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DYNAMIC BALANCING OF FORAGING AND DEFENSIVE EFFORT CONTRIBUTE TO THE OPTIMALITY OF THE HONEY BEE ROBBING STRATEGY

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THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By

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ABSTRACT OF THESIS

DYNAMIC BALANCING OF FORAGING AND DEFENSIVE EFFORT CONTRIBUTE TO THE OPTIMALITY OF THE HONEY BEE ROBBING STRATEGY

The optimality of a foraging strategy shifts in response to dynamic ecological conditions and the need to devote effort to other tasks. Nest defense and foraging effort in the honey bee may trade off as both tasks are performed by a shared workforce of physiologically-specialized individuals in exclusive roles. Honey robbing is a foraging strategy predicted to benefit from simultaneous increases in foraging and defensive effort, but may be constrained by workforce specialization. We developed a methodology to induce robbing behaviors with uninhabited bait hives. We used this methodology to evaluate foraging and defensive effort before and during robbing by measuring forager activity and guard defensive behavior. We then assessed three cues as potential indicators guards use to determine colony robbing status. We assessed changes in identifying odor through laboratory assays of comb exposure, robber behavior through a genomic analysis of aggression biomarker genes, and field studies of the correlation between forager activity and guard defensiveness. Our results indicate colonies can simultaneously increase defensive and foraging effort when participating in robbing. We determine guards likely respond to multiple cues, with strong evidence for robbing nestmate behavior and some evidence for forager activity as signals. These results show colonies are able to dynamically balance the trade-offs of worker specialization to facilitate optimal foraging through complex social cues.

KEYWORDS: honey bee, honey robbing, optimal foraging, trade-off

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03/25/2020
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DYNAMIC BALANCING OF FORAGING AND DEFENSIVE EFFORT CONTRIBUTE TO THE OPTIMALITY OF THE HONEY BEE ROBBING STRATEGY

BY

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Clare C. Rittschof, PhD.
Director of Thesis

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03/25/2020
Date

I would like to dedicate this thesis to my grