanisms that play a role in the division of labor of eusocial insects.

In all, my dissertation explores a very important thermoregulatory behavior in honeybees. Not only is this behavior critical for the survival of the colony, but it also serves as a model system by which to study the interaction of individual and group behavior. This research helps is to understand how societies respond to environmental perturbations, and also how these group responses emerge from a physiological and individual level. Throughout my dissertation, I have created a new behavioral assay that is effective yet accessible to study the fanning response. I have also added to the literature on the honeybee, an invaluable pollinator. Overall, understanding more about how societies are organized can not only help manage honeybees more effectively, but can be used as a framework by which to study many complex biological systems and societies, including our own.

CHAPTER 2

Social Context Influences The Initiation And Threshold Of Thermoregulatory Behaviour In Honeybees[1]

2.1 ABSTRACT

Interactions between individuals in a society are the basis of effective task allocation. Division of labor plays a critical role in the ecological efficiency of social insect societies. In this study I test whether social context, specifically the number of workers present, affects thermoregulatory task performance in honeybees, *Apis mellifera*. I report here that worker bees assayed singly are significantly less likely to initiate fanning behaviour in response to elevated temperature than bees assayed in small groups of three or ten workers. Bees assayed in groups also exhibit lower response thresholds than those assayed alone. The likelihood for fanning behaviour varies significantly among behavioural castes, while thermal response thresholds do not. These results suggest that worker task performance depends on the presence of other workers, and offer another method by which division of labor in societies is organized.

[1] This paper was published in Animal Behaviour with Michael D. Breed

[2] This paper is currently in review in *Insectes Sociaux,* including co-authors Sharif A. Durzi, Kelsey Scheckel, and Michael D. Breed.

2.2 INTRODUCTION

A defining feature of an animal society is the constant interaction among its members. These interactions are crucial to the organization of work and transmission of information within the society. In social insect societies, worker activities are coordinated so that the work is accomplished in an efficient manner (Wilson, 1976). The mechanisms underlying this coordination include temporal polyethism, in which worker age determines task specialization (Camargo et al., 2007; reviewed in Robinson, 1992), dominance hierarchies (Bonabeau, Sobkowski, Theraulaz, & Deneubourg, 1991; Donnell, 1998; Honk & Hoegweg, 1981; Powell & Tschinkel, 1999), wherein rank determines task performance, and physical castes, in which worker size and/or shape specialization determines task type (Hölldobler & Wilson, 1990; Oster & Wilson, 1978). These simple devices are species level characteristics that provide much of the basic framework for variation in task specialization among individuals in societies. However, these models do not fully explain variation in effort between colonies or variation within colonies of social insects.

In addition to these fundamental factors affecting the division of labor, there are a number of mechanisms that facilitate adjustments in work allocation in response to factors like colony ontogeny, seasonality, and environmental stressors such as low food availability, drought, or pressure from predators and parasites. Key regulatory mechanisms include variation in response thresholds to tasks (Page et al., 1998), information feedback loops (Seeley, 1982), “foraging for work” (Pinter-Wollman et al., 2011; Tofts & Franks, 1992; Tofts, 1993), genetic variation among workers (Jones et al.,

2004a), and nutritional status of workers (Toth et al., 2005; Toth & Robinson, 2005). Depending on the species in question, several of these factors may interact to predict the behavior of workers. Variation among workers in response threshold, genetics, nutritional experience, and hormonal status may play particularly key roles in driving task specialization in honeybees (Beshers & Fewell, 2001; Johnson, 2010). Here, I test the novel hypothesis that social context, i.e. the number of conspecifics present, influences the division of labor of thermoregulatory behavior in honeybees, *Apis mellifera*.

Honeybees maintain a relatively constant temperature of 36°C within their colonies when rearing brood (Fahrenholz, Lamprecht, & Schricker, 1989; Himmer 1927; Lindauer, 1955). In the winter, when brood is absent, temperature is also regulated (Kronenberg & Heller, 1982; Stabentheiner, Kovac, & Brodschneider, 2010; Stabentheiner, Pressl, Papst, Hrassnigg, & Crailsheim, 2003). Several behaviors contribute to thermal regulation, including fanning to circulate air and remove excess heat (Parkin & Cohen, 2001), heat shielding (Siegel et al., 2005a; Starks & Gilley, 1999), and foraging for water that is then used for evaporative cooling (Kühnholz & Seeley, 1997). In colder ambient temperatures, honeybees shiver to produce thermal energy (Heinrich & Esch, 1994; Starks et al., 2005) and will press their abdomens onto the surface of brood comb or even enter cells to more effectively spread the heat generated (Kleinhenz et al., 2003). Honeybees also regulate carbon dioxide (Seeley, 1974) and humidity (Human, Nicolson, & Dietemann, 2006). For an overview of social

insect thermoregulation see Jones & Oldroyd (2007). The thermoregulatory behavior on which I focus in this study is fanning (Egley & Breed, 2013).

Fanning behavior is best studied in bumblebees (Heinrich, 1993). Recent work by Duong & Dornhaus (2012) in *Bombus impatiens* found that worker responsiveness, in terms of threshold for initiation of fanning behavior, did not change with age or experience. This differed from the findings of Weidenmuller (2004) and Westhus et al. (Westhus, Kleineidam, Roces, & Weidenmüller, 2013) in *Bombus terrestris* in which experience decreased thermal response thresholds. Gardner et al. (2007) studied colony thermoregulation by workers and found that nest climates were more consistently maintained when brood was present. Engels et al. (1995) found a similar mechanism for nest temperature regulation, including fanning, in a stingless bee, *Scaptotrigona postica*. Fine thermoregulatory control is crucial for survival in many social insects, and it is important to understand all mechanisms by which this may be happening.

I first tested the hypothesis that honeybees respond to a thermal threshold to commence fanning behavior. I then tested the hypothesis that bees are more likely to fan when in groups than when solitary. I also tested whether the thermal fanning threshold decreased as group size increased. Because I could identify distinct behavioral task groups among honeybee workers (nurses, guards, entrance fanners and foragers), the final experiment tested whether these task groups differ in their probability of fanning and thermal thresholds. Taken together, the results from this study

address how individual behavioral thresholds can interact with social context to shape division of labor in social insects, as well as in animal societies in general.

2.3 METHODS

General

Ten *Apis mellifera L.*, "Italian" colonies on University of Colorado campus were used for these experiments. Colonies were maintained in 10-frame wooden hive bodies with plastic frames. Supplemental feeding of a 1M sucrose solution was performed at the beginning of the season due to dry conditions. All experiments were conducted between May 1st and October 1st, 2012.

Collection/Task Groups

These experiments required bees from four distinct task groups: nurses, guards, fanners and pollen foragers, which were defined using established behavioral criteria, described below. The focus was on behavioral role, rather than chronological age of the bees. Behavioral castes were determined by observing the behavior of bees in colonies.

Nurses were identified as a bee seen with her head in a brood comb cell. This method follows the methods of Sakagami (1953), Huang et al. (1994), and Wagener-Hulme et al. (1999). While it is possible that not all bees I categorized as nurses were providing care, for the purpose of identifying nurses I felt this method was reasonable.

Guards were identified as a subset of the bees on the entrance landing board. Guards exhibit a distinctive posture with their wings spread and the their abdomen slightly tilted upward. They are also active in examining incoming bees. This method of

identifying guards has been used extensively in studies of this task group. Moore et al. (1987) gave a detailed description of guard behavior and subsequent studies include Downs & Ratnieks (2000), Hunt et al. (2007), and Pacheco & Breed (Pacheco & Breed, 2008). Breed et al. (2004) reviewed defensive behavior of honeybees and give an overview of the role of guards in honeybee colony defense.

Fanners were also a subset of the bees collected on the entrance landing board. These bees fan their wings to ventilate the colony. Their distinctive posture and orientation relative to the entrance distinguished them from foragers that might briefly fan before departing, bees signaling using their Nasanov gland (Free, 1987) or other defensive behaviors that may occur in the presence of intruders (Yang, Radloff, Tan, & Hepburn, 2010). Egley and Breed recently described entrance fanning for ventilation in honeybees (2013). For this study I identified a bee as a fanner only after it had performed the fanning behavior for at least ten seconds. I recognize that bees in other locations in the colony may also fan for ventilation purposes, but I focused on entrance fanners because they were easily collected in a field context, and Egley & Breed (2013) suggest that entrance fanners are relatively uniform in age.

I used one type of forager, **pollen foragers**, in this study. Pollen foragers are easily identified because they fly back to the nest with corbiculae (pollen sacs) full of pollen (Huang et al.,1994; Pankiw & Page, 2001; Wagener-Hulme et al., 1999). Excluding other forager types in this way reduces the task variance among bees in the experiment, as nectar foragers may represent a broader range of ages than pollen foragers (Pankiw & Page, 2001). Also, nectar foragers are difficult to identify without

expressing the crop contents, a method that may disrupt subsequent behavior. Bees returning to the colony without pollen loads include nectar foragers, water foragers, guards that have made short flights, and younger bees on orientation flights. To collect pollen foragers, I used steel mesh placed over the colony entrance to keep bees from entering the colony. Pollen foragers were then easily identified and collected.

Treatment groups

The experiments required isolation of one, three, or ten bees for testing in the laboratory. For any given replicate, each isolated individual or group came from the same task group and hive. Thus I had, for example, single isolated guards, guards in groups of three and guards in groups of ten. I collected bees opportunistically, as I observed a bee performing one of the focal tasks

I collected bees from a chosen task group one at a time using forceps, and placed them into a mesh cage (4cm x 4cm x 4cm). During collection, I randomly placed bees into the three treatment groups of individuals, three or ten bees. I then transported them back to the laboratory. Time and date of collection were recorded at this time. The sample size was 20 of each of the treatment group sizes for each task group and I attempted to maintain approximately equal colony representation across task group and treatment group size. The overall sample size was 240 treatment groups.

Temperature Regime and Behavioral Assay

The overall experimental design assessed the frequency of fanning and the temperature at which fanning was initiated in the treatment groups. The mesh cage

containing the bees was placed into a two-liter glass container (9cm x 24cm), which sat on a heating unit. The bees were allowed to acclimate for 25 minutes, which was chosen based on the amount of time required in preliminary trials for the activity level of the bees to stabilize. After the acclimation period, I began to increase the jar temperature at a rate of 1°C per minute, starting at room temperature (an average of 28°C). Temperatures were taken at approximately the center of the jar using a Cole Parmer high accuracy (± 0.3°C) digital thermometer probe that was fed through a fitted hole through the top of the jar.

Bees were observed continuously during the heating regime. Fanning during the heating regime was characterized as an individual standing still but fanning her wings for at least 10 seconds; this is the same criterion applied when entrance fanners were collected in the field. I recorded the initial temperature at which any individual bees fanned (hereafter, “thermal threshold”) and the proportion of bees fanning in a treatment group. I use the first temperature at which bees fanned as the threshold because 1) I wanted to focus on the initial response of the bees and 2) typically, once a bee fanned, others either joined in during that initial bout or did not fan during the entire trial.

Statistical Analysis

I used a generalized linear mixed model to analyze both probabilities of fanning and thermal thresholds. Both models used four task groups and three group sizes (one, three or ten bees), both categorical factors. Group size and task group are fixed effects, while colony is a random effect. This representation of colonies was important to control

for colony level effects, so colony was included in the statistical analysis as a random effect.

I used a binomial error distribution (link=logit) for the probability analysis and I used a two-column response variable of number of bees that did fan and the number of bees that did not fan in the group. I used a Gaussian error distribution for the temperature threshold analysis, as the response variable was temperature. I started with a full model to examine two-way interactions between treatment group size and task. I used backward selection; therefore, when an interaction was not significant (alpha=0.05), it was dropped from the model and the model was re-run. To explore the magnitude of the effects of each treatment variable, I performed a type II ANOVA (Wald chi square tests), and for each of the significant main effects, I performed a post hoc (Tukey) analysis. I used R version 2.15.0, for all data analysis (R Development Core Team) and library lme4 for generalized linear mixed model analysis (Bates, Mächler, & Bolker, 2014).

2.4 RESULTS

Probability of fanning depends on group size

Group size was a significant predictor of probability of fanning (Table 1). Worker honeybees were significantly more likely to fan in groups of 10 compared to when they are singly assayed (Tukey: z=3.088, p=.00533, Figure 1). 15 out of 80 (18.8%) of the bees fanned while isolated, 79 out of 240 (32.9%) in groups of 3, and 381 out of 800 (47.6 %) in groups of 10. Fanning was observed in 68 out of 80 trials of groups of 10, compared to 15 out of 80 in isolated bees, and 45 out of 80 trials in groups of three.

Model Term	ChiSq	df	p-value
Group Size	24.917	2	**0.000003885**
Caste	44.248	3	**0.000000001337**
Group Size * Caste	23.94	6	**0.0005357**

Table 2.1: **ANOVA table (Type II Wald Chi Square) testing the factors influencing probability of fanning**
Probability of fanning is the response variable. There were a total of 240 observations from three group size treatment groups and four behavioral caste groups. Hive is controlled for as a random variable. Only significant interactions are shown.

Model Term	ChiSq	df	p-value
Caste	4.3029	3	0.2306
Group Size	27.1107	1	**0.00000214**

Table 2.2: ANOVA table (Type II Wald Chi Square) testing the factors affecting thermal threshold for fanning behavior

The response variable is thermal threshold in degrees Celsius. Data were collected from 240 observations, which included three treatment group sizes and four behavioral caste groups. Hive is controlled for as a random variable. Interactions were tested but were not significant, so they are not included in the final model.

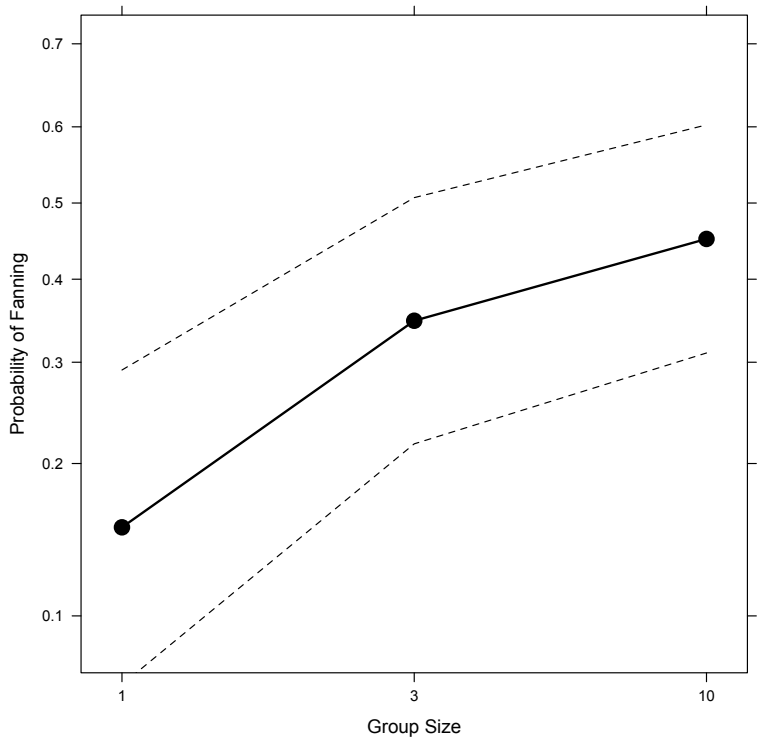


Figure 2.1: The probability of fanning as related to group size. The points are averaged probabilities, while the dotted lines are 95% confidence limits. Letters indicate significance as analyzed in a post hoc (Tukey) test.

Thermal threshold depends on group size

Group size was the only significant predictor for thermal threshold (Table 2). As group size increased, thermal threshold significantly decreased (Figure 2). Bees in groups of ten, on average, fanned at 38.92°C (SE= 0.766, N=68), a significantly lower temperature than bees in groups of three, which fanned at 42.56°C (SE=0.83; Tukey: z=-2.951, N=42, p=0.00849). Bees in groups of ten also fanned at a significantly lower temperature compared to single bees, which initiated fanning at a mean temperature of 47.97°C (SE=.524; Tukey: z=-4.820, N= 15, p=.0001). Additionally, bees in groups of three fan at a significantly lower temperature compared to single bees (Tukey: z=-2.699, p=0.01826).

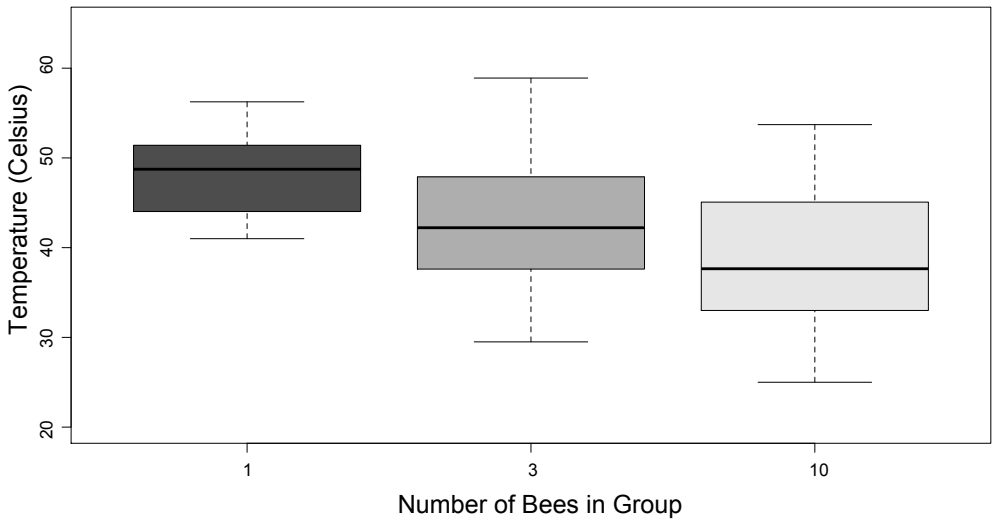


Figure 2.2: Thermal fanning threshold by treatment group size. The boxplots show median, quartiles, and range (there were no outliers). Letters indicate significance as analyzed in a post hoc (Tukey) test.

Probability of fanning depends on caste

Behavioral caste was a significant predictor for fanning probability (Table 1). Across all treatment group sizes, fanners were the most likely to fan (Tukey: z=3.795, n=60, p<0.001), while foragers are the least likely (Tukey: -6.636, n=60, p<0.001; Figure 3). The ANOVA based on the generalized linear model also showed a significant interaction between group size and caste (Table 1). This is because foragers did not have an increased fanning probability while in the treatment groups of 10 (GLMM: z=-2.219, p=0.02652). No other group size – caste pairwise interaction showed a significant effect.

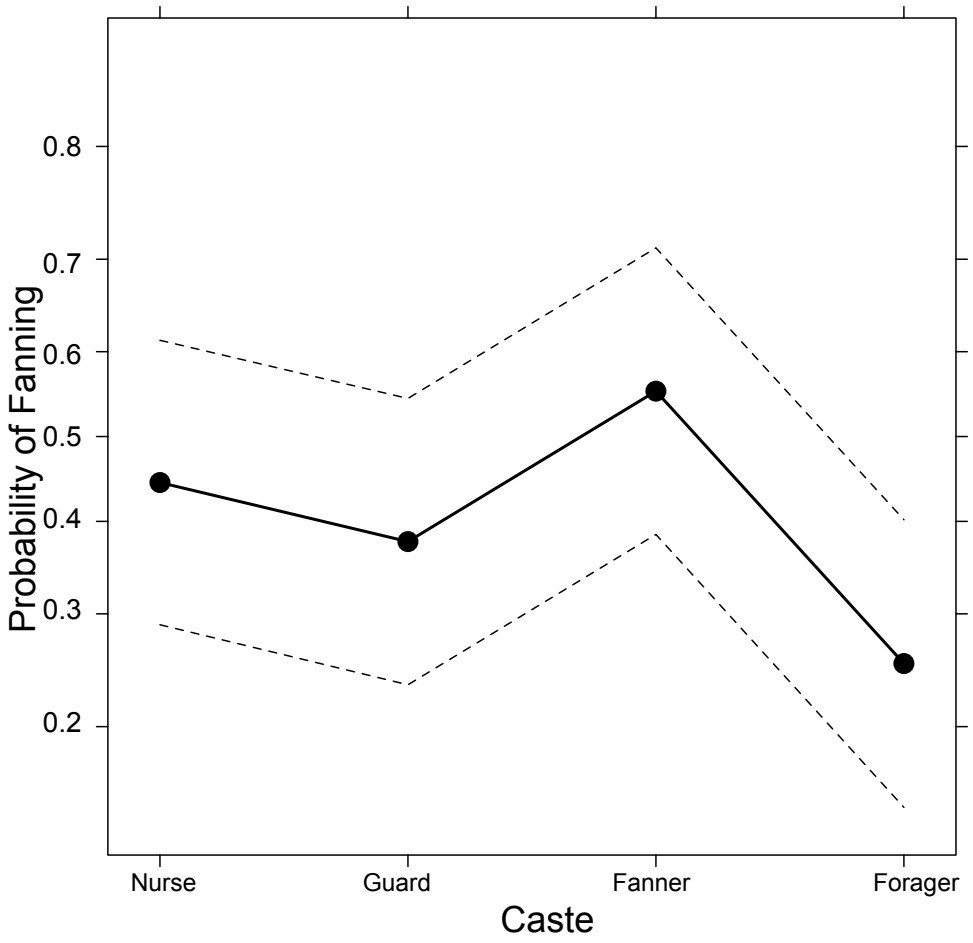


Figure 2.3: Probability of fanning as related to caste. All treatment group sizes are included. Points are averaged probabilities, while dotted lines are 95% confidence limits. Letters indicate significance as analyzed in a post hoc (Tukey) test.

Thermal threshold depends on task group

A generalized linear model reveals no significant effect of behavioral caste on thermal threshold, however there is a trend for a higher threshold for foragers (Figure 4). Further analysis of these data by isolating the thermal threshold data by caste found that there was indeed no significant effect of caste on thermal threshold. I then analyzed only treatment groups of ten bees, as this type of group was where I had observed the

most fanning behavior. Foragers in groups of ten fanned at the highest mean temperature, 42.08C (SE±1.44, N=16). Conversely, nurses fanned at 36.84C (SE±1.30, N=20), with guards (n=15) and fanners (n=17) in between at 37.07C ± 1.68 and 40.02C ± 1.54, respectively, but these differences were not significant.

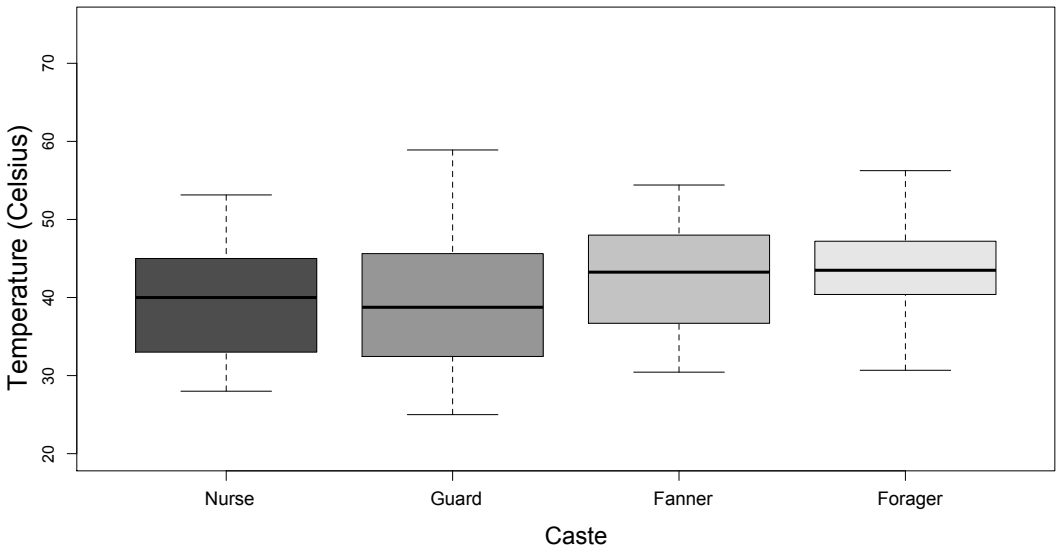


Figure 2.4: Average thermal fanning thresholds by caste. Boxplots show median, quartiles, and range (there were no outliers). Letters indicate significance as analyzed in a post hoc (Tukey) test.

2.5 DISCUSSION

My results show that social context influences worker bee performance of a critical thermoregulatory behavior. Honeybees in groups are significantly more likely to initiate fanning than bees that are alone. Bees in these small groups also exhibit significantly lower thermal thresholds than isolated bees. While fanners are most likely to fan, foragers are least likely to fan. Effects of probability of fanning within behavioral

castes are independent of thermal threshold, as there was no significant effect of caste on fanning threshold. These results suggest that social context may play a more important role in the division of labor in societies than previously believed.

At the colony level, honeybees exhibit a thermal response threshold at which fanning behavior commences (Jones et al. 2004). In previously published work this conclusion was based on observations of bees in entire colonies (Egley & Breed 2013). The finding that the response threshold for fanning is dependent on the presence of other bees is, to my knowledge, unique in studies of honeybee division of labor. This result suggests that response thresholds for other behavioral tasks should be examined to determine if expression depends on social context. Furthermore, Pacala et al. (1996) found that, compared to small groups, larger groups were more efficient at tracking changing environments. These results are consistent with the theme that worker interaction rates are important in division of labor (Fewell, 2003; Gordon, 1989).

My results also show that there was a significant difference in probability of fanning across castes. For all treatment group sizes, foragers had the lowest probability of fanning, while fanners had the highest. Specifically, even foragers in larger groups were less likely to fan than the other groups. While castes differed in their probability of fanning, they did not differ in their thermal response thresholds. In the context of crucial hive behaviors, this makes sense; individuals can vary in the likelihood that they will perform some important behavior, but to effectively accomplish the task, the ones that perform the behavior must be coordinated at some level. Given the highly efficient nature of colonial thermoregulation in honeybees, these results provide further evidence

of this coordination (Southwick & Moritz, 1987). A future direction may be to explore thresholds and probability of fanning in reserve bees that aren't seen performing a specific task (Johnson, 2002).

Environmental cues, such as temperature and concentration of gasses, play a key role in influencing fanning behavior (Egley & Breed, 2013; Seeley, 1974), as indicated by bees fanning before a destructive temperature or CO_2 concentration is reached. However these results may indicate the ability for worker bees to use the presence of other individuals to evaluate if and when they should perform a task. Honeybees have an extensive social communication repertoire, including pheromones (Pankiw & Page, 2003), vibrations from other bees (Donahoe, Lewis, & Schneider, 2003), and antennal contact with others (Cao, Hyland, Malechuk, Lewis, & Schneider, 2007; Gordon, 1989). These results suggest that worker bees utilize both environment and social cues when making behavioral decisions, although my data leave open the question of what cues are eliciting fanning, or whether bees actually make an assessment of the number of bees around them.

Other studies have investigated the role that the presence of other individuals has on behavior. Ruel, Cerdá, & Boulay (2012) found that below a critical number of workers in the colony, a queen was not likely to be replaced if she was lost. They also found that smaller and larger colonies produced around the same number of queens, however the larger colonies had a better chance at rearing a successful new queen (Ruel et al., 2012). Nest size and caste ratio has also been implicated in division of labor among workers. Individual workers are seen doing more specialized tasks in larger

colonies, suggesting that a more strict division of labor becomes established as group size increases (Holbrook, Barden, & Fewell, 2011). Another study also found that in smaller colonies, foragers spent more time looking for food and eating alone, while workers in larger colonies were more likely to recruit others when they located a food source (Burkhardt, 1998). Alternatively, Sempo and Detrain (2010) found that there were no significant change in behavioral repertoire or activity level in major workers when minor workers were depleted, although they do speculate that regulation of colony function may take place as active workers switch to accomplish tasks important for colony survival. These studies indicate that workers in social groups can alter their behaviors based on the number of individuals around them, and perhaps based on colony need.

Honeybee division of labor appears to be largely structured by age (reviewed in Robinson, 1992), response thresholds to stimuli (Robinson, Page, Strambi, & Strambi, 1989), and the physiological state of individual bees (Toth & Robinson 2005, Toth et al. 2005). In these models, task choice depends more on the priming of workers for task performance than on physical proximity to work that needs to be done. In contrast, a "foraging for work" model for division of labor suggests that workers should engage tasks that need performing based on their physical proximity to the site of task performance (Tofts & Franks 1992, Tofts 1993, Pinter-Wollman 2011). I found that honeybee workers shared thermal threshold regardless of the task they were performing when collected. These results suggest that fanning may not be a distinct task for a specialized group of bees, and workers can switch from other tasks to fanning as

needed. This more closely fits a foraging-for-work model for division of labor than the temporal and genetic models often thought to apply to honeybees.

Honeybees exhibit fanning behavior in a variety of locations within the nest. A recent study (Egley & Breed 2013) found that fanners at the colony entrance often transition to guarding and that the frequency of fanning is correlated with ambient temperature. While Egley & Breed (2013) treated entrance fanners as a distinct group, fanning in other spatial contexts may not fit this model. These results indicate that members of tested behavioral castes can perform fanning, although the bees that I behaviorally labeled as fanners were more likely to fan than the rest of the castes I distinguished. Further experimental test are needed to understand the specific role fanning behavior plays in the division of labor.

Studies have not explored the effect of learning in honeybee thermoregulation, though this has been explored in different species of bumblebees. Duong & Dornhaus (2012) found that *B. impatiens* workers had no change in temperature threshold if the bees have previously fanned, indicating that the bees do not use a self-reinforcement model for thermal threshold. However, they conclude that the differences in observed response thresholds could be due to an increased probability of performing a given task, instead of an exhibited variation in perceived thermal threshold for a perceived stimulus (Duong & Dornhaus 2012). This high variation of fanning threshold among the bees likely allows the colony to more efficiently thermoregulate than if there was little variation among fanners (Jones et al. 2004, Jones & Oldroyd 2007). Additionally, O'Donnell &

Foster (2001) found that although *Bombus bifarius nearcticus* workers did differ in their thresholds, they did not seem to specialize in thermoregulation.

Furthermore, while social learning has been extensively studied in social animals (reviewed in Galef & Laland, 2005), social influence has not. Social influence is when behavior is altered by the presence of conspecifics (Whiten & Ham, 1992). While task specialization can be socially induced in solitary bees (Jeanson et al., 2008), the increase in efficiency of a particular task based on the interaction of nest mates has not been explored in the context of social influence. Webster & Fiorito (2001) further parse out social influence into several more specific categories, including social facilitation and social support. Social facilitation involves the initiation of a behavior based on a conspecific performing the behavior, while social support posits a situation where simply the presence of another individual is enough stimuli to trigger a change in its motivational state (Whiten & Ham 1992, Webster & Fiorito 2001). Further experiments are needed to explore whether the initiation of fanning is being induced because of social support or because of social facilitation.

The bees used in this study were removed from their normal nest environment, a procedure that could affect their behavior. Removals in this way are very much a part of the experimental procedures used in studies of behavioral thresholds, with a large literature having developed around assays of sucrose response thresholds (integrated sometimes with olfactory thresholds) in single harnessed bees (Pankiw & Page, 1999; Scheiner, Page, & Erber, 2004). The behavior of bees in my assays corresponded well to the observed behavior of fanning bees in colonies, with the typical thermal thresholds

in my assays corresponding to thermal responses of fanners at colony entrances documented by Egley & Breed (2013).

If response thresholds are being considered as organizing features, or factors that drive self-organization in societies, then the social context in which the threshold is measured must be considered. For fanning by worker honeybees, these results show that shifting thermal threshold and group size effects could have non-linear outcomes in models of labor allocation. A number of studies have addressed how the rate of social interactions affect behavior and task performance in social insects (Cole & Cheshire, 1996; Pinter-Wollman et al., 2011). This study differs in that I explore how social context can alter the methods by which individuals respond to environmental stimuli. While it is unclear what social cues operate in the honeybee system, the social modulation of response thresholds should be explored in other behavioral contexts and in other social insect species. These results also provide insight into how analogies might be drawn between social insect societies and the importance of social awareness in other animal societies.

CHAPTER 3

Larvae Influence Thermoregulatory Fanning Behavior in Honeybees (*Apis mellifera l.*)[2]

3.1 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, I explore the thermoregulatory fanning behavior in honeybees performed to maintain colony temperatures in the presence of larvae. I found that honeybees are more likely to fan when larvae are present, but need direct contact with larvae to fan. I found no evidence that exposure to brood pheromone plays a role in stimulating fanning behavior. Finally, I 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.

[2] This paper is currently in review in *Insectes Sociaux,* including co-authors Sharif A. Durzi, Kelsey Scheckel, and Michael D. Breed.

3.2 INTRODUCTION

For many species, care of offspring is a critical component of offspring survival. This care comes in many varieties and can range from choosing where to lay eggs, to defending young, to food provisioning (Smiseth, Kölliker, & Royle, 2012). While offspring care has been extensively studied in vertebrates (Balshine, 2012), social insects also provide an effective study system, as offspring are typically obligately dependent on relatives for care. As offspring care can be costly, caregivers often monitor a variety of information to insure that investments are made judiciously. For example, in some species, adult ants may rely on begging behavior by young to assess when they are hungry (Hölldobler, Stanton, & Markl, 1978; Mas & Kölliker, 2008). Caregivers must then balance foraging efforts with other environmental information, such as risk of predation and trade-offs between caring for current versus future reproduction. In this paper I explore how cues from offspring coupled with cues from the environment shape thermoregulatory care of offspring in a eusocial honeybee.

Care of offspring, defined as parental investment that increases current reproduction at the expense of future reproduction (Wittenerger 1981, Zeh et al. 1985), is surprisingly widespread in insects, occurring in 10 orders (Zeh, Smith, Zeh, & Smith, 1985). In most orders, however, parents perform offspring care facultatively to increase survival of their offspring, but the young can survive without it (Mas & Kölliker, 2008). Eusocial insects have obligate offspring care and larvae will not survive without adult investment (Mas, Haynes, & Kölliker, 2009). Larval begging by use of movements is known in some ants, such as *Novomessor* (Hölldobler et al. 1978) and *Myrmica*

(Creemers, Billen, & Gobin, 2003), as well as *vespid* wasps (J. H. Hunt, 1991; Ishay & Landau, 1972; Ishay & Schwartz, 1973). More often, though, chemical cues communicate larval status to adult caregivers. For example, adult bumblebees assess hunger status using larval cuticular hydrocarbons (Den Boer & 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 & 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 & Shine, 2008). For example, leaf-cutting and grass-cutting ants select appropriate depths in soil for brood chambers (M. Bollazzi & Roces, 2002; Martin Bollazzi & Roces, 2007), fire ants move brood as nest temperatures vary throughout the day (Penick & Tschinkel, 2008), while termites create elaborate architecture and nest orientation for regulation of air movement in their mounds (Jacklyn, 2010; Korb, 2003). 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.

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; Himmer 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 (Human et al., 2013; Seeley, 1974; P T Starks & Gilley, 1999). 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 & Esch, 1994; Kleinhenz et al., 2003; P T Starks & Gilley, 1999). Eusocial insects provide an interesting system by which to study how caregivers can effectively provide this extensive care.

I 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 & Oldroyd, 2007). This includes spreading water on comb to evaporatively cool it (Kühnholz & Seeley, 1997), heat shielding, where bees use their bodies to absorb then dissipate excess heat (Bonoan, Goldman, Wong, & Starks, 2014; P T Starks & Gilley, 1999), and fanning behavior, used to circulate air through the colony (Cook & Breed, 2013; Egley & Breed, 2013). Fanning behavior is of particular interest because of the group dynamics needed to be effective, and is the focus of this study. Overall, these

behaviors effectively maintain the proper climate for larval development and have major implications for the overall success of the colony.

Here, I tested the previously unexplored question: how do larvae influence the thermoregulatory fanning behavior in adult honeybees? I hypothesized that the presence of larvae would affect fanning behavior. Specifically, I predicted that honeybees would fan more when they are in the presence of a larva. I 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 I also predicted that brood pheromone would increase fanning behavior. Finally, if I observe effects of the presence of larvae on adult behavior in the lab, I should also see variation in the thermoregulatory response of bees across the season, as honeybees diminish or stop larval production in the winter. I predicted adult honeybees sampled from hives with larvae would fan more than bees from winter hives. The goal is to provide a deeper understanding of the role the presence of larvae plays in this critical thermoregulatory behavior.

3.3 METHODS

Collection of Workers and Larvae

I 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 the colonies. I 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, I collected only a single bee and a single larva. While bees are most likely to fan in groups (Cook & Breed, 2013), I controlled for social effect by using single bees with a single larvae to directly test effect of presence of larvae. I collected fanners as discerned by location on landing platform, distinct upright but curved abdomen position, and rapid wing movement for a period of at least 10 seconds without changing position and orientation relative to hive opening. Studies on bumblebees use 10s of sustained fanning to define a fanner (Weidenmuller, 2004). This posture and time distinguishes 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., 2010). I 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. I collected a single fanning adult honeybee by grabbing a leg with forceps. I then opened the hive to collect larvae. I opened the hives as carefully as possible and with no smoke to ensure some continuing

of normal behavior with such a large disturbance, however when I opened hives, I did not use them again for 2 hours, until normal hive behavior returned (Personal Obs.). I 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, I transported the bee and larva in the cage to the lab, where I performed the behavioral assay. I performed 163 trials with a larva and a single bee and 112 trials with no larvae.

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, I 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, with the additional chamber added on, so as to not change the volume to which the bee is confined. I performed the same protocol for collecting a fanner and a larva as previously stated, except I placed the larva into the auxiliary chamber, separated by 1 inch from the adult fanner bee. Again, I transported these cages into the lab to perform the behavioral assay. As adult bees could not interact with the larva, I did not collect this data. I 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. I acquired synthesized brood

pheromone (Super Boost, Contech Enterprises Inc.). Super Boost is comprised of 10 fatty acid esters (Le Conte, Arnold, Trouiller, & Masson, 1990). Since I saw a response of increased fanning behavior in the presence 5 larvae, I wanted to use a 5 larvae equivalent dose to account for any evaporation or degradation. I kept Super Boost frozen (-18°C) until use, then let thaw for 5 minutes at room temperature. I vortexed the Super Boost for 1 minute, then put .112g into 10mL of hexane. I then vortexed this mixture for 1 minute. This stock solution was serially diluted 3 times until I had 200 doses of BP per 10mL solution. When not in use, these solutions were kept frozen at -18°C.

I placed 250 micro liters of BP solution onto filter paper (Whatman #2 42.5mm) 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.

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

I placed filter paper into color-coded cages (only CC 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 KS or

another lab assistant, CR, watched the trials and recorded data. Similar to my other experiments, bees were allowed 25 minutes to acclimate before beginning the heating regime and behavioral assay. Brood pheromone experiments were conducted from June to September 2013. I performed 48 trials with brood pheromone and 49 controls.

Presence of Larvae At Hive

Larvae Present (Spring/Summer)

I identified fanners at the hive using the protocol described above and placed them into mesh cages in groups of ten. I 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, Wille, Gerig, & Luscher, 1976; Huang & Robinson, 1995; Pearce, Huang, & Breed, 2001). Therefore, I collected groups of ten bees opportunistically at the entrance of the hive. I chose groups of ten because single bees are not likely to fan (Cook & Breed, 2013). Bees were randomly collected using forceps and placed into individual wire mesh cages (cylindrical, 5cm x 2.5cm). 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, I then brought them into the lab. I placed the cage into a two-liter glass container (9cm x 24cm), and then loosely sealed the top. I then set the container on a heating unit (Proctor Simplex). I 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, I left the bee alone for 25 minutes to acclimate. This amount of time allowed for the bee's activity level to steady after being caught and transported (Cook & Breed 2013, Preliminary Trials). I then began the heating regime, starting at room temperature (an average of 25.24° C $\pm$ .110° C), by heating the air inside of the jar 1°C per minute. I 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. I recorded bees as fanning if they began fanning their wings while standing still for at least 10 seconds, which is the same criteria I used when I collected fanners from the hives. Additional data I recorded consisted of the temperature at which bees began to fan (herein, thermal threshold), as well as any behavioral interactions with the larvae the adult bees had, including antennation, touching with legs, probing with proboscis, and carrying in mandibles.

I recorded occurrence of fanning behavior, worker and larvae interaction, and corresponding temperatures were recorded until the bees reached their thermal maximum and ceased all activity. I recorded fanning behavior by 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 seconds without changing position (Egley & Breed, 2013). I 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. I performed 20 assays for each season, for 60 total assays.

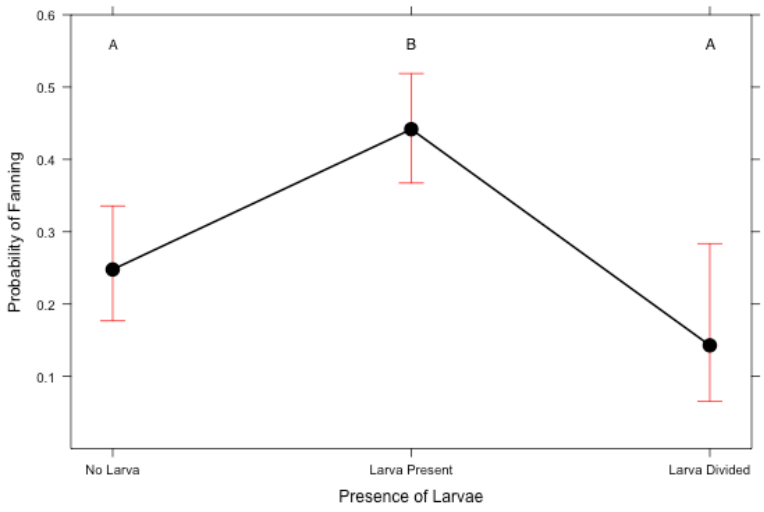
Statistical Analysis

To test all hypotheses I used a generalized linear mixed model and treated hive as a random effect. I used both probability of fanning and thermal response threshold as the response variables. This gave us a more comprehensive perspective of how fanning behavior could be modulated by larvae. To look at probability of fanning, I performed a logistic regression with a binomial error distribution (link=logit). To explore thermal response threshold, I performed a linear regression with a Gaussian distribution. To evaluate the magnitude of a significant effect, I used a post hoc (Tukey) test. In all models, I treated presence of larvae in the hive, presence of larva in a trial, separation, and pheromone as categorical predictor variables. With all of the models, I started with the most inclusive model, including to additional factors: whether the hive was in the sun (binomial) and total trial time (continuous). If predictor variables were insignificant (alpha=0.05), I dropped them from the model. I used R and R Studio, version *0.98.1103* and the package LME4 or generalized linear mixed model analysis (Bates et al., 2014).

3.4 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; 1a). 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; Figure 1b). When performing these behavioral assays I 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 experimental container. 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.



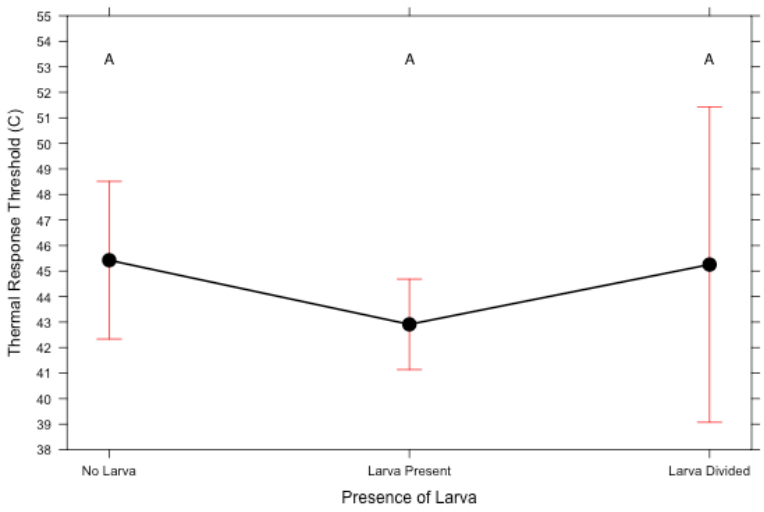


Figure 3.1 (A) The mean probability of fanning ± the 95% confidence intervals of fanning with no larva, presence with direct contact of larva, and presence but no contact with larva (divided). (B) The mean thermal response threshold ± the 95% confidence intervals of fanning with no larva, presence with direct contact of larva, and presence but no contact with larva (divided).

Effect of separation of adult and larvae on fanning behavior

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; Figure 1a). 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= Z=1.385, p=0.166; Figure 2a).

Effect of Brood Pheromone on Fanning Behavior

Honeybees were not more likely to fan when exposed to brood pheromone as compared to controls (N=98: Z=-0.492, p=0.6226; Figure 2a). 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°C ± 2.9°C, whereas bees that were not exposed to brood pheromone fanned at 24.68°C ± 1.61°C (N=46: F=3.457, df=1, 29, p=0.0731; Figure 2b).

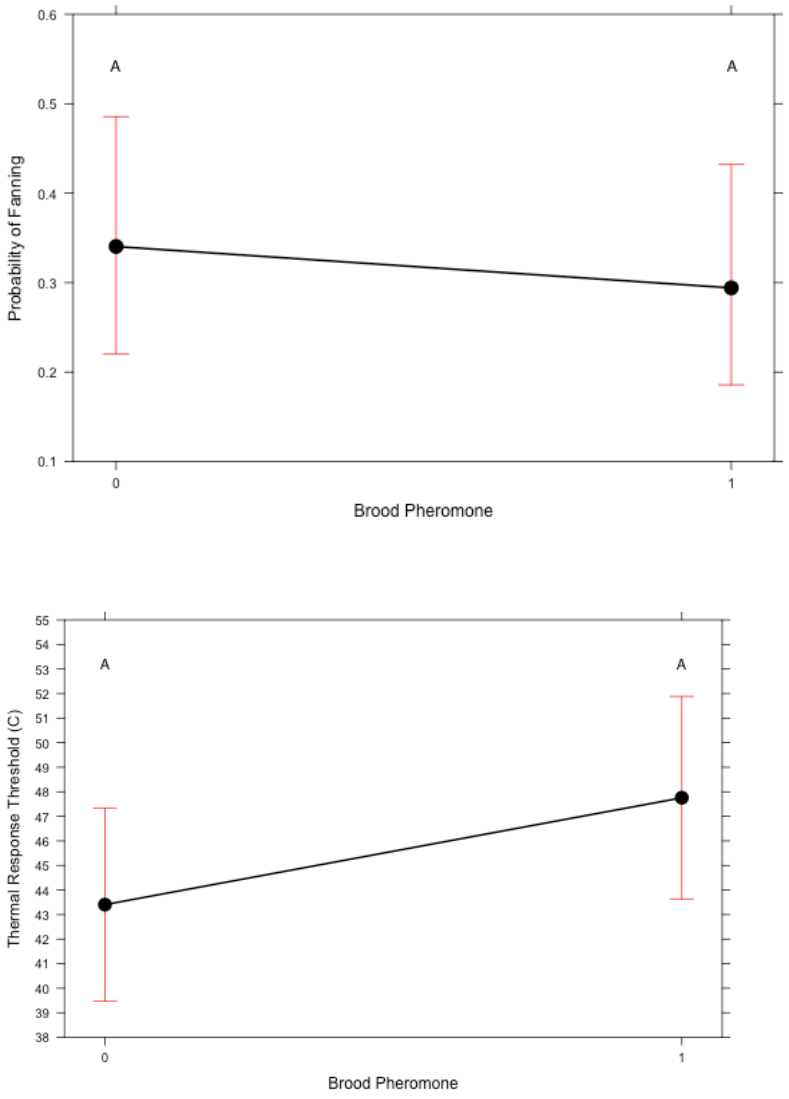
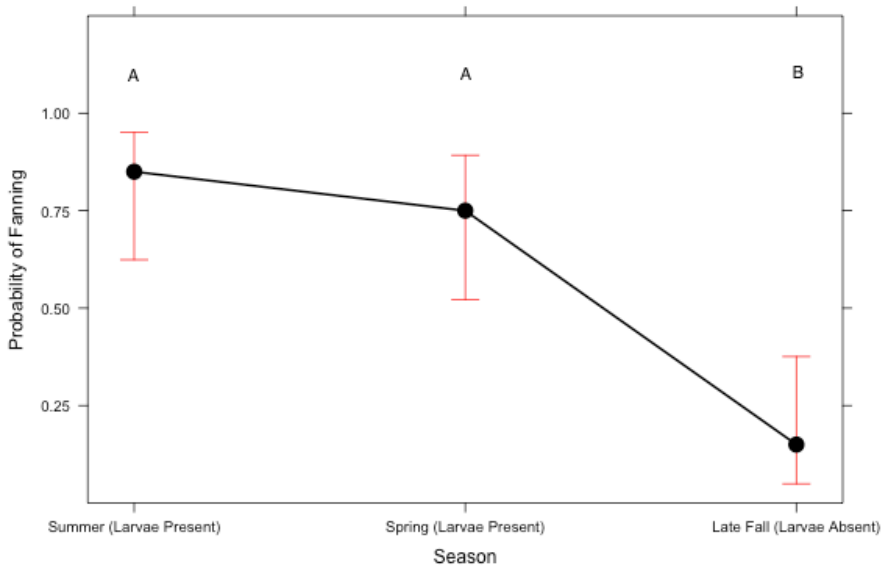


Figure 3.2 (A) The mean probability of fanning ± the 95% confidence intervals of fanning when exposed to brood pheromone or not. There was no significant difference in probability of fanning when honeybees were exposed to brood pheromone. **(B)** The mean thermal response threshold ± the 95% confidence intervals when exposed to brood pheromone or not. There was no significant difference in thermal response threshold when bees were exposed to brood pheromone.

Effect of Season on Fanning Behavior

Season significantly influenced whether bees fan. Bees were most likely to fan in the summer (brood present) or in the early spring (brood present) (N=60, Spring-Summer: Z=-0.784, p=0.433) and least likely 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; Figure 3a). During the fall only 15% (N=20) instances of fanning were observed, whereas 80% (N=20) during the spring and 85 % (N=20) in the summer. Bees in early spring began fanning at significantly lower thermal response thresholds than bees in the summer (N=40, t=-3.021, p=0.00492), where as there was no significant difference in fanners between spring and late fall (N=40, t=0.179, p=0.85), and summer and late fall (N=40, t=1.87, p=0.07; Figure 3b). This was likely because I saw so few fanners in the fall, and therefore the variance across thermal thresholds is higher.



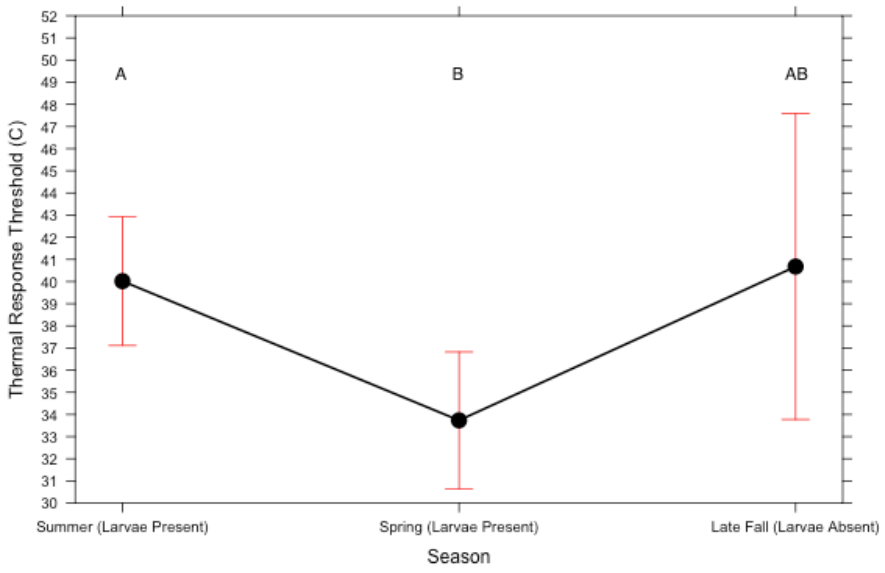


Figure 3.3 (A) Honeybees sampled from hives with larvae present are significantly more likely to fan compared to honeybees sampled from hives without larvae present Mean probability of fanning ± 95% Confidence intervals across larvae presence at the hive. Temperatures and forage were similar in spring and fall. **(B)** Bees in the spring fanned at significantly lower thermal thresholds than bees in the summer and bees in the fall. Mean thermal response thresholds ± 95% Confidence intervals across larvae presence at the hive. Temperatures and forage were similar in spring and fall.

3.5 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. Surprisingly, 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 (Tanya Pankiw et al., 1998), vibrations (Cao et al., 2007; Donahoe et al., 2003), 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 ants, direct physical contact between a reproductive individual and workers inhibits ovary growth in workers (Tsuji, Egashira, & Hölldobler, 1999). However, Tsuji et al. showed that preventing contact did not inhibit ovary development, and therefore the signal was not volatile. My results further indicate that direct contact between individuals sharing a colony disperses important cues that influence behavior.

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). I found, however, that brood pheromone had no affect 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. I allowed bees to come into direct contact with brood pheromone, as it is unknown how it is distributed throughout the hive (Tanya Pankiw et al., 1998). Brood pheromone also plays an important role in the division of labor in honeybees (Sagili et al. 2011). Sagili and others (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.

I 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 & 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 & 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, 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 my lab results, I decided to evaluate whether I would see the same patterns in the field. I 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. These results 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.

Organisms that perform obligate offspring care must utilize feedback from their environment to effectively do so. This information comes from both the environment and from young. Caregivers can balance physical or chemical cues from offspring in the context of environmental information to know what kind and how much care to provide (Mas & Kölliker, 2008). While much attention has been paid to vertebrates, there are restrictions to studying them, particularly the types of experiments that can be performed in the laboratory and in the field (Balshine, 2012). Eusocial insect colonies offer useful systems by which to study care of offspring. Individuals other than parents provide offspring care, care is performed by a group (Wilson, 1971), and often times, genetic lineages are known (Jones, Myerscough, Graham, & Oldroyd, 2004b). With these factors, social insects can provide extensive insight into the trade-offs that

caregivers must evaluate to know how and when to provide essential care to young. My study shows that larvae directly influence honeybee thermoregulatory behavior. Further, I 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.

CHAPTER 4

Rapidly Changing Environment Modulates A Decentralized Thermoregulatory Fanning Response in Honeybee Groups[3]

4.1 ABSTRACT

Social insect societies maintain homeostasis through decentralized collective effort. In quickly changing environments, homeostasis can be difficult, as information may promptly become outdated. How do decentralized social insect groups respond to rapid environmental changes? Honeybee (*Apis mellifera L.*) workers employ thermoregulatory fanning behavior as part of their repertoire to maintain nest temperatures below 36°C, as larvae can develop malformations and die if temperatures surpass this threshold. Here, I determine if honeybees alter their fanning behavior when experiencing different rates of thermal change. I found that honeybee fanners were significantly more likely to fan when experiencing rapidly increasing temperatures, but this response was only seen in larger groups of bees. Additionally, fanners responded at significantly lower temperatures when temperatures were increased quickly, but again, only when they were in larger groups. My results show a statistically significant interaction between fanning response and group size. These findings illustrate the importance of exploring both response thresholds and probability of response of animals in social groups experiencing changing environments, as both factors affect homeostatic responses. Understanding how animals employ self-organized systems to maintain homeostasis provides insight into decentralized organization across many biological systems.

[3] This paper is currently in review at *Animal Behaviour*, with co-authors Rachael E. Kaspar and Michael D. Breed

4.2 INTRODUCTION

All animals use homeostatic mechanisms to perform optimally in a changing environment. Many aspects of homeostasis are not controlled centrally (Vodovotz, An, & Androulakis, 2013), which leads to questions about how it is effectively maintained. For example, the mammalian immune system mostly operates peripherally with individual cells responding according to local information (Parkin & Cohen, 2001). Several parallels exist between the immune system and homeostatic mechanisms, such as thermoregulation, in social insect societies (Eberl, 2010; Jacob, Steil, & Bergmann, 2006). Many social insect species utilize decentralized homeostasis to maintain the climate and structure of their nests (E. Wilson, 1971). The extent to which a system can maintain appropriate conditions depends on how rapidly or slowly the external environment changes. For example, the rate of temperature change of an environment actually alters the critical thermal limits of insects (Ribeiro, Camacho, & Navas, 2012; Terblanche, Deere, Clusella-Trullas, Janion, & Chown, 2007). These induced physiological changes in individuals can scale in a non-linear fashion to shape group-level responses to changing environments (E. Bonabeau, Theraulaz, & Deneubourg, 1996; Mangel, 1995; Pacala et al., 1996). However, very little empirical work has been done to explore how non-linear effects can alter critical homeostatic responses of groups.

Social insect societies give us opportunities to explore the emergence of homeostatic behavior at levels ranging from individuals to the entire colony. By responding to perturbations, individual workers can trigger many animals in the group to

perform the same homeostatic behavior. This then affects the environment of the colony by returning conditions to a set point. For example, ants initiate foraging depending on the rate at which they encounter other successful foragers (Gordon, 2010). Termites follow this model when they repair mound breaches in colony defense (Emerson, 1956). Honeybees collectively thermoregulate in cold (Heinrich & Esch, 1994) or hot ambient temperatures (Egley & Breed, 2013; Jones & Oldroyd, 2007). These individual reactions to local changes lead to decentralized homeostatic group responses.

However, rapidly changing environments pose a particular challenge to self-organized groups. If conditions change too quickly, information may become outdated by the time a response is mounted, rendering the response ineffective. Furthermore, the roles of workers within division of labor in social insects are often triggered by response thresholds, which are internal response points at which a task-specific behavior is performed (Robinson, 1992). Quickly changing environments may overshoot these thresholds before an individual can respond effectively. Rapidly changing temperatures have even been shown to change critical thermal maxima and minima (Ribeiro et al., 2012; Terblanche et al., 2007). Social insect societies theoretically can utilize their relatively large size to collect as much information as possible to buffer against these effects (E Bonabeau, Theraulaz, & Deneubourg, 1998). Empirically, ants make increasingly effective foraging decisions when more individuals gather information (Pacala et al., 1996) and honeybees forage in patches more efficiently with larger hive populations (Donaldson-Matasci, DeGrandi-Hoffman, & Dornhaus, 2013). Honeybees can better choose thermal optima within a larger group of workers (Szopek, Schmickl,

Thenius, Radspieler, & Crailsheim, 2013), while larger colonies can regulate carbon dioxide within a much narrower range than small colonies (Seeley, 1974). Larger bumblebee colonies respond to increasing temperatures more quickly than smaller ones (Weidenmuller, Kleineidam, & Tautz, 2002). More populous groups may thus have a regulatory advantage. As decentralized responses emerge from the interaction of many individuals with slightly different information, do larger groups mount more effective responses in a rapidly changing environment?

Honeybee colonies potentially face dramatically changing temperatures throughout a day, which can be particularly difficult to buffer against. Despite this, honeybees are highly effective at keeping hive temperatures within a very narrow range, between 33-35°C (Jones & Oldroyd, 2007; J. Tautz, Maier, Groh, Rossler, & Brockmann, 2003), never letting temperatures surpass 36°C as larvae can die (Groh, Tautz, & Rössler, 2004; Himmer, 1932; Winston, 1991). There are several thermoregulatory behaviors in which honeybees engage, such as cooling by heat shielding (Bonoan et al., 2014; Siegel et al., 2005a; P T Starks & Gilley, 1999) which involves bees pressing their bodies against comb then dispersing to remove heat, spreading water for evaporative cooling (Kühnholz & Seeley, 1997), warming by shivering (Heinrich & Esch, 1994; Siegel et al., 2005a), and shivering inside brood cells (Kleinhenz et al., 2003) (for a review of insect thermoregulation see (Jones & Oldroyd, 2007)). In thermoregulatory fanning behavior, a honeybee moves its wings rapidly to circulate air within the nest or at the nest entrance. This fanning response occurs in response to increases in temperature (Egley & Breed, 2013) and is more likely to occur

in groups of workers, with the fanning response being initiated collectively (Cook & Breed, 2013). This makes fanning an ideal behavior for studies of individual and group responses to rapidly changing environments.

In this study I tested whether honeybee groups alter their fanning response depending upon differentially changing thermal environments. First, I hypothesized that the rate at which honeybees are heated would affect the probability of initiating fanning behavior. I predicted that honeybees would be more likely to fan the more rapidly temperatures were increased. Second, I hypothesized that rate of temperature increase would affect the thermal response threshold, or the temperature at which honeybees begin to fan. I predicted that bees that are heated at a faster rate would exhibit thresholds for fanning at lower temperatures. Third, I explored how ambient temperature at time of collection influenced probability and thermal response threshold of fanning. I predicted that honeybees would be more likely to fan when they are collected from warmer temperatures, compared to cooler temperatures. Overall, given the efficiency of groups, I predicted that all of these responses would be more prominent in larger groups. Taken together, I hope to elucidate how a changing environment influences homeostasis in complex societies.

4.3 METHODS

General Beekeeping

Twelve *Apis mellifera* colonies on University of Colorado's East Campus were used for these experiments. Colonies were maintained in 10-frame wooden Langstroth

hives with plastic or wood frames. Bees were supplemented with 1M sucrose and pollen patties (Mann Lake) as needed. All experiments were conducted from June-September 2014.

Collection of Fanner Honeybees

Fanners are easily identified from their unique posture and orientation at the entrance (Cook & Breed, 2013). Other fanners occur throughout the colony of the hive, however I only sampled entrance fanners as they were easily identified and collected. I selected bees that were observed fanning for at least 10s (Weidenmuller, 2004). These identification protocols ensure I am not collecting bees that are Nasanov fanning. Nasanov fanners are distinguished by the straight posture of their abdomen and exposure of the Nasanov gland while fanning (Free, 1967). These criteria were also used to identify fanning during the behavioral assay. I also avoided fanners which had pollen on their corbicula, as Cook and Breed (Cook & Breed, 2013) found that pollen foragers were significantly less likely to fan in heating assays. Only nestmates were used within one experimental cage (cages described below). Upon collection bees were immediately brought into the laboratory so that no longer than 10 minutes elapsed from the time of collection to when transportation to the laboratory was complete.

Set up of experimental groups

I had two treatment variables: group size and rate of heating. As I collected fanning bees, I randomly placed them in 3 different group sizes (1, 3, or 10) into

individual mesh cages (cylindrical: height: 20cm, radius: 6cm). I chose ten as the largest group size because it is the largest group with which I could distinguish and analyze individual behavior. While small with respect to the typical size of honeybee colonies, interactions within groups of ten bees mimic those found in larger groups (Cook & Breed, 2013) and is typical of a group size of fanners at the hive on a summer day (personal obs.). However, I have seen a large range of fanning bees at the hive, from the rare single bee to over 50 bees. For each collection event, hives were randomly selected but collection was distributed uniformly across hives. When sampling, I recorded outside temperature, whether the hive was in the sun or the shade, the time of collection, and the date. I then transported them into the laboratory.

Temperature Regimes, Experimental Apparatus, and Behavioral Assay

Once in the lab, I placed caged bees into 1-gallon glass jars (Specialty Container Inc.). Cages were propped on wooden stilts so the cages did not touch the sides or bottom of the jar. Jars sat on top of a single heating apparatus (Corning or Simplex Proctor hot plate). I inserted temperature probes (Cole Parmer High Accuracy (±0.3C) Digital Temperature Probe) into the jar and gently secured the lid. I allowed bees to acclimate in each jar for 25 minutes before the heating regime began. This acclimation time is based on time required for the bees to become less behaviorally agitated, as observed in preliminary tests, and on the protocol from (Cook & Breed, 2013). After 25 minutes, I recorded the initial air temperature of the chamber (which was on average

25.24° C ± .110° C) and the trial start time. Temperatures were taken at approximately the center of the chamber where bees were restricted in the cage.

The second treatment variable, heating rate, was randomly assigned before the trial began. I treated bees with one of three temperature ramping regimes: 0.5°C/minute, 1°C/minute, and 2°C/minute. Rate was controlled by the heat settings on the hotplate; I placed hotplates on higher settings earlier for faster rates. I chose rates of change that I acknowledge are extreme but still fit within the upper limits of what bees might experience in natural environments. When exploring critical thermal temperatures in beetles, Allen et al. (Allen, Clusella-Trullas, & Chown, 2012) used temperature rates from 0.05°C/minute to 0.5°C/minute. Ribeiro and others (2012) use rates between 0.17°C/minute and 2°C/minute to assess maximum critical temperatures in leaf cutter ants (*Atta sexdens rubropiosa*). These insects live in a thermally buffering substrate, and therefore are likely experiencing less dramatic temperature changes as compared to honeybee hives in the sun. The assigned target rate I aimed for was often close, but not exact, so the actual ranges included rates from 0.37°C/minute to 0.749 (mean and standard deviation: 0.533±0.109) in The 0.5°C/minute rate, 0.75°C/minute to 1.49°C/minute (1.039±0.222) in The 1°C/minute, and 1.5°C/minute to 3.35°C/minute in The 2°C/minute rate (2.080±0.436). Rate was calculated as:

(End Temperature°C – Start Temperature°C) / Total Trial Time in Minutes.

In a group, bees initiate fanning virtually simultaneously. Because they are starting to fan at the same time, I am measuring probability and response threshold at the first initiation of group fanning (Cook & Breed, 2013). Therefore, I measured fanning as a group level response. The response variables are the proportion of fanners out of the total number of bees in the group and the temperature at which bees begin to fan (hereafter thermal response threshold). After the initial fanning bout, I continued the trial to see if fanning occurred later in the trial and recorded that, as well, however I treated that data separately (and is not included in this study). Trials concluded when the last bee reached lethal temperature and died, which I also recorded. Time of trial conclusion was recorded and actual rate of temperature change was calculated.

Statistical Analysis

The null hypothesis about the group size is that honeybees fan independently of each other. Under this null hypothesis, individual honeybees in groups would have the same probability of fanning as solitary honeybees, and the observed number of fanning should follow a binomial distribution with parameters p and n, where p is the probability of observing a solitary bee fanning during a trial and n is the group size. To test this null hypothesis I estimated p – from the observations of solitary honeybees to be 0.135 (95 CI: [0.0313, 0.238]; sample size of 52 solitary bees). I then performed a goodness of fit test in R (using the function “goodfit( )” provided by the package “vcd”) with parameters p = 0.135 and n = group size (3 or 10) to compare the observed distributions from the

groups of 3 and 10 bees to the expectation that the numbers fanning fit a binomial distribution.

To analyze the data for the two hypotheses postulating effects of group size, heating rate, and their interactions, I used a generalized linear mixed model. Specifically, I examined the probability of fanning using a mixed model logistic regression (link=logit) with the glmer function (LME4 package in R) on a binomial error distribution. Probability of fanning was calculated from a two-column response variable of bees that fanned and bees that did not fan. When a two-column proportion is run through a logit transformation, the logit function calculates a probability.

I analyzed thermal response threshold using a mixed linear model using the lmer function (LME4) on a Gaussian error distribution, then used an ANOVA (Type II Wald Chi Square) to analyze the magnitude of each main effect. For categorical factors, I used a Tukey post-hoc test to analyze the effect within the variable.

I approached each of these models with backward selection using AIC (Symonds & Moussalli, 2011). The main predictor variables in all of these models were rate of ramping (which were The three categorical target rates: 0.05°C/minute, 1°C/minute, and 2°C/minute), group size (1, 3, 10), outside temperature, and presence of sun on hives, as well as the interactions of rate of ramping, group size, and outside temperature (Table 1). I treated hive as a random effect, which allowed us to control for the inherent differences between hives, which I acknowledge likely exist, but are not relevant to the hypotheses. Once I arrived at a model with only significant predictor variables or interactions (Table 2), I performed a model fit analysis to directly compare predictive

value of the models and ensure I relied on the best one. I used R (version 3.0.2) and R Studio (version *0.98.1103)* and the package LME4 (Bates, 2008).

4.4 RESULTS

1) Do honeybees fan independently of each other?

Honeybees in groups do not fan independently of each other. Using the probability of observing fanning from the solitary honeybee experiments (0.135; 95% CI: [0.0313, 0.238]), applied to a binomial distribution, I found that the distribution of honeybees fanning in groups does not fit a predicted binomial distribution (groups of 3: Pearson: $\chi^2 = 506.817$, df=3, p =$1.589e^{-109}$; Likelihood Ratio: $\chi^2 = 57.81$, df=3, p=$1.726e^{-12}$. Groups of 10: Pearson: $\chi^2 = 10651.23$, df=10, p =$0.00e^{+00}$; Likelihood Ratio: $\chi^2 = 295.23$, df=10, p=$1.585e^{-57}$, Figure 1a and 1b). The differences in fanning behavior seen in groups is not due to individual probabilities or thresholds, but due to the influence of other bees in that group.

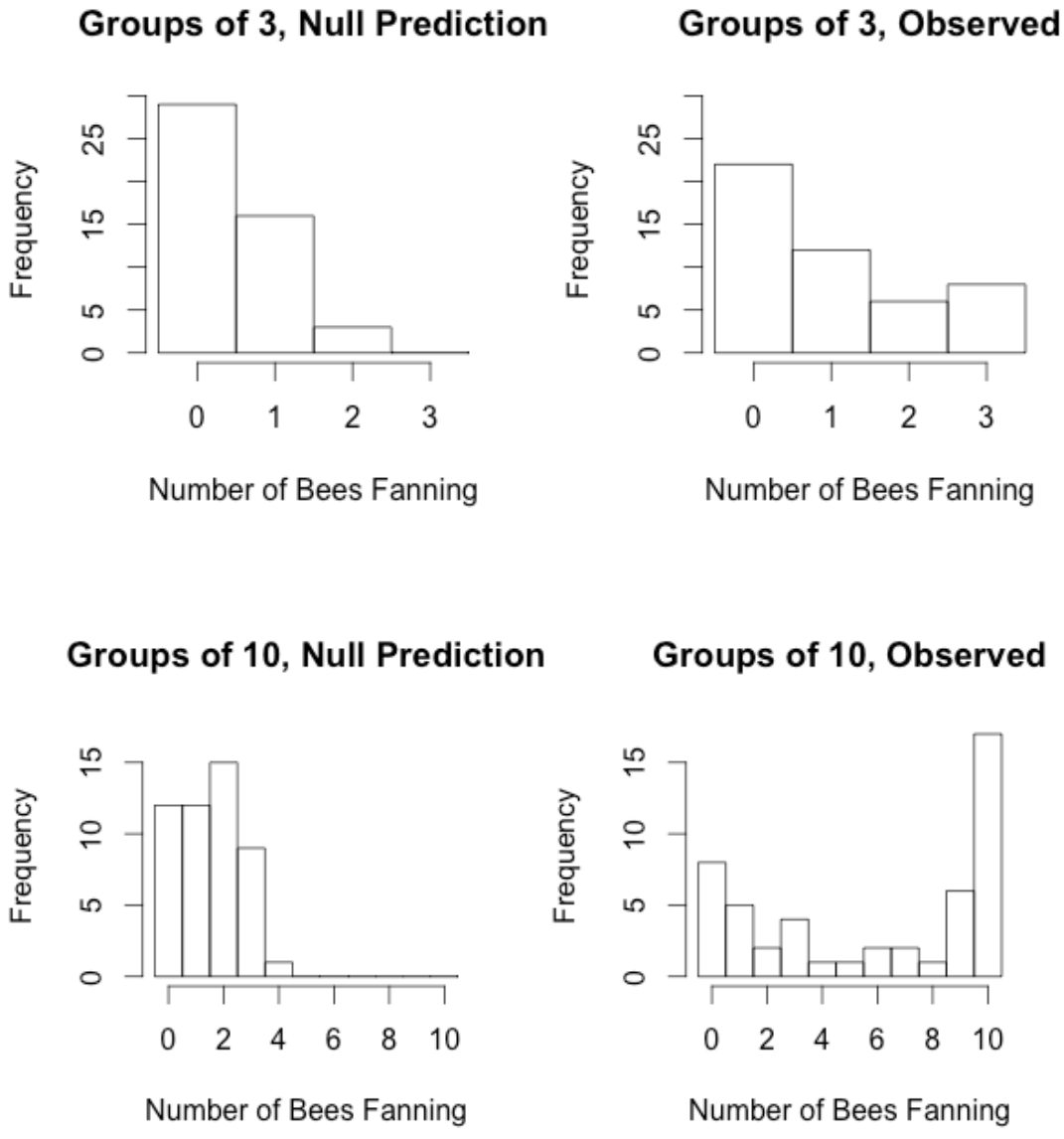


Figure 4.1a and 4.11b: Expected And Observed Distributions Of The Fanning Response in Groups of Three and Groups of Ten Honeybees
Frequency plots of the expected and observed distributions of a) groups of three and b) groups of ten. The expected distribution is a binomial distribution based on the independence of honeybees behaving as though they were independent.

2) Factors Affecting the Probability of Fanning

Following the model selection procedure outlined above, the best model (Table 1) included the following predictors (Table 2): (i) the interactions between group size

and rate of temperature increase (N=148, Z=5.331, P < 0.0001), (ii) the interaction between group size and outside temperature (Z= 5.050, P < 0.0001), and (iii) group size (Z=-4.159, P < 0.0001). The significant interactions are particularly interesting. The significant interaction between group size and rate of temperature increase shows that honeybees were significantly more likely to fan when being heated at a faster rate, but only when they were in the largest group of 10 bees. I observed fanning in 17 out of 19 trials with 10 bees heated at 2°C/minute, 9 out of 20 for groups of 3 bees at 2°C/minute, and only 4 bees out of 20 at 2°C/minute. For groups of 10, fanning occurred in 13 out of 16 trials at 0.5°C/minute, 11 out of 14 trials at 1°C/minute, and 17 out of 19 trials at 2°C/minute (Figure 2). I report the significant interaction between ambient temperature and group size result in the "*Effect of Ambient Temperature on Fanning Probability and Thermal Threshold"* section.

TABLE 4.1: The Model Comparison

Main Effects	Interactions	AIC
Rate Group Size Sun	Rate*GroupSize OutsideTemp*GroupSize OutsideTemp*Rate	635.2

OutsideTemp	OutsideTemp*Rate*GroupSize	
Group Size	**Rate*GroupSize** **OutsideTemp*GroupSize**	630.737

Comparison of full model to simplest, most predictive model (ANOVA: p = 0.018). The response variable is probability of fanning. Bold text indicates a significant response variable effect.

TABLE 4.2: The Significant Effects Predicting Probability Of Fanning In The Best Model.

Main Effect	**Intercept**	**Slope**	**Z Value**	**P Value**
Group Size	-0.543	0.242	-2.575	0.010*
Interactions			**Z Value**	**P Value**
Rate*GroupSize	0.085	0.016	3.049	0.0023*
OutsideTemp*GroupSize	0.007	0.0014	2.764	0.0057*

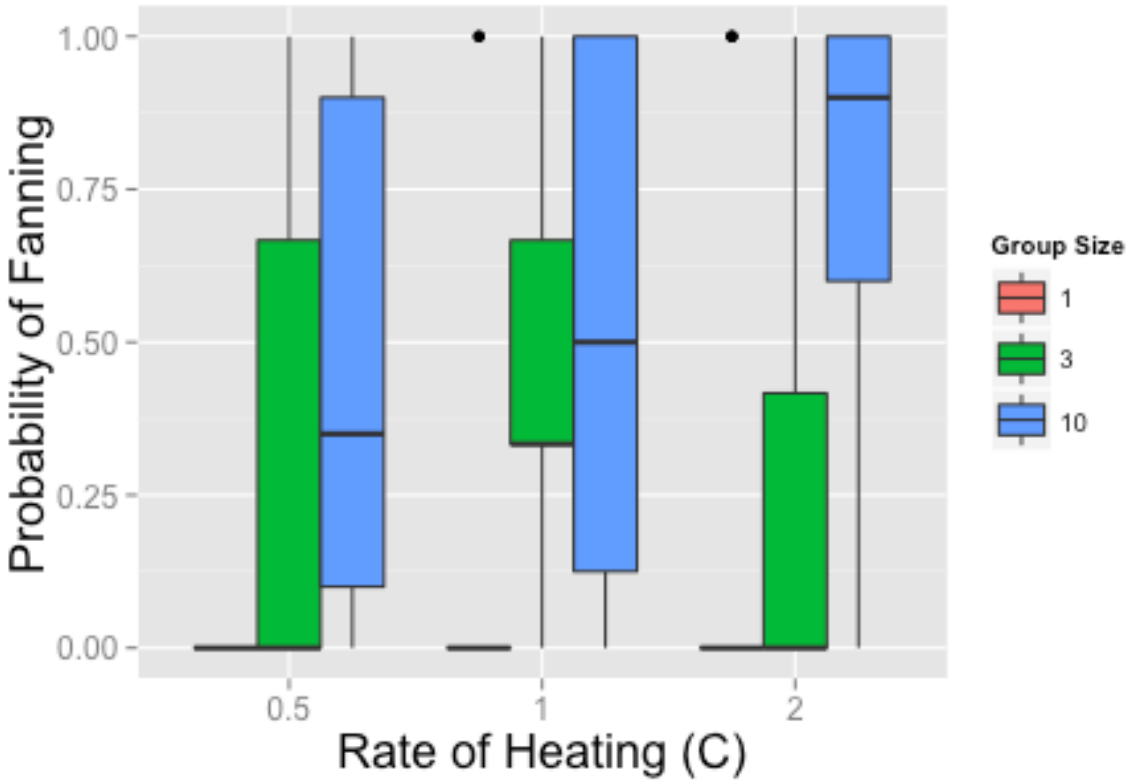


Figure 4.2: Probability Of Fanning Across Groups and Heating Regimes
Boxplot of probability of fanning. Bees were significantly more likely to fan when they were in groups of 10 in all heating regimes. Horizontal bars are medians, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers (N=148). Created using R Package ggplot2.

3) Factors Affecting the Thermal Response Threshold

The best model for thermal response threshold included rate of temperature increase and group size as significant predictor variables. Honeybees fanned at significantly lower temperatures when they were in groups of ten compared to when they were alone (N= 74, χ^2=11.554, p = 0.0031). They also fanned at significantly lower temperatures when they are being heated at faster rates, compared to slower ones (χ^2=7.566, p = 0.023; Figure 3). Bees who were in groups on ten and were heated at 0.5°C/min fanned on average at 36.3°C, ten bees heated at 1°C/min fanned at 35.3°C on average, and 10 bees that were heated at 2°C/minute fanned at 29.14°C on average. Zero single bees fanned when being heated at 0.5C/minute, which is why there is no single bee boxplot for that rate. The main effects were significant, whereas the interactions were not. This shows that for thermal response threshold, bees were likely cueing in on group size and heating rates independently. There was no significant difference among other group sizes or rates.

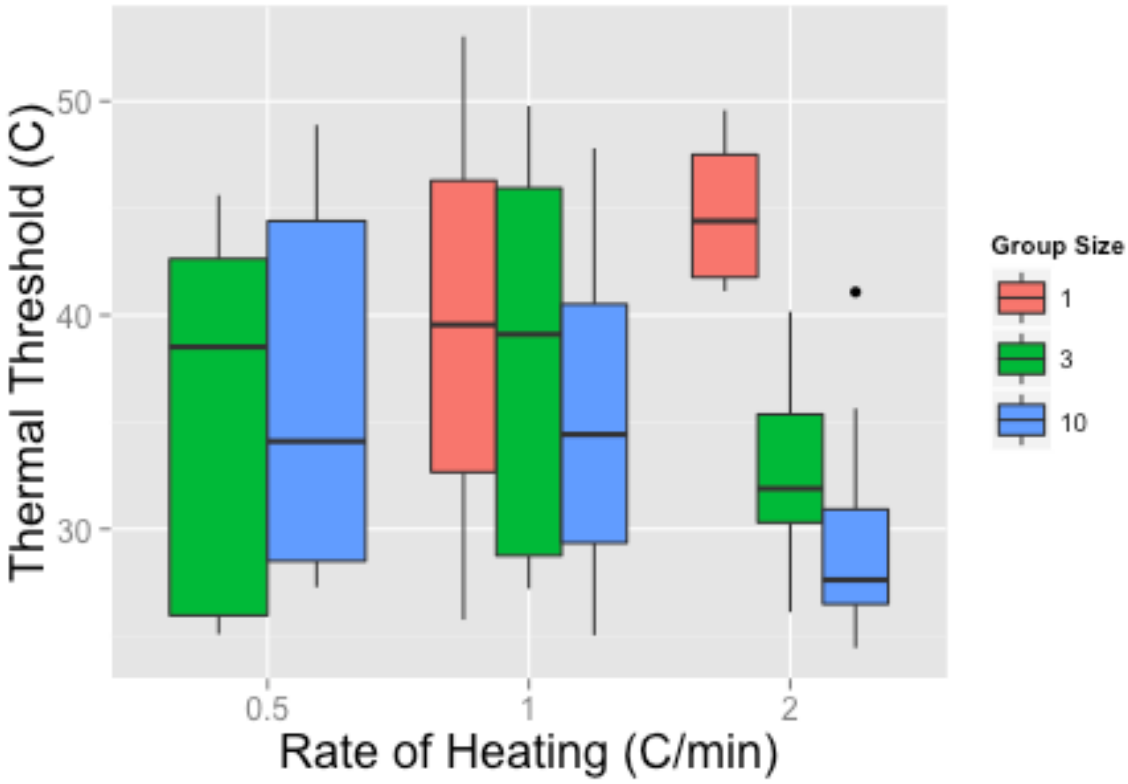


Figure 4.3: Thermal Response Threshold Across Group Size And Heating Regime When bees were being heated at 2°C/min, bees in groups of 10 fanned at even lower temperatures than when they were being heated at 1°C or 0.5°C per minute. Zero solitary bees fanned when they were heated at 0.5°C/minute, which is why there is no single bee boxplot at that rate. Horizontal bars are medians, boxes are 25 – 75th percentile, lines are 1.5 * IQR, points are Tukey outliers (N=74). Created using R Package ggplot2.

4) Effect of Ambient Temperature on Fanning Probability and Thermal Threshold

The interaction between group size and ambient temperature in predicting the probability of fanning was also significant. Honeybees were significantly more likely to fan when they were collected in higher ambient temperature conditions, but again, this

was only seen for bees who where then placed in groups of 10 (Figure 4). I made sure that this was not an artifact of collection, as warmer days allowed us to collect more bees; group sizes were evenly distributed across all ambient collection temperatures. Finally, there was no significant effect of outside temperature on thermal response threshold (N=73, T= -0.637, p=0.526).

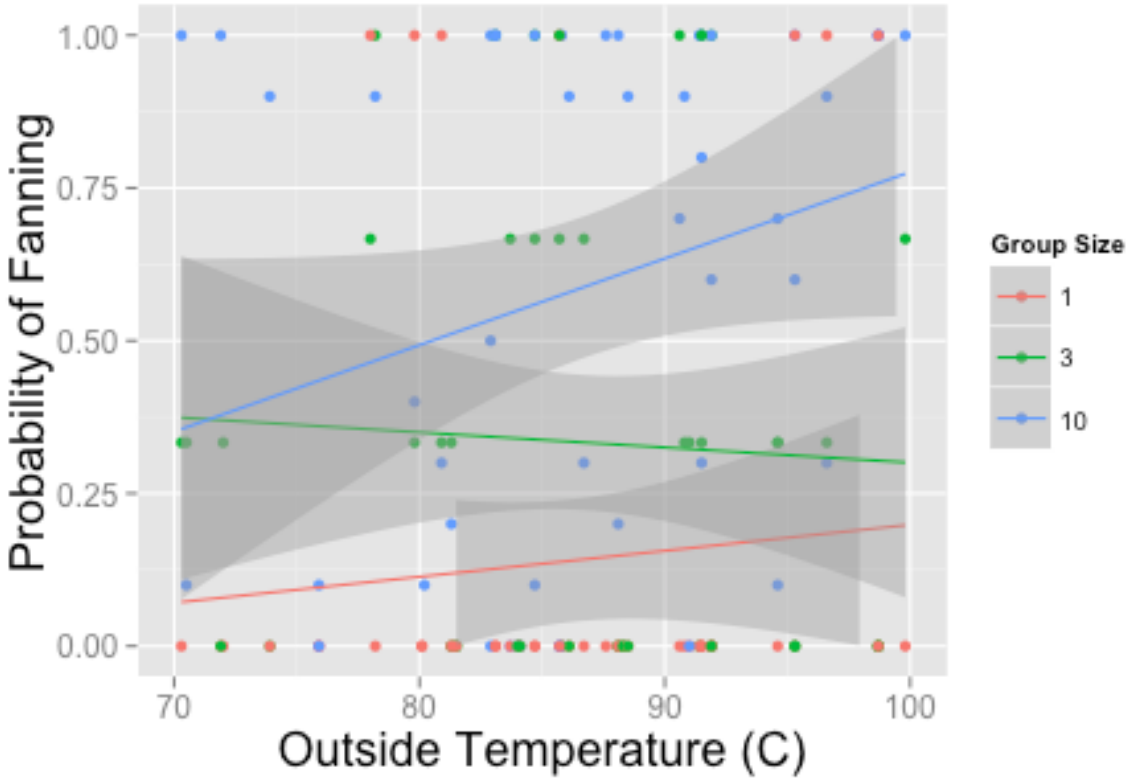


Figure 4.4: Outside Temperature And Group Size Interact To Influence Probability Of Fanning
Bees that experienced high outside temperatures and then heated in groups of ten were significantly more likely to fan compared to bees heated in groups of ten experiencing lower outside temperatures. Probability of Bees heated in groups of three or by themselves was not affected by higher outside temperatures. Shaded area around lines is 95% confidence (N=74). Created using R Package ggplot2.

4.4 DISCUSSION

Rapidly changing environments can be especially challenging for maintaining homeostasis. Despite this, honeybees maintain a tightly controlled thermal environment inside the hive. Here, I show that honeybees behave differently when temperatures increase more rapidly, but that the differences depend upon social context – group size. When experiencing a temperature increase of 2°C/minute, honeybees are significantly more likely to begin to fan than honeybees that are being heated slowly. I saw this result only in larger groups, which consisted of ten bees. The rate of temperature increase had no effect on the probability of fanning for a bee by herself or in a smaller group. I verify that honeybees do not fan independently; when in groups, honeybees fan more than predicted from the behavior of single bees and fanning in groups does not fit a binomial distribution. While I know that honeybees are more likely to fan and fan at lower temperatures in groups of ten (Cook & Breed, 2013), the results show that they are also cueing in on how quickly temperatures are changing. Theoretically, larger groups should be able to respond to rapidly changing environments more effectively, as they collect and synthesize more information more quickly [17, 32]. When honeybees experience quickly increasing temperatures, they are more likely to respond, but only in larger groups.

My results show that honeybees begin to fan at significantly lower temperatures when temperatures are increased at faster rates, but again, only when bees are in

larger groups. For the collective fanning response to be effective, a certain number of bees must initiate fanning in high ambient temperatures. But participation is not enough – the temperature at which a bee begins to fan, their response threshold, plays a role in the impact they will have on temperature control. Starting to fan at too high of a temperature will not be as effective as starting at lower temperatures. Bees that are in smaller groups or by themselves are not as likely to respond, even when being heated quickly. This is seen in bumblebees (*Bombus terrestris*) as well; Weidenmuller and others (Weidenmuller et al., 2002) found that larger bumblebee colonies respond faster to increasing temperatures. Even on a smaller scale of only a group of ten, I show a significant effect of decreased thermal threshold with a group size of only 10 bees. Response thresholds are often considered as being a static characteristic; an individual simply reaches that threshold and responds (Jones et al 2004, Beshers & Fewell 2001). In bumblebees, response thresholds are modulated by both rate and previous experience (Westhus et al., 2013). My results show that when groups of ten bees are heated at fast rates, they are both more likely to begin fanning and fan at lower temperatures, essentially anticipating rapidly increasing temperatures.

I found that honeybees that were brought into the lab were significantly more likely to fan if they were collected in hot ambient temperatures, compared to bees collected in cooler temperatures. This effect was only seen when bees were heated in larger groups, compared to small groups or by themselves. While ambient temperatures differed across trials as they were performed at different times of the day and throughout the summer season, temperatures at which trials started were consistent, as

they were performed in a laboratory setting. Bees collected in hotter ambient temperatures then brought into the lab experienced a more dramatic temperature change to acclimate. However this likely did not play a role in their increased fanning response, as bees collected in hot ambient temperatures but placed solitarily or heated more slowly did not show an increase in fanning probability or thermal response threshold.

In many social organisms, a group response does not occur until a quorum of individuals is readied by collective information (Rangel & Seeley, 2008; Sumpter & Pratt, 2009). In larger groups, information is shared by more individuals who are synthesizing information and potentially readying for a response (Donaldson-Matasci et al., 2013; Page & Mitchell, 1998). For the fanning response, honeybees that are exposed to higher temperatures outside may be more likely to fan based on the thermal information they acquired before being collected. The larger group size of fanners could allow them to reach a critical amount of thermal information more quickly, meaning they were hotter already, hence the increased chance of fanning seen only in larger groups of bees.

Self-organization occurs in many biological systems. While it is critical to explore how self organization occurs, understanding the rules organisms use can provide insight into created systems, and vice versa (Seeley, 2002). Social insect societies offer opportunities to test hypotheses about decentralized homeostasis. These groups are diverse, open, self-organized systems whose colonies range in worker specialization and population size. All of these societies function on some level with many individuals collecting information and responding to environmental perturbations. Further, group

size is known to increase efficiency of many decentralized tasks in social insects, including foraging (honeybees: (Donaldson-Matasci et al., 2013), ants: (Pacala et al., 1996) wasps: (Jeanne & Bouwma, 2002)), thermoregulation (bumblebees: (Weidenmuller et al., 2002), honeybees: (Cook & Breed, 2013)), nest site selection (Sasaki, Granovskiy, Mann, Sumpter, & Pratt, 2013), and overall colony organization (Naug, 2009). These systems can be explored from many different levels of organization, thus providing information about how regulation of group behaviors in societies occurs.

Decentralized biological systems have similar organizations as some engineered systems, specifically computational (Kitano, 2002; Vodovotz et al., 2013) and chemical systems (Androulakis, 2014). These systems are often modeled with the assumption that every unit is the same, whereas in reality, units are diverse (Camazine et al., 2001; Kitano, 2002). Furthermore, stochastic events could affect self-organized systems differently (Cohen & Harel, 2007) as well as differentially affect small versus large systems (Jeanne & Bouwma, 2002). While modeling decentralized systems provides a way to generate hypotheses of how they will behave, exploring established self organized biological systems offer a powerful comparison as they provide insight into stochastic events or emergent properties not predicted by mathematical models [47, 48]. By integrating mechanisms from all of these perspectives, researchers can improve upon hypotheses, predictions, models, and methods by which to explore decentralized systems.

Response thresholds and probability of performance are critical organizing components of division of labor in social insects (Jeanson & Weidenmüller, 2014). These behavioral responses are modulated by both how quickly the environment changes and the social environment an individual experiences; so much so that these two contexts show strong interactive effects. This study emphasizes the necessity of exploring self-organization in the context of changing environments, which is inevitably influences the organization of biological systems.

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CHAPTER 5

Social Cues Involved in the Fanning Response in Honeybees

5.1 ABSTRACT

Social information is critical for the performance of tasks by groups of animals. Workers in honeybee societies coordinate to accomplish many jobs, and their collective effort keeps the colony as a whole functioning. One of those jobs is nest thermoregulation. Honeybees are effective keeping their hive cool during hot summers, never letting temperatures in the interior of the nest surpass 36°C. To do this, honeybees fan to circulate hot air out of the hive. Typically, several bees perform this behavior together. Previous work has shown that as temperatures are experimentally increased, the rate of fanning happens depends on the number of bees in the group, with larger groups fanning more. What social cues are important in priming bees to fan? By restricting social interactions among fanners, I found that honeybees are likely to be responding to tactile cues that affect their likelihood of fanning. Workers likely do not use air movement from other fanners to prompt fanning. Finally, I show that fanning in the laboratory is performed, regardless of whether bees are together with nestmates or with non-nestmates. I rule out several cues that honeybees could use to know when to effectively fan; these include vibration, volatile pheromones, visual cues, and air movement. This helps us to understand how this critical thermoregulatory behavior is regulated, and increases our knowledge of important cues that inform division of labor in social insect societies.

5.2 INTRODUCTION

Information is required for social groups to effectively function. Some social groups are so large that individuals are unable to assess what needs to be done on a large scale. Therefore many complex social groups utilize local information to behave (Camazine et al., 2001). Information can be acquired directly, with individuals specifically sharing information about the task, such as when fish observe another fish making a turn in a school (Rowland, 1999). This can also occur indirectly, with individuals using local information to infer what needs to occur, such as schools of fish that use water displacement, measured by the lateral line, to know how its neighbor is moving (Partridge, 1982). Behaviors can range from group movements, like a school of fish, to specific tasks that a group must coordinate to accomplish, such as ants collectively carrying food (McCreery & Breed, 2014). Social information is critical for the performance of many group behaviors.

Eusocial insects divide dozens of tasks between thousands of individuals. These tasks include building nests, foraging for enough food for the entire colony, and thermoregulation. Division of labor is based on information gained by individuals responding to their local environment and to interactions between other individuals in the colony (Robinson 1992). Some eusocial insects exchange fluid (trophallaxis) in which a myriad of information is conveyed, such as food quality (Farina & Nunez 1991) and floral odors (Farina et al. 2007). Honeybees directly communicate locations of forage by performing the waggle dance (Frisch, 1965; Lindauer, 1971). Most eusocial insect colonies utilize cuticular compounds to evaluate whether the individual is a

nestmate or a non-nestmate, while worker ants use pheromone trails to mark the path to food (Wilson, 1971). Because of these mechanisms that enable efficient division of labor, eusocial insects are some of the most ecologically successful organisms on the planet (Hölldobler & Wilson 2008).

Social environment strongly influences eusocial division of labor. Huang and Robinson (1992) found that honeybees kept in isolation began foraging 2 weeks earlier than those exposed to other honeybees. Dor et al. (2005) found that honeybees housed in isolation did not develop ovaries, and those who were housed with just one other bee, with no contact, did. In fact, ants (*Camponotus fellah)* that are kept alone exhibited significantly higher mortality than ants kept in small groups of 10 (Koto et al., 2015). Cook and Breed (2013) showed that isolated honeybees are significantly less likely to begin to fan than bees in groups of ten, even when experiencing increasing temperatures. Fanning is a critical component of hive thermoregulation, as developing larvae can die if hive temperatures exceed 36°C. But increasing temperatures is not enough; the social context is necessary for fanning to occur. While we know that social environment is important for certain tasks to occur (Cook & Breed 2013), much less is known about the important information being exchanged during these social interactions. Therefore, in this study, I asked what social cues are important for fanning behavior to occur?

Honeybees utilize a myriad of modalities to communicate, including tactile (Cao et al., 2007; Gordon, 1989), vibrational (Donahoe et al., 2003), and pheromonal cues (Pankiw & Page, 2003). To disentangle which of these cues are necessary for fanning

behavior to occur, I prevented aspects of interactions from occurring. Specifically, I used single screen mesh divisions of groups of bees that allowed visual cues, perception of vibration, perception of volatile pheromones, but which restricted tactile cues, such as trophallaxis and antennation. I also used double screen mesh divisions that allowed visual cues, perception of vibration, and perception of volatile pheromones, but eliminated tactile cues. Based on extensive observation of honeybees interacting during previous experiments, I first hypothesize that tactile cues are the most important for fanning behavior. Therefore, I predicted that when honeybees were prevented from touching one another, they would fan less.

Secondly, Winston (1991) hypothesized airflow induces fanning behavior. Using different types of mesh, I tested the effect of airflow through these dividers on fanning behavior. Based on observations and preliminary experiments, I hypothesized that airflow does not play a significant role in fanning behavior. I predicted that there would be no change in fanning behavior across the different mesh types, but there would be an overall reduction in fanning behavior compared to non-divided cages.

Finally, I tested whether the fanning response was altered if the honeybees were in a cage with non-nestmates rather than nestmates. I hypothesized that non-nestmates would affect the fanning response. I predicted that there would be a reduction in fanning, because the tactile cues honeybees could potentially be using may be interrupted by unfamiliar nestmate recognition cues. These experiments provide insight into the important social information honeybees utilize to know when to fan.

5.3 METHODS

General Honeybee Keeping

Ten Italian *Apis mellifera L.* colonies were kept at the University of Colorado apiary. The hives were kept using standard beekeeping techniques in Langstroth hive boxes. I supplemented their foraging with 1M sucrose solution *ad libitium*, depending on conditions.

Identification of Fanners

Fanners at the hive were identified as honeybees that were standing still, rapidly moving their wings for at least 10 seconds, head down with a curved abdomen. This is different from Nasanov fanning to spread pheromone (Free 1987), and other behaviors such as some defense (Yang et al., 2010) or for orientation flights. I focused on fanners that were stationed at the entrance because: 1) they are more likely engaging in thermoregulation than fanners that may be distributed throughout the hive who are fanning to evaporate water from honey and 2) they are relatively easy to identify and collect. I collected bees off the entrance “porch” by grabbing their back legs with forceps. I then placed them into cages. Individuals were never used more than once.

Cages

Single vs. Double Mesh

Using methods established by Mann & Breed (1997), Katzav-Gozansky et al. (2004), and Dor et al. (2005) who used divided containers to test olfactory cues, I created 3 cage types: not divided, single mesh divided, and double mesh divided. Each cage was a mesh cylinder (height: 5cm x radius: 2.5cm). The dividers were 5 rectangles (height: 5cm x length: 2.5cm). The dividers were made of the same cage mesh. To create the double mesh, a drop of hot glue was placed on the corners and allowed to dry, then melted slightly to glue the second layer of mesh. All dividers were secured on one side.

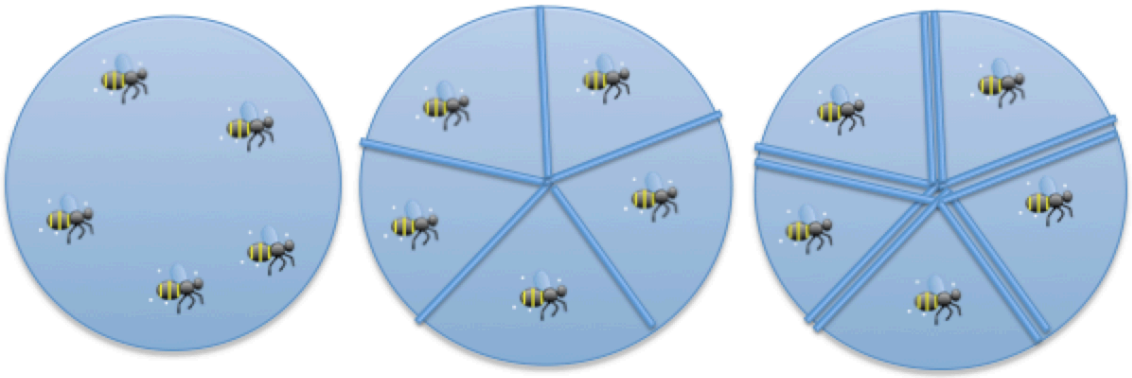

Figure 5.1: The design of the cages. A) The non-divided cage allows for relatively normal social interactions. B) The single layer mesh dividers allows for visual, pheromonal, and vibrational cues, while allowing limited physical interaction, and therefore limits tactile cues. C) The double layer of mesh allows visual, pheromonal, and vibrational cues, while also preventing physical contact and completely preventing tactile cues.

Different Size Mesh

Cages were again mesh cylinders (height: 5cm x radius: 2.5cm). The dividers were three different mesh types: mosquito netting, 1/6 mesh, and ¼ mesh. To prevent

contact, each divider was a double layer as described above, but I took great care in aligning the two dividers up so that the openings were true to their measurements. For the mosquito netting, I created a rectangular frame (height: 5cm x length: 2.5cm x width: 0.5cm), then placed two layers of mesh on either side of the frame.

Each cage had 5 compartments, and each compartment contained 1 honeybee in all experiments.

Nestmate vs. Non-nestmates

I collected 5 fanner honeybees from the entrance of the hives. I collected and ran both a non-nestmate treatment and a nestmate treatment concurrently. I collected all 5 nestmates in one trial from the same hive. I collected all non-nestmates from 5 different, randomly selected colonies. While collection from hives was random, I made an effort to sample evenly across the 10 colonies.

Behavioral Assay

Once collected into cages, I transported the cages with the honeybees into the lab. I then placed them into the heating apparatus, which consisted of 1-gallon glass jars (Specialty Container Inc.). Cages were propped on wooden stilts so the cages did not touch the sides or bottom of the jar. Jars sat on top of a single heating apparatus (Corning or Simplex Proctor hot plate). I inserted temperature probes (Cole Parmer High

Accuracy (±0.3°C) Digital Temperature Probe) into the jar and gently secured the lid. I allowed bees to acclimate in each jar for 25 minutes before the heating regime began. This acclimation time is based on time required for the bees to become less behaviorally agitated, as observed in preliminary tests, and on the protocol from (Cook & Breed, 2013). After 25 minutes, I recorded the initial air temperature of the chamber (mean temperature ± SD: 25.24° C ± .11°C) and the trial start time. Temperatures were taken at approximately the center of the chamber where bees were restricted in the cage. I then began to heat only one group at a rate of 1°C/minute (Cook & Breed, 2013, Cook et al., in review).

Statistical Analysis

To analyze probability of fanning, I performed a logistic regression on proportion of fanning out of five bees. I used a two-column response variable, consisting of number of positive (fanning behavior observed) and negative (fanning behavior not observed) responses. I used a binomial error distribution (link=logit) for the analysis. I used the "glm( )" function in R (version 3.0.2) and R studio (version *0.98.1103*).

To analyze the thermal response threshold, I used an ANOVA, as the predictor variables were categorical. This was evaluated using a Gaussian distribution. I used the "aov( )" function. I then performed a post-hoc test (Tukey) to analyze each pairwise comparison.

To compare the thermal response threshold of nestmates and non-nestmates, I performed a student's t-test, as there were only two categorical groups.

I used the function "ggplot( )" from the package ggplot2 to create all graphs. All analyses were compared to an alpha = 0.05 for significance.

5.4 RESULTS

Hypothesis 1: Social Cues

Fanners were significantly less likely to begin fanning if they were in separate compartments, whether they were divided by single mesh (n=118, Effect size: -1.35, z=-6.525, p<0.0001), or double mesh (n=105, Effect Size: -1.788, z=-6.937, p>0.0001) compared to a non-divided cage. However, there was no significant difference in the probability of fanning between single mesh and double mesh cages (n=101, Effect size: 0.436, z=1.527, p=0.127; Figure 1).

Honeybees that were divided by a single layer of mesh began fanning at significantly higher temperatures than bees in undivided cages. Honeybees in non-divided cages initiated fanning at 32.7°C ± 1.122°C (mean ± SE), whereas bees in single mesh divided cages began fanning at 38.5°C ± 1.64°C (n=118 total, Effect size=5.848, t=3.142, p=0.0025). A trend indicated that there was an increase in fanning onset temperature between bees in non-divided cages and bees in cages with double mesh dividers, which fanned at 34.0°C ± 1.55°C, although this was not significant (n= 105 total, Effect size: 4.456, t=1.916, p=0.059). There was no significant difference in

thermal response thresholds between bees in single mesh and double mesh cages (n=101 total, Effect size: 1.392, t=0.644, p=0.52; Figure 2).

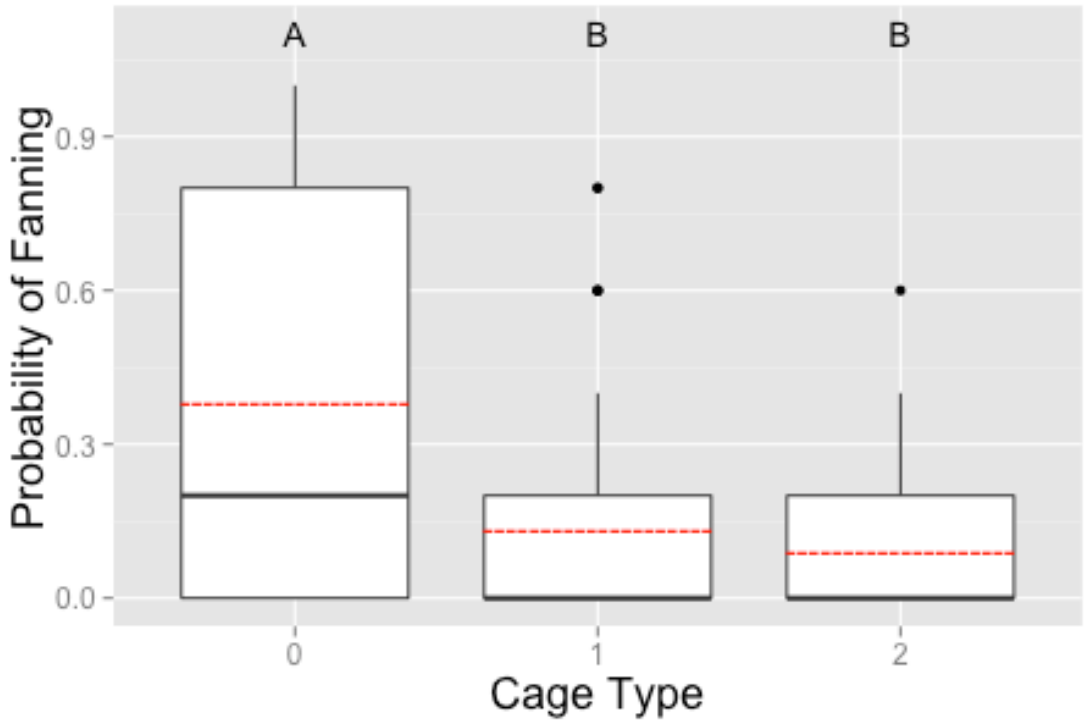

Figure 5.2: A boxplot showing the probability of fanning across non-divided cages, single mesh divided cages, and double mesh divided cages. Honeybees in non-divided cages were significantly more likely to fan than single mesh or double mesh cages. Horizontal bars are medians, red dashed lines are means, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers. Letters indicate statistical significance. Created using R Package ggplot2.

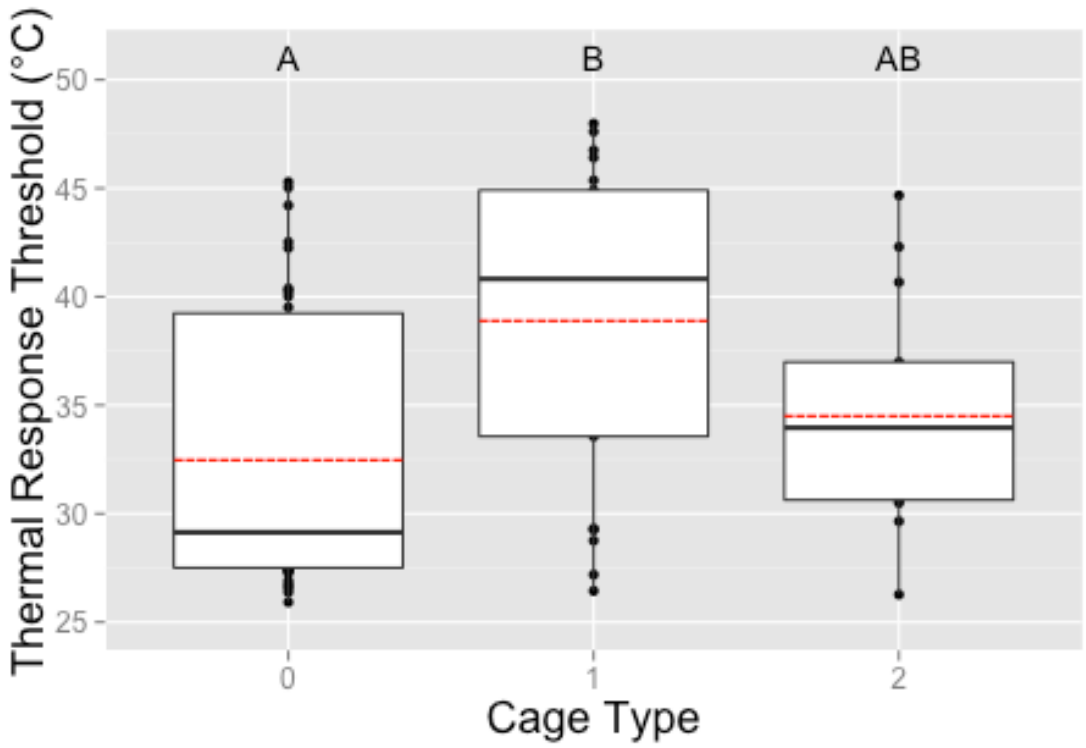

Figure 5.3: A boxplot showing the thermal response threshold across non-divided cages, single mesh divided cages, and double mesh divided cages. Honeybees in non-divided cages fan at significantly lower temperatures than bees divided by single mesh, however there was no significant difference between non-divided cages and double mesh dividers. Horizontal bars are medians, red dashed lines are means, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers. Letters indicate statistical significance. Created using R Package ggplot2.

Hypothesis 2: Airflow affects fanning behavior

Similar to the comparison between single and double mesh divided cages, we found that honeybees are significantly more likely to fan in non-divided cages compared to any of the different-sized mesh divided cages (Non-divided-Large Mesh: n=30 total, Effect size= -1.79, z= -4.361, p>0,001; Non-Divided – Medium Mesh: n=30 total, Effect size= -1.15, z=-3.188, p=0.00143; Non-Divided-Small Mesh: n=30 total, Effect size=-2.12, -4.828, p>0.001). However, there was a significant difference between the small and medium mesh sizes (Small-Med: n=30 total, Effect size: -0.970, z=-2.092, p=0.036). There was no significant difference in probability of honeybees fanning in the small compared to the large, or the medium compared to the large. (Small-Large: n=30 total, Effect Size= -0.325, z= 0.645, p= 0.519, Med- Large: n=30 total, Effect size= 0.644, z= 1.473, p=0.414; Figure 3).

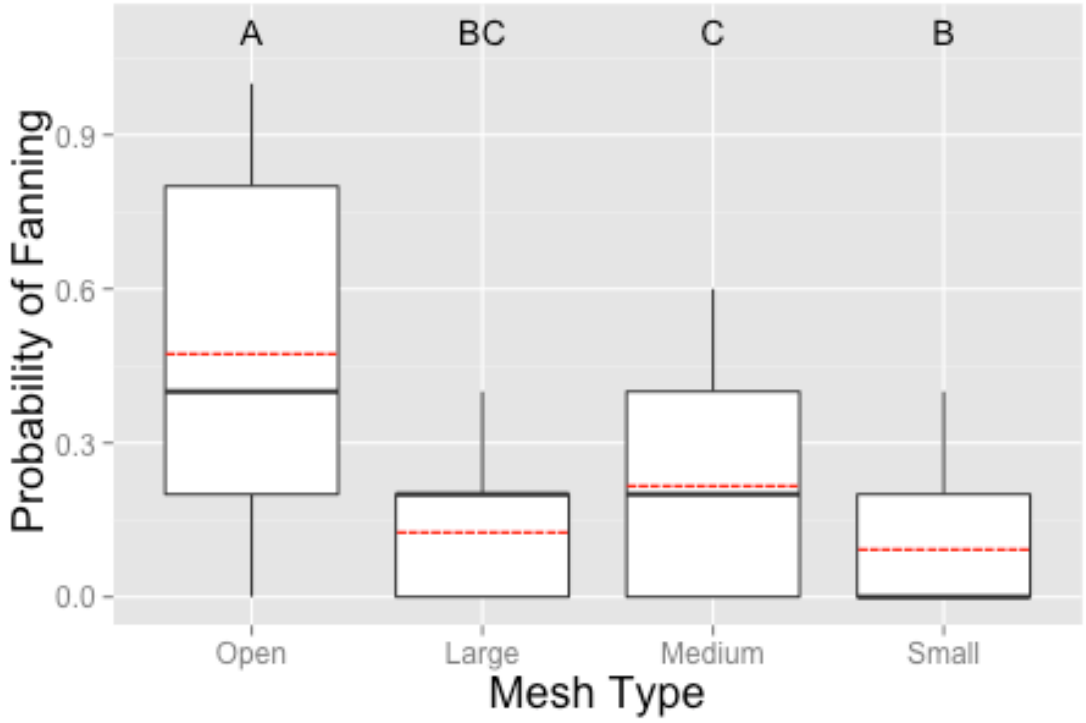

Figure 5.4: A boxplot showing the probability of fanning across Non-Divided cages, small mesh, medium mesh, and large mesh divided cages. Honeybees in Non-Divided cages are significantly more likely to begin fanning than small, medium, or large mesh divided cages. There was no difference across divided cages. Horizontal bars are medians, red dashed lines are means, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers. Letters indicate statistical significance. Created using R Package ggplot2.

Honeybees in non-divided cages initiated fanning at significantly lower temperatures than honeybees that were separated by larger mesh (n=30 total, Effect size= 7.313, t=-2.216, p = 0.0342; Figure 4). In non-divided cages, honeybees began to fan at 35.8°C ±1.7°C while bees in large mesh cages fanned at 43.1°C ± 2.0°C. Honeybees that were divided with small mesh fanned at 38.99 ± 4.18, while bees

divided by medium mesh began to fan at 38.6°C ± 2.9°C. However, there was no significant difference across the non-divided cages, or any of the divided cages (Non-divided-Small: n=30 total, Effect size=3.157, t=3.865, p=0.42; Non-divided-Med: n=30 total, Effect size= 2.786, t=0.875, p=0.388; Small-Med: n=30 total, Effect size = 0.371, t=0.091, p=0.928 ; Med-Large: n=30 total, Effect size=4.527, t-value=1.269, p=0.214; Small-Large: n=30 total, Effect size=4.156, t=0.993, p=0.329; Figure 4).

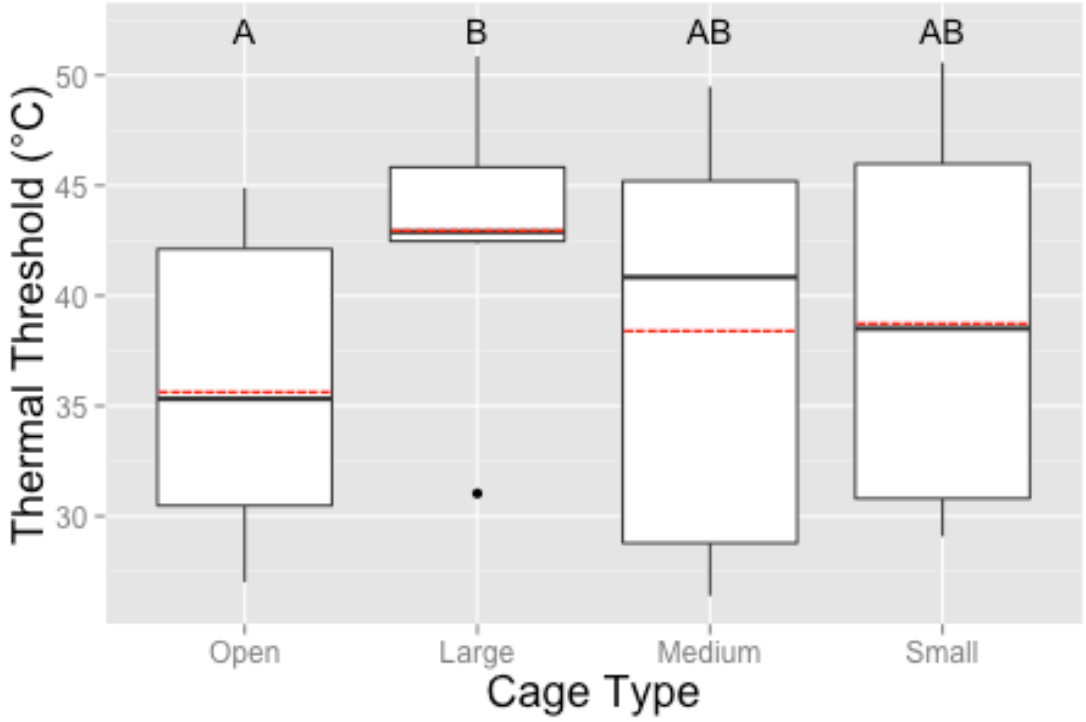

Figure 5.5: A boxplot showing the thermal response threshold across non-divided, small, medium, and large mesh size divided cages. Honeybees in large mesh divided cages fanned at significantly higher temperatures than non-divided cages. There were no significant differences in thermal threshold between any of the other treatments. Horizontal bars are medians, red dashed lines are means, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers. Letters indicate statistical significance. Created using R Package ggplot2.

Hypothesis 3: Nestmate Status Affects The Fanning Response

We found no significant difference in the probability of fanning between nestmates and non-nestmates (n=82 total, Effect size-0.1975, z=0.913, p=0.361; Figure 5).

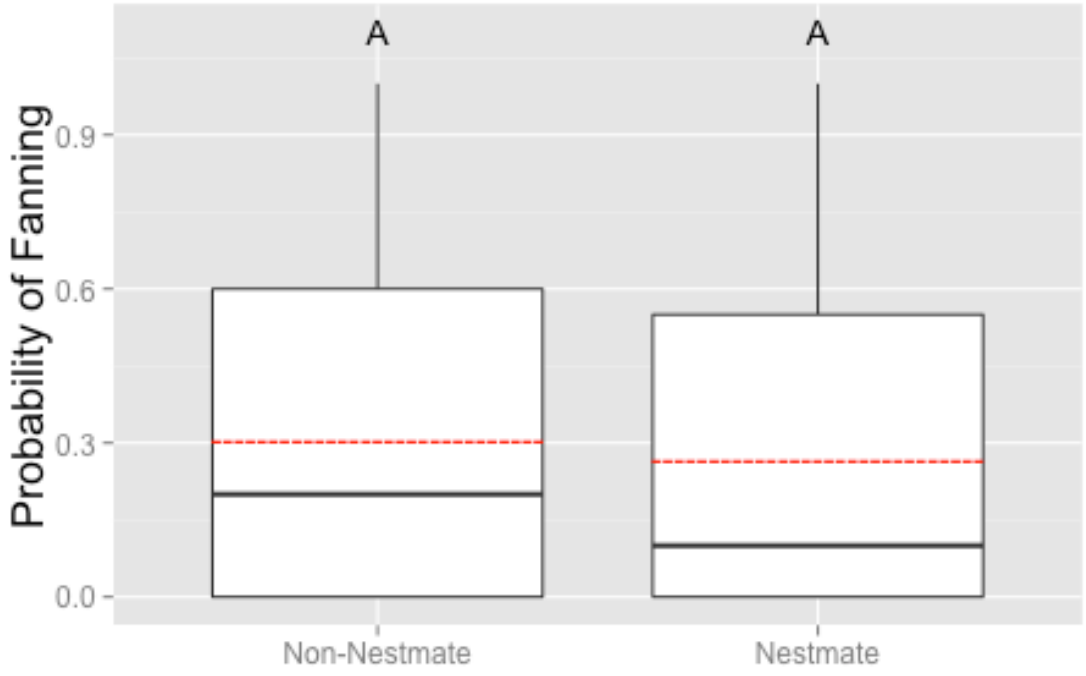

Figure 5.6: A boxplot showing probability of fanning in trials with nestmates compared to non-nestmates. There was no significant difference in the probability of fanning between groups of nestmates and non-nestmates. Horizontal bars are medians, red dashed lines are means, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers. Letters indicate statistical significance. Created using R Package ggplot2.

Nestmates began to fan at 35.4C ±1.52, while non-nestmates began to fan at 38.2± 1.366. However, there was no significant difference in the thermal response threshold between these two groups (n=82 total, df=47 t=-1.36, p=0.18; Figure 6).

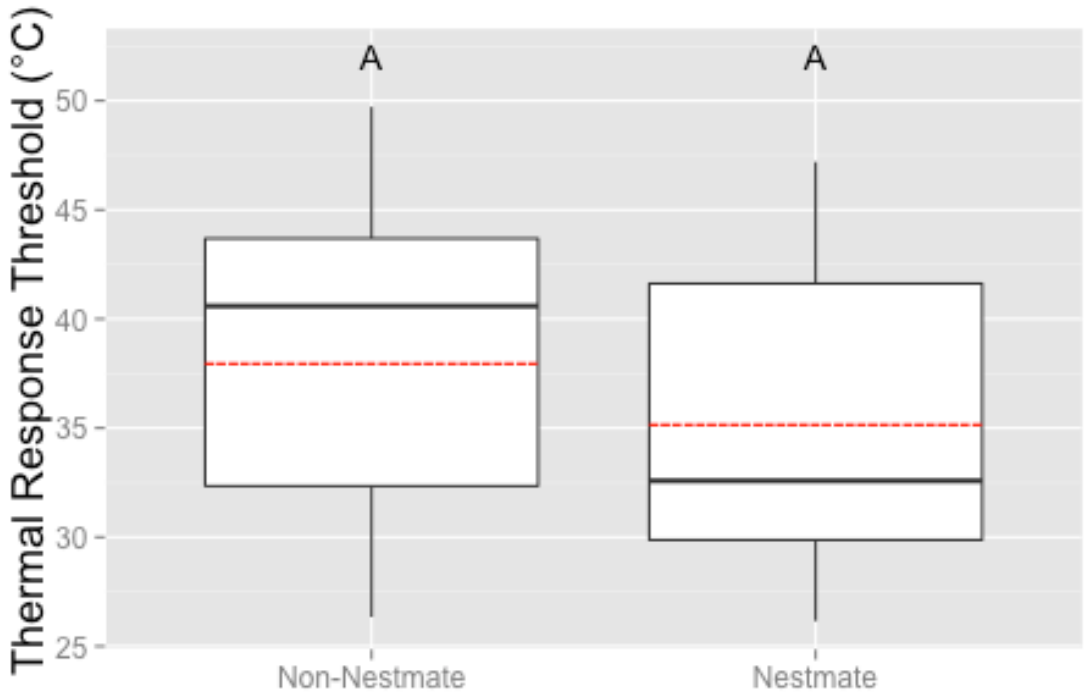

Figure 5.7: A boxplot showing the thermal response threshold in trials with nestmates compared to non-nestmates. There was no significant difference in the thermal response threshold between groups of nestmates and non-nestmates. Horizontal bars are medians, red dashed lines are means, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers. Letters indicate statistical significance. Created using R Package ggplot2.

5.5 DISCUSSION

Transfer of information among individuals is critical for a group to perform a shared task effectively. Here, I explored the methods honeybees could be using to

assess their roles in cooling their colony. In the cages that were divided with a single layer of mesh, honeybees were able to see each other, perceive pheromones, feel vibrations, and have limited tactile contact. The single layer of mesh allowed for slight physical contact, while double layer of mesh eliminated the tactile contact while still permitting pheromonal and vibrational information to pass from bee to bee. There was no statistical difference in the fanning response between these two types of divided cages. My results support the importance of tactile cues in playing a role in the initiation of fanning behavior.

Another cue that was modified in the divided cages was the amount of air movement among fanners. To explore this factor, I designed cages with different sized mesh to disrupt airflow at different rates. Again, in the divided cages, honeybees fanned less and began fanning at higher thermal response thresholds if they were divided, regardless of size of mesh. If airflow played a role in the fanning response, I would see a higher rate of fanning in the large mesh, which let more air through. However, I found that honeybees fanned at significantly lower probabilities than non-divided cages, regardless of the type of divider. This supports my hypothesis that airflow is not a significant cue for honeybees to use to assess when to fan. Winston (1991) hypothesized that airflow plays a larger role in influencing fanning behavior than temperature. I show that this is likely not the case, as airflow does not seem to influence the fanning response, at least in a laboratory setting. In fact, honeybees in non-divided cages were significantly more likely to fan and fanned at lower response thresholds than honeybees in any of the divided cages.

Honeybees are equally as likely to begin to fan whether they are in groups comprised of nestmates or non-nestmates. Honeybees use cuticular hydrocarbons in nestmate recognition, many of which come from the wax they use to make their comb (Breed & Buchwald, 2009; Breed et al., 1995; Couvillon et al., 2007). If a honeybee that doesn't belong, based on the cuticular hydrocarbon profile, tries to come into the hive, the interaction can become aggressive; guard honeybees will bite, sting, and try to remove the intruder (Breed et al., 2004). However, when honeybees were sampled from these different hives and assayed together, I saw no instances of conflict. By personal observation, I saw fewer interactions overall, but honeybees from different hives were not aggressive. Couvillion et al. (2013) found that when honeybee guards were removed from the nest entrance, they behaved less aggressively toward non-nestmates, compared to when they were at the nest entrance. This shows that honeybees become less defensive and discriminatory when they are not at their nest. In the assay, as temperatures increased during the trial, they even fanned at statistically identical rates and thermal thresholds. Away from the nest there is not a need to engage in costly defensive behaviors.

There were slight differences between the different sizes in probability of fanning between the small and medium sized mesh cages. I found that honeybees in the small mesh were less likely to fan than bees in the medium mesh, but there was no difference between the small mesh and the large mesh, or the medium mesh and the large mesh. The small mesh, because the holes are much smaller, might be obscuring other potential cues, such as visual cues, which could confound my results. However, the

large mesh is so wide and is letting the most air through, but still inhibits the fanning response. Fanning is stimulated by both increasing temperatures and social interactions. Furthermore, those social interactions need to have a tactile component, not just airflow, for fanning behavior to occur.

In honeybee communication, tactile cues are a major part of the information conveyed in the hive (Erber et al., 1997; Erber, 2015). Tactile information can come in many forms for honeybees, including antennal contact and proboscis contact. For example, when a honeybee is gathering information from another honeybee performing a waggle dance, a significant amount of that information comes from the receiving bee touching the other as she waggles. Rohrseits & Tautz (1999) found that receivers have antennal contact with a waggler for more than 60% of the time the dancer is waggling. Honeybees also exchange fluid, or trophallax, with each other constantly. While this is often viewed simply as food exchange, much information, such as food quality (Farina & Nunez, 1991; Tezze & Farina, 1999) and floral odors (Farina et al., 2007) can also be conveyed. More directly related to this study, Farina and Wainselboim (2001) found that hotter bees unloaded nectar faster than cooler bees. While the exact mechanism for the fanning response is unknown, thermal information could play an important role in what is exchanged during these physical interactions.

Thermoregulation in the honeybee hive is critical for the survival of the colony. If the temperature exceeds 36C, the developing larvae inside can die (Himmer, 1932; Martin Lindauer, 1952)). While there are some mechanisms of thermoregulation that do not require communication, such as swarm clustering in cold ambient temperatures

(Lemke & Lamprecht, 1990; Myerscough, 1993; Ocko & Mahadevan, 2014; Omholt, 1987; D J T Sumpter & Broomhead, 2000), fanning does not fit this hypothesis. In models of clustering to keep warm, the group behavior can be predicted by individual responses to ambient temperature: if a honeybee is cold, she moves to the inside of the cluster. If she is warm, she moves to the outside (Watmough & Camazine, 1995). However, in fanning, when a honeybee is warm and around others, she begins to fan, which increases her own temperature. The group fanning response does not come from individuals following simple rules, but seems to require more sophisticated communication. It would therefore make sense that whatever is being communicated is effective and elicits a rapid response when needed. While I have ruled out several methods by which honeybees may be communicating, such as volatile pheromones and airflow, I still don't know the exact cue or cues honeybees utilize to fan. Regardless of the method of assessment, I have shown that perception of information among bees, rather than just ambient temperature, is crucial for initiation of the honeybee fanning response.

Information transfer of this type is key to homeostatic functions in social insect colonies. Fewell (2003) showed that certain individuals can act as hubs, spreading information to other individuals. Interactions of this type among individuals cause the formation of social networks. Networks are often established by direct, tactile, contact, and ensure that colony-sustaining jobs such as foraging or brood care, are performed efficiently (Bonabeau et al., 1997). Often times, social networks are sustained via basic types of contacts, such as intensity of interaction (O'Donnell 2001) or number of

interactions (Pacala et al. 1996) and can influence the jobs that are performed. It is largely unknown if qualitative information is being exchanged, but several theoretical and empirical studies (Bonabeau et al.,1998; Bonabeau et al. 1996; Bonabeau et al., 1997; Detrain et al., 1999) have shown extreme modulation of task performance based on these fleeting interactions. Understanding how interactions, information transfer, and communication function in social groups furthers our understanding of how coordinated behaviors can evolve.

CHAPTER 6

Octopamine and Tyramine Regulate the Thermoregulatory Fanning Response in Honeybees (*Apis mellifera L.*)[4]

6.1 ABSTRACT

Biogenic amines regulate the proximate mechanisms underlying most behavior. These molecules cause the physiological changes in the individual that lead to a behavior. While the role of biogenic amines is relatively understood in individuals, how biogenic amine-mediated individual responses influence group behavior is not fully understood. Here, I explore how changes in biogenic amines can modulate the performance of a group-performed thermoregulatory fanning behavior in honeybees. The concentrations of two biogenic amines, octopamine and tyramine, are significantly lower in active fanners than in non-fanners. I then establish a causal relationship by demonstrating that honeybees treated with these biogenic amines showed decreased fanning responses, but only when both amines were included in the treatment. This is the first evidence that fanning behavior is influenced by these two biogenic amines and this finding is consistent with other studies of the role of these amines in regulating insect behavior. This exploration of the proximate physiological mechanisms that mediate fanning behavior increases our understanding of what triggers individuals to behave and how individual behavior coordinates with group responses.

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6.2 INTRODUCTION

Chemical messengers, including biogenic amines, coordinate the behavioral responses of animals (Nelson 2010). Biogenic amines often serve as neurotransmitters or neurohormones and play a significant role in proximate mechanisms of behavioral regulation in all animals, including insects (Blenau & Baumann, 2001; Roeder, 2005; Scheiner et al., 2006; Verlinden et al., 2010). The behavioral roles of biogenic amines are particularly interesting in studies of social insect societies, as behavioral changes induced by these chemicals can be observed at the individual level, but they can also alter the collective behavior of the colony. Collective behavior in social insects can translate into division of labor in task performance, and exploration of the proximate mechanisms that control individual behavior has the potential to lead to a deeper understanding of the regulation of division of labor.

Honeybee societies have emerged as a model system to study how changes in biogenic amines affect individual behavior (Fussnecker et al. 2006; Lehman et al., 2006; Pankiw & Page, 2003; Sagili et al., 2011; Schulz & Robinson, 1999; Schulz & Robinson, 2001a). Four biogenic amines have been identified as playing significant roles in the honeybee division of labor: dopamine (Agarwal et al., 2011), serotonin (Harris & Woodring, 1992), tyramine (Fussnecker et al., 2006; Matsuyama et al., 2015), and octopamine (Barron et al., 2007). For example, honeybee workers treated with octopamine show an increase in overall activity (Fussnecker et al., 2006b) and an increase in octopamine is correlated with the onset of foraging by the workers (Schulz & Robinson, 2001b). Furthermore, Pankiw and Page (2003) found that honeybees treated

with octopamine had significantly lower sucrose response thresholds than controls. This is important as sucrose response thresholds dictate when and what food a forager collects (Barron et al., 2002; Scheiner et al., 2004). Seemingly small physiological changes in individuals can have major implications for the entire colony because such changes are magnified through cascading social interactions within the social group.

I use honeybee thermoregulation to further explore how biogenic amines regulate behavior in social insects. Thermoregulation is one of the critical behaviors that must be performed within honeybee colonies to ensure adult and larval survival in honeybees (Fahrenholz et al., 1989; Himmer, 1927; Lindauer, 1952). When brood is present, honeybee workers regulate the temperature of the hive at 36°C. During the summer, honeybees use foraged water to spread on the wax honeycombs to evaporatively cool the brood (Kühnholz & Seeley, 1997). They also form heat shields by pressing their bodies on comb to absorb heat then disperse (Siegel et al., 2005; Starks & Gilley, 1999), and workers fan to move hot air out of the colony and allow cool air to flow in (Egley & Breed, 2013). I focus on fanning behavior because it is a group-performed behavior that is influenced by individual response thresholds (Jones et al., 2004; Cook and Breed 2013).

To explore the influences biogenic amines have on fanning behavior, first I had to identify if there were any differences in fanning bees compared to non-fanning bees. I hypothesized that honeybees that were actively fanning would have different concentrations of neurotransmitters than those who were not fanning. I found differences in the concentration of specific neurotransmitters in association with fanning

behavior, which led to experiments to verify a causal relationship by treating honeybees with biogenic amines. I hypothesized that those bees treated with specific biogenic amines would show a significant difference in fanning behavior when compared to controls. Finally, I hypothesized that there would be differences in biogenic amines between guards and fanners, which are of similar age and are collected from the same location in a colony, but performing different jobs. This multi-faceted approach of measuring biogenic amine levels and treating bees with those amines helps to elucidate the role biogenic amines play in the fanning response of honeybees.

6.3 METHODS

General Honeybee Keeping

I used honeybees collected from 10 European honeybee colonies at the Honeybee Research Laboratory at the ASU Polytechnic Campus in Mesa, Arizona, 85212. All collections took place in May 2015.

Comparing Induced Fanners Not Induced Bees in the lab

I wanted to compare the biogenic amines of bees that were actively fanning to bees that were not fanning. I collected 2 groups of 10 fanners at the hives. Fanners were identified as honeybees that were standing still, rapidly moving their wings for at least 10 seconds, with a curved abdomen. This is distinct from Nasanov fanning which serves to spread a pheromone (Free 1987). In groups of ten, honeybees are relatively likely to begin to fan (Cook & Breed, 2013). Fanning is a group response that is almost

always initiated all at once, so I therefore only used trials where all ten honeybees fanned. I collected fanners by grabbing them by their back legs using forceps, and placing them into a containment cage. This cage was made of wire screen, formed into a cylindrical shape (5cm x 2.5cm). I brought the two cages with ten bees each into the lab. I then placed each group of bees into a heating apparatus. This apparatus consisted of a 1 gallon glass jar placed on a hotplate (Proctor Simplex). The hotplate's coils were lined with aluminum foil to buffer against heat. I used bamboo skewers to prop up the cages so they did not come into contact with the bottom or edges of the jar. I used high accuracy digital thermometers (±0.3°C, Cole Parmer) to take air temperatures. The probe was placed at approximately the center of the jar, where the cage and honeybees were sequestered. These collections took place in May 2015.

Heating Regime

Once the groups of honeybees in cages were placed into the jars, I allowed them to acclimate for 25 minutes (Cook & Breed, 2013). After the acclimation period, I recorded the start temperature and time. I then began to heat only one group at a rate of 1°C/minute (Cook & Breed, 2013, Cook et al. 2015***). I identified fanners using the same criteria as above. The bees that did not fan experienced the same environment as the fanner bees, except for the heating regime. Once honeybees in the heating regime began to fan, I randomly (either fanners or non-fanners first) removed the cages from the jars and placed the cages directly into liquid nitrogen as quickly as possible. All ten

honeybees had to fan to be included. Honeybees that were not induced to fan never fanned in their trials.

Collection of Fanners and Guards at the Hive

To compare the biogenic amine concentrations of honeybees from the hive environment, I sampled both fanners and guards on the hive porch. Fanners and guards belong to the same age cohort (Egley & Breed, 2013) and are statistically as likely to begin fanning (Cook & Breed, 2013). The criteria for identifying fanners both in the assay and at the hive were the same: fanners at the hive were identified as honeybees that were standing still, rapidly moving their wings for at least 10 seconds, with a curved abdomen. This is distinct from Nasanov fanning to spread a pheromone (Free 1987), and other behaviors such as some defense (Yang et al., 2010) or taking off for orientation flights. I focused on entrance fanners for two reasons: 1) they are more likely engaging in thermoregulation than fanners that may be distributed throughout the hive who are fanning to evaporate water from honey and 2) they are relatively easy to identify and collect.

I collected honeybees by grabbing them by their back legs with forceps. I then submerged them immediately in liquid nitrogen to snap freeze them. The dewar (Cole Parmer Transport Dewar) I used was divided with mesh so that I could collect both fanners and guards at the same time but not mix them up. I alternated, randomly selecting a guard, then a fanners, back to a guard. I collected 150 bees total, across 10 hives.

Collection of Honeybees Across Their Lifetime

I established marked cohorts of honeybees within each colony by collecting two frames of brood comb. I selected frames that appeared to have ready-to-emerge worker bees, and removed all already-eclosed workers. I placed the frames into an enclosed observation container (wood frames, two long sides of Plexiglas), then placed the entire container with frames into an incubator. The incubator was set at 33°C. I let bees emerge from 10am until 6pm. I then collected all emerged bees, then marked them with a color unique to that date and hive, using a dot of paint (Testor's) on the dorsal side of their thorax. For each cohort, I marked between 100-120. I then transported them back to their hives, releasing the marked bees and replacing the brood frames.

Cook and Breed (2013) established that fanning behavior changes across the behavioral task groups of the worker honeybee. Honeybees that were fanners were the most likely to begin to fan, whereas foragers were the least likely to fan. Nurses and guards were slightly less likely to fan compared to fanners. These task groups are correlated with age (Huang & Robinson, 1996). Therefore, I wanted to establish how fanning behavior changed at specific ages. I sampled bees from the marked cohorts at days 1, 5, 10, 15, 20, 25, and 30 when possible. In some instances, I had to collect bees ±1 day to account for access to hives, liquid nitrogen, and dry ice (which will be discussed later).

After the honeybees were snap frozen in liquid nitrogen, I placed them into marked zip top baggies and stored them whole in a -80°C freezer until transportation

from the ASU Honeybee Laboratory to the USDA ALARC. For transport, I placed baggies in a cooler with dry ice. Immediately upon arriving to the USDA facility, the honeybees went back into a -80°C freezer until dissection.

Dissections of brains

For dissection, I chose 3 bees at a time for each individual sample. I removed the head from the body and placed the head face-up on a chilled wax surface that sat on top of dry ice. The head was pinned down using 2 pins – one through the clypeus and one through a compound eye. I then dissected the frons - or front face plate - to expose the hypopharangeal glands. The hypopharangeal glands, while frozen, are easily scraped off. Next, I cut the optical lobes. I then scooped the brain out using forceps and placed them into a 1.5 mL Eppendorf tube with the 20 μL of PCA solution. I pooled 3 brains from the same treatment and collection. These analyses took place in May 2015.

I extracted the biogenic amines from the brains using 20 μL of solution, made of 0.2M perchloric acid and two internal standards, DHBA and Synephrine (100000pg/μL). Brains were homogenized in this solution for 1 minute, then further sonicated in an ice bath for 5 minutes. I let the tissue extract further while sitting in an ice bath for 15 minutes. After extraction, I spun the samples in a refrigerated centrifuge (4°C) for 10 minutes at 12,000 RCF.

Samples were kept on ice and covered until being run on the HPLC. A maximum of 6 samples were prepared at a time to reduce the amount of time they sat around,

which can degrade the amines. I used 10 μL of the supernatant to determine the amine concentrations.

HPLC Analysis of Neurotransmitters

I used High performance liquid chromatography (HPLC) to measure the biogenic amine concentration in the samples. The HPLC system (ESA, Chelmsford MA, USA) was a Coularray model 5600A with a 4-channel electrochemical detector (ED). It is comprised of a model 582 pump and a reverse –phase catecholamine HR-80 column. I injected samples using a manual injector (Rheodyne 9125, Rohnert Park CA, USA) with a 20 μL loop. Channel 1 was set at 650mV for octopamine and tyramine, while channel 2 was set at 425mV for dopamine and serotonin, and channel 3 was set at 175mV. The mobile phase consisted of 15% methanol, 15% acetonitrile, 1.5mmol l^{-1} sodium dodecyl sulfate, 85 mmol l^{-1} sodium phosphate monobasic, 5mmol l^{-1} sodium citrate, and polished water. I adjusted the pH of the solution to 5.6 using phosphoric acid. I set the mobile phase flow rate at 1mL min^{-1}. All of my results are presented as concentrations per brain, so I divided measured concentrations by 3. I compared peaks from honeybee brain samples to a set of standards (DA, OA, 5-HT, TA; Sigma Aldrich) that were run before and after every 6 to determine the quantity in the samples.

Treatment with Biogenic Amines

To test the role of biogenic amines in fanning behavior, I fed honeybees solutions from 11 colonies from the University of Colorado apiary. I chose feeding to treat the

honeybees because it is just as good as injections (Barron, Maleszka, Vander Meer, Robinson, & Maleszka, 2007) but I believe it has more controllable behavioral impact but not potentially perturbing the behavior by piercing their exoskeleton.

Biogenic Amine Treatments

I treated honeybees with one of 3 biogenic amine treatments, 1 positive control, and 1 negative control. The negative control was 2M sucrose solution. For the positive controls and treatments, I used a concentration of 2mg/mL biogenic amines. I chose this concentration because it was found to be effective in influencing honeybee behavior via feeding (Barron et al., 2007). The biogenic amines I used were in powder form, so I measured 2mg into a glass scintillation vial, and added 1mL of 2M sucrose solution right before administering to bees. The positive control was a biogenic amine, synephrine, which is used as an internal standard in the HPLC analyses. This amine has never been shown to influence insect behavior. Octopamine and tyramine were also prepared this way. For the octopamine + tyramine treatment, I used 2mg each. I did this because octopamine and tyramine act independently on separate g-coupled protein receptors (Roeder, 2005). Powders were kept shielded from light using aluminum foil sleeves & covers, and solutions were kept in aluminum foil sleeves to protect them from light, as well as being on ice, inside a covered cooler. New solutions were made every morning, and not more than 3 trials were run with the same solution.

Administering Biogenic Amines to Honeybees

Once solutions were made, I had a lab assistant who was not involved in the experiment blind the solutions by color-coding the vials. I brought the solutions out to the apiary, where I randomly selected a hive that had fanners present on the entrance. Biogenic amines take about 30-40 minutes to influence behavior but degrade quickly after (Fussnecker et al., 2006b). I decided to use a group size of 5 honeybees to also minimize the amount of time the first honeybee within a group was waiting, metabolizing the amines.

I identified fanners as stated above. I collected fanning honeybees by grabbing them by their two back legs. As I held them with the forceps, I aliquated 10 μL of solution into a pipette. I then touched the pipette to the antennae of the honeybee until she extended her proboscis and drank the entire droplet. If the honeybee did not drink within 30 seconds, or if she did not drink the entire droplet, I released her and chose another fanner.

I then placed each bee into the cage for transportation back into the lab. Each cage was color-coded to match the blinded color of the treatment vial for that day, so that I knew which cage was treated with each solution in the color-coded vial, but I did not know which treatment was being administered. To further minimize the amount of time elapsed through collections, myself and one other research assistant performed feeding and collection for the 5 treatments at the same time (with the person doing the collections the quickest moving on to the 5th treatment, when I had 5). For one round of all 5 treatments, I collected all bees from the same hive to control for inherent hive differences.

I transported the cages into the lab, and then placed the cages containing the honeybees into the heating chamber. There, they acclimated for 20 minutes. I used the same heating protocol and behavioral assay as stated above. I recorded if fanning occurred, the proportion of honeybees fanning out of 5, and the temperature at which they begun to fan. All treatments took place July - September 2015.

Statistical Analysis

To analyze the neurotransmitter concentrations of induced fanners to non-induced fanners in the lab, as well as in fanners and guards at the hive, I performed a Mann-Whitney rank sum test. I did this because the biogenic amine concentrations were not normally distributed, as tested for by the function “qqnorm( ) “in R. I used the “wilcox.test( )” function in the R base package to perform the Mann-Whitney test. In both of these instances, the null hypothesis is that biogenic amine concentrations will be equal in the two treatment populations. All analyses were performed on a per brain concentration, so the measured concentration divided by 3. We pooled samples for a more robust HPLC analysis, but wanted to explore the biogenic amines per individual brain, so as to have biologically relevant data.

To analyze the effect of biogenic amine treatments, I looked at both the proportion of fanners and the thermal response threshold of fanning as response variables. I used a generalized linear mixed model to perform a logistic regression (link=logit), using the “glm( )” function in the base package R. I performed a logistic regression as the response variable is proportion of fanners. To analyze the thermal

response threshold, I used a generalized linear model with a Gaussian distribution, as the response variable was temperature, a continuous variable. Again, I used the function “glm( )” in R.

I analyzed neurotransmitters across the lifetime of honeybees. I compared the concentration of biogenic amines on day 1, 5, 10, 15, 20, and 25 using an ANOVA using the “aov” function in R. The predictor variable was day, and I treated it as a categorical variable. The response variable was per brain concentration of biogenic amines. I then performed a post-hoc (Tukey) analysis using the “TukeyHSD” function to evaluate which pairwise comparisons were significantly different. I made graphs using the ggplot( ) function in R (version 3.0.2) and R Studio (version *0.98.1103).*

6.4 RESULTS

HPLC Analysis of induced versus not induced fanners

I compared the 4 neurotransmitters (octopamine, dopamine, tyramine, and serotonin) of induced fanning honeybees and non-induced honeybees. Induced fanners had significantly lower concentrations of octopamine (MW rank sum test: T=319, n=40, p=0.014; Figure 1) and tyramine (MW rank sum test: T=306.5, n=40, p=0.005; Figure 2) than non-induced bees. There was no difference between induced fanners and non-induced workers for dopamine (MW: 392, n=40, p=0.636) or serotonin (MW: 373.5, n=40, p=0.330).

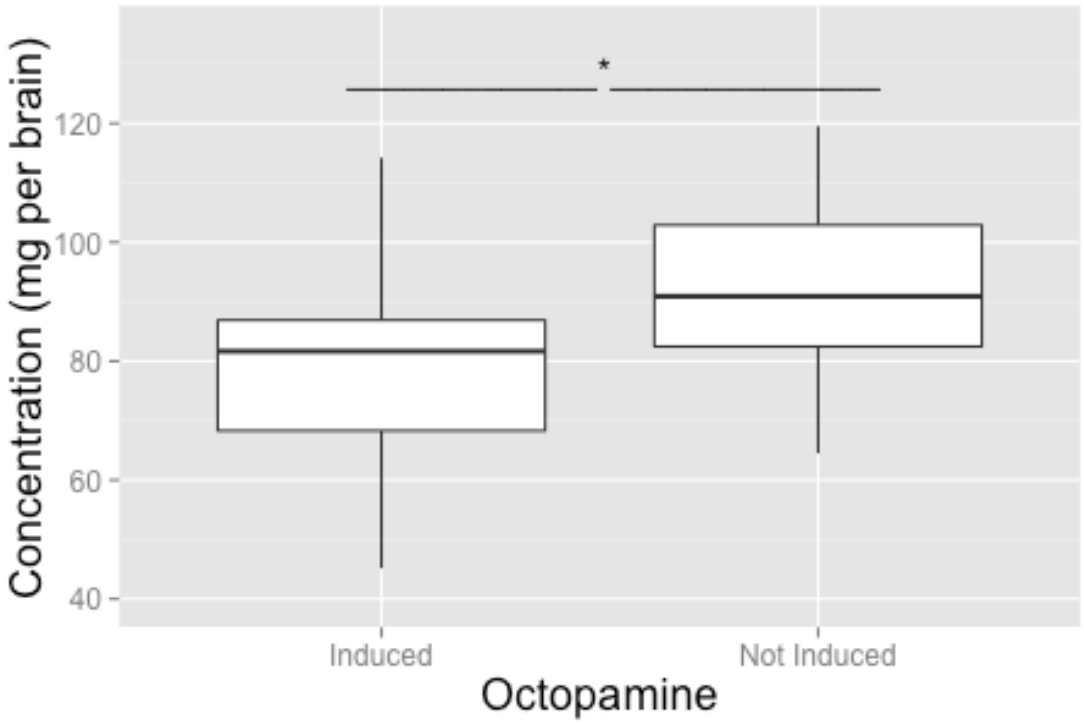

Figure 6.1: A boxplot showing the significant decrease in Octopamine in induced fanners compared to non-fanning honeybees. Horizontal bars are medians, boxes are 25 – 75th percentile, lines are 1.5 * IQR, points are Tukey outliers (N=74). Created using R Package ggplot2.

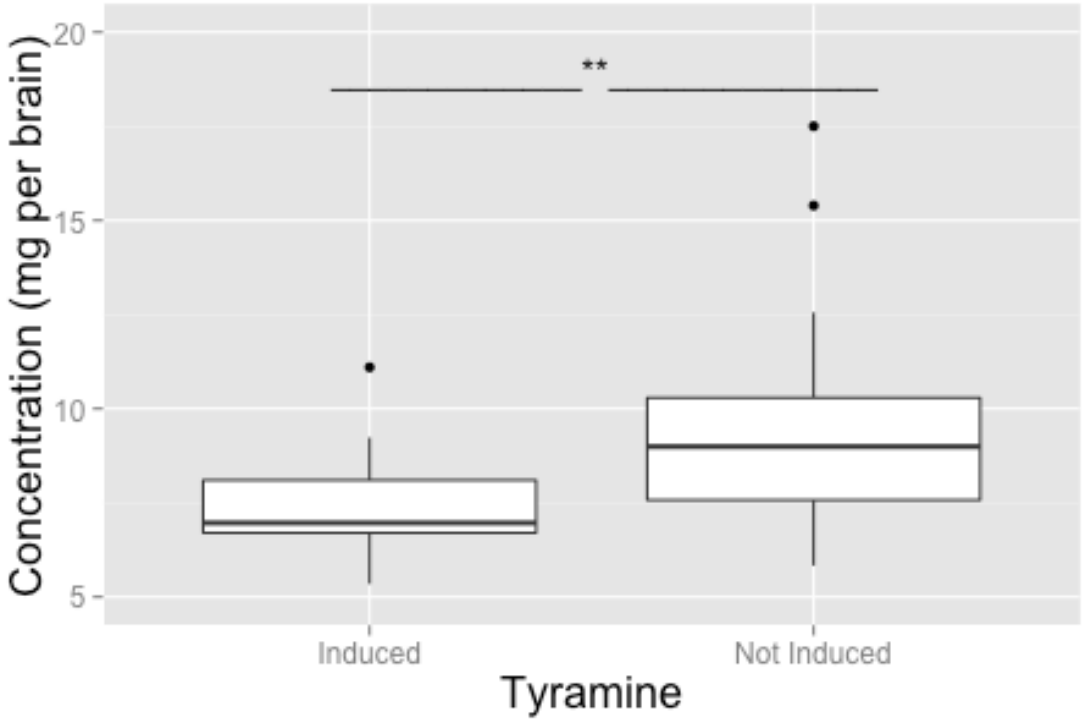

Figure 6.2: A boxplot showing the significant decrease in tyramine in induced fanners compared to non-fanning honeybees. Horizontal bars are medians, boxes are 25 – 75th percentile, lines are 1.5 * IQR, points are Tukey outliers (N=74). Created using R Package ggplot2.

Treatment with Biogenic Amines

To test for a causal relationship for octopamine and tyramine in fanning behavior I treated fanners with these biogenic amines. Honeybees that were treated with both octopamine + tyramine were significantly less likely to begin fanning than controls (Sucrose: Effect size = -0.79, Z= -2.966, p=0.003; Synephrine: -0.6325, z=-2.338, p=0.0194). Additionally, octopamine treated bees were significantly more likely to fan than those treated with tyramine (n=52, Effect size=0.523, z=2.033, p=0.0421), and those treated with octopamine + tyramine (n=52, Effect size=0.822, z=1.225, p=0.002). There was no significant difference in probability of fanning between the octopamine

and controls (Sucrose: n=52, Effect size = 0.031, z=-0.123, p=0.90; Synephrine: n=51, Effect size=0.189, z=0.7245, p=0.456), or tyramine treatment and the octopamine + tyramine treatment (Effect size = 0.298, Z = 1.089, p=0.276). There was also no significant difference in probability of fanning between tyramine and the controls (Sucrose: N=52, Effect size=0.49, z=1.91, p=0.0561; Synephrine: n=51, Effect size=0.33, z=1.277, p=0.201).

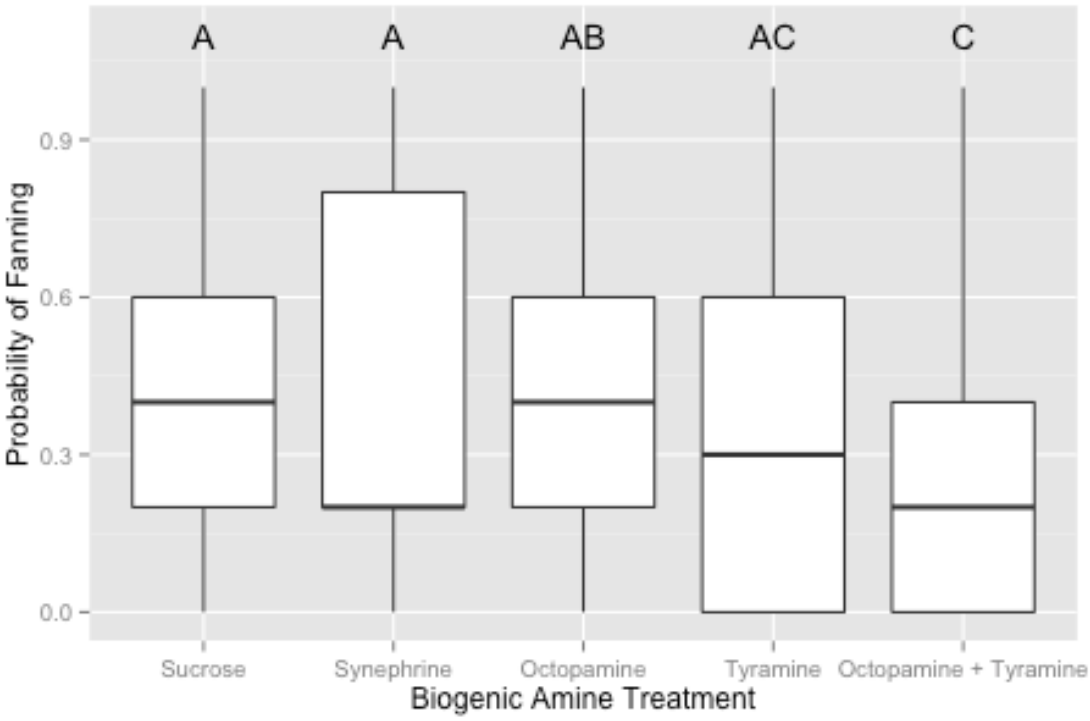

Figure 6.3: A boxplot showing the probability of fanning across biogenic amine treatments. Octopamine + Tyramine treated bees fanned at a significantly lower rate than controls and the octopamine treatment, but was not significantly different from the tyramine treatment. All other treatments were not significantly different from controls. Horizontal bars are medians, boxes are 25 – 75th percentile, lines are 1.5 * IQR, points are Tukey outliers (n=74). Created using R Package ggplot2.

Biogenic Amines of Fanners and Guards

Because Cook and Breed (2013) found that guards and fanners are the most likely task groups to fan, I next tested the hypothesis that differences in the biogenic amines levels exist between guards and fanners. There were no significant differences in any of the measured biogenic amines between guards and fanners (MW rank sum test: Octopamine: T=608.5 n=48, 0.68; Dopamine: T=518, n=48, p=0.152; Tyramine: T=606, n=48, p=0.718; Serotonin: T=534.5, n=48, p=0.274).

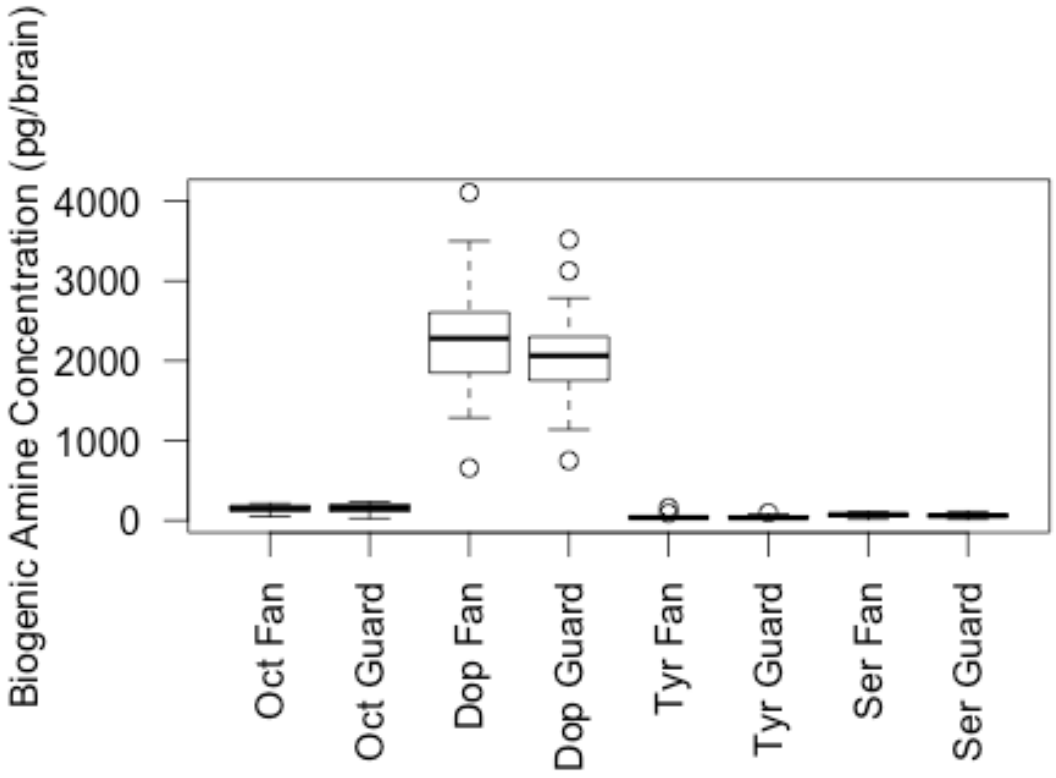

Figure 6.4: A boxplots comparing the 4 biogenic amines in fanners and guards collected at the hive. There was no significant difference in any of the biogenic amines measured between these two groups. Horizontal bars are medians, boxes are 25 – 75th percentile, lines are 1.5 * IQR, points are Tukey outliers (N=74). Created using R Package ggplot2.

HPLC Analysis of Honeybee Workers Across Lifetime

I found a significant difference in octopamine on day 20 compared to day 1 (n=40, Effect size: 0.008, t=2.707, p=0.0113). There was no significant difference between any of the other days. There were no significant differences across dopamine, serotonin, or tyramine (Data not shown). However I did see some trends, which compared to previous experiments (Schulz & Robinson, 1999) that shows similar changes.

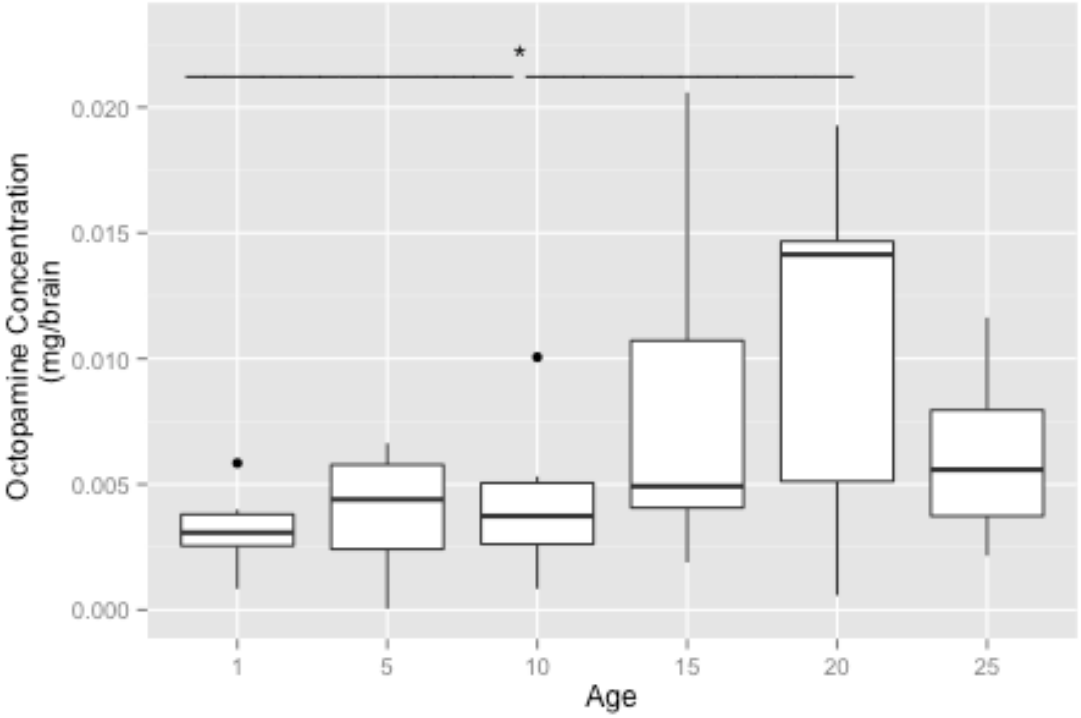

Figure 6.5: Changes in Octopamine across the lifetime of worker honeybees. Horizontal bars are medians, boxes are 25 – 75th percentile, bars are 1.5 * IQR, points are Tukey outliers (n=148). Created using R Package ggplot2.

6.5 DISCUSSION

Neurotransmitters play a significant role in many aspects of honeybee behavior. Here, I report that octopamine and tyramine play an important role in expression of the thermoregulatory fanning behavior. Using HPLC (Hartfelder et al., 2013), I measured a decrease in both tyramine and octopamine in fanning honeybee brains. Honeybees induced to fan in the lab had significantly lower concentrations of both of these biogenic amines than controls. Based on these results, I tested for a causal relationship with those biogenic amines and fanning behavior by using an established protocol of feeding biogenic amines to honeybees (Barron et al., 2007). I found that honeybees treated with both octopamine + tyramine fanned significantly less than honeybees fed either of these chemicals by themselves and controls. While there was not a significant difference from the controls, tyramine appears to play more of a role in inhibiting the fanning response than octopamine (Figure 1). Treatment with both octopamine and tyramine inhibited the fanning response to levels that are significantly lower than that of the controls. This could indicate that the synergistic effects of octopamine and tyramine together influence fanning behavior. These findings suggest these two biogenic amines play a significant role in eliciting the fanning response.

While I do see a difference in fanners induced in the lab compared to non-induced fanners, I found that there was no significant difference in the four measured biogenic amines between guards and fanners collected at the hive. While fanners were actively fanning, guards are also present at the entrance of the hive, experiencing the

exact same environment. Cook and Breed (2013) found that there was no significant difference in the probability of fanning between guards and fanners (Cook & Breed, 2013). My results show that there was no significant physiological difference between these two task groups, and provide further support of my previous findings. The environmental similarities of guards and fanners may be playing a larger role in their similar physiologies than the behavioral differences.

Why is there a difference in actively fanning honeybees in the lab, but not from the field? Guards and fanners are known to switch between these two tasks (Egley & Breed, 2013), with both task groups primed for fanning as they are experiencing similarly hot temperatures. While high temperatures may cause an amine reduction in both guards and fanners, only in fanners does this decrease dip below a critical threshold to release the fanning response. The lower concentrations of tyramine and octopamine in induced fanners may trigger a release of fanning behavior in response to a change in temperature. In an analogous regulatory mechanism, foraging is regulated by the sucrose receptor expression differences, which correlate with whether a honeybee will be a pollen or a nectar forager (Hunt et al., 1995; Erber et al., 1998). A similar process may be at work influencing the fanning response in middle-aged worker honeybees, which may be the reason I see the differences in induced bees in the lab, but not those at the hive. Recent research shows differences in gene expression of octopamine receptors and division of labor (Reim & Scheiner, 2014). Further analysis should explore octopamine and tyramine receptor expression in relation to specific behaviors.

Biogenic amines show significant correlations with division of labor of honeybees (Schulz & Robinson, 2001). Worker honeybees go through a substantial shift in behavior around day 20 of their life. At that point, many workers shift from hive duties to foraging duties (Winston, 1991). Prior to this shift, workers typically perform jobs on the periphery of the hive, such as fanning (Schulz et al., 2003). While a small sample size may have contributed to the largely insignificant results, I did measure a significant increase in octopamine around day 20. Octopamine is a main biogenic amine in invertebrates (Roeder, 2005), and plays a critical role in honeybee behavior (Bateson et al., 2011; Farooqui, 2007; Giray et al., 2015). Much is known about the broad influence of biogenic amines in division of labor in honeybees (Schulz et al., 2003; Wagener-Hulme et al., 1999). These results provide further insight into how this critical thermoregulatory fanning behavior is also largely regulated by biogenic amines.

The optimal performance of tasks in social insect societies relies on differences individual response thresholds (G E Robinson, 1992). Octopamine is known to play a role in modulating the honeybee sucrose response threshold; honeybees that are treated with octopamine have significantly higher sucrose response thresholds (T Pankiw & Page, 2003). Honeybee fanners respond to increasing temperatures; there are more fanners at the hive on hotter days (Egley and Breed 2013). However, Cook and Breed (2013) found that the social environment modulates this thermal response threshold; honeybees fan at lower temperatures when group size is larger. While I didn't find a significant effect of biogenic amines on the thermal response threshold for fanning, I did show that the likelihood of fanning is decreased by treatment of

octopamine + tyramine. Jeanson and Weidenmuller (2014) emphasize probability of response, as sometimes, individuals just don't respond. Here, I show that while these biogenic amines didn't alter the thermal response threshold, it did alter the probability of fanning. This inhibition of a behavior can have major implications in the division of labor in a honeybee colony.

Thermoregulatory fanning in honeybees occurs as a group response. Cook and Breed (2013) have demonstrated that larger social groups increase the fanning response. However, when honeybees were fed octopamine + tyramine, they were less likely to begin to fan, even in a social group. Even if one bee was still likely to begin to fan, it did not set the other bees fanning, as typically happened in the untreated groups. This inhibition of behavior shows that a physiological change in individuals can have major implications for the performance of fanning for the entire group. Fanning is most effective when multiple bees are performing the job, and therefore, this alteration of the group response by modulating biogenic amines may have implications for the temperature regulation of the hive as a whole.

There are many factors that could influence the initiation and threshold at which fanning behavior begins. These experiments are the first to link the environmental and social cues with the proximate mechanisms that influence this critical fanning behavior. By exploring the relationship between physiological mechanisms and performance of behavior I help to provide the framework by which to explore how individual variation among many workers optimizes task allocation and the division of labor in eusocial insects.

CHAPTER 7

Summary and Conclusions

7.1 SUMMARY OF KEY FINDINGS

Honeybee division of labor has been extensively studied, informally for many centuries, formally for many decades. However, exploration of the mechanisms of the fanning response was emphatically lacking. My dissertation not only fills that gap, but also provides a model system by which to more thoroughly explore the group, individual, and physiological mechanisms of an important thermoregulatory behavior. I show that the honeybee fanning response does occur with increasing temperatures, but only when honeybees are in groups. I also show that some behavioral task groups within the colony, such as fanners and guards, are more likely to perform fanning than others, such as foragers (Chapter 2). Because precise thermoregulation is critical for the development and survival of larvae, I provide evidence that a single honeybee, while not likely to fan by herself, is significantly more likely to fan when experiencing increasing temperatures along with a larva. However, this only occurs when the adult honeybee can directly contact the larva. Brood pheromone does not seem to play a role in the fanning response (Chapter 3). I then illustrate that honeybee groups are actually cueing in on rate of temperature change, as honeybees in groups of ten fan significantly more and at lower temperatures when being heated fast, compared to smaller groups or slower rates of temperature change (Chapter 4). In addition to using tactile information from larvae, adult honeybees also use tactile information from other adults to know when to fan. Regardless of how honeybees are separated, their fanning response is

dampened when prevented from having full contact with other bees. Honeybees will also still fan at similar rates, even if they are being heated with a group of non-nestmates (Chapter 5). Finally, I show that actively fanning honeybees in the lab have lower concentrations of octopamine and tyramine, compared to non-fanning bees. I then experimentally verify that higher concentrations of octopamine and dopamine actually inhibit the fanning response (Chapter 6). The fanning response is a socially complex behavior, which I am excited to be able to provide more information on. These experiments elucidate what environmental and social information is important for the performance of this important behavior, as well as begin to disentangle the interactions occurring to make this behavior most efficient.

7.2 FUTURE DIRECTIONS

I hope to further explore both the social cues that honeybees use to know when to fan, as well as what social contexts may be influencing biogenic amine changes, and thus fanning, in honeybee brains.

The next question that should be addressed is what is the important tactile cue that honeybees are utilizing to know when to fan? Throughout the many trials, I observed the extensive interactions that honeybees engage in that seem to increase with temperature. Several hypotheses emerged from these observations. First, the number of interactions could be what is influencing the fanning response. It has proven difficult to video record these interactions in the heating chamber, but changing the number of interactions by changing the cage size, for example, could test this hypothesis. During

these observations, the interaction that I saw occur most frequently was trophallaxis. This is likely because the honeybees are getting hot and using the fluid to cool themselves, as well as sharing it with others. Thermal information could also be contained in the fluid coming from another bee.

Future studies could feed honeybees hot versus cool liquid, then assay them to see if they are more or less likely to fan after being fed the hot liquid. I actually performed an experiment where I fed warm 1M sucrose solution (40°C) and room temperature 1M sucrose solution (25°C) to honeybees, then heated them in my heating and behavioral assay to see if 1) they would fan upon drinking a warm liquid and 2) would be more likely to fan or fan at different thermal response thresholds. At first, my results were promising; I elicited a fanning response in 5 honeybees fed warm sucrose. However, due to my uncontrolled method of heating the sucrose solution, I was unable to replicate these results over 50 more treatments and 50 more controls. Unfortunately, I was unable expend further resources to more effectively test this hypothesis.

My biogenic amine experiment yielded exciting results that I would like to pursue. Dr. Colin Brent and I are already planning several follow up experiments to further explore the role these biogenic amines play in fanning behavior. First, I used a concentration of 2mg/mL of both octopamine and tyramine, but these biogenic amines may be active at other concentrations. I will establish a dose response curve for these biogenic amines in regard to fanning behavior to see what limits these chemicals have for eliciting a change in the fanning response. This will help us to understand which biologically relevant concentrations play a role in honeybee behavior. I am also

interested in if the inhibition of fanning is reversible. I will treat honeybees with octopamine and tyramine together, measure their fanning response, then let the honeybees metabolize them. After several hours, will measure their fanning response again to see if it returns to the levels of that of the controls. Finally, Cook and Breed (2013) also found that honeybees were not likely to fan when they were by themselves. I would also like to treat honeybees with octopamine+tyramine, and then heat them singly. This would help to parse out whether it is the biogenic amine changes that induce fanning behavior, or if there needs to be a social influence that mediates the proximate fanning response.

Understanding the honeybee society more fully has implications for honeybee conservation in several ways. First, colony collapse disorder is a complex issue that likely has many interacting causes. A more full perspective of how the entire society is responding to perturbations in our environment can help disentangle the mechanisms that are causing the collapse of the honeybee hive. Second, our planet is facing dramatic climate change. Because both increasing temperatures and rate of change influence the fanning response, ecologically, it will be interesting to see how honeybees respond to global climate change. This is particularly fascinating, given the different levels through which to explore how a complex society effectively deals with ecological change.

7.3 CONCLUSIONS

Eusocial insect societies are complex, yet incredible ecologically successful. Studying these groups offer multiple levels of exploration, including physiology that leads to individual responses, individual responses that are not static but change given ecological and social conditions, as well as group responses that must occur effectively for the colony to survive. Eusocial insects are both pollinators and pests. Regardless of their role in human's lives, understanding how their societies function will allow us to better manage their populations. Many of their behaviors can be explored theoretically to establish hypotheses, and then tested empirically, both in the laboratory and in the field. Furthermore, I believe the basic behavioral mechanisms that I've explored here can be applied across taxonomies and considered when exploring behaviors in many organisms. Overall, studying the complexities in behavior that scale up, from individuals to populations, provides insight into our own complex society.

CHAPTER 8

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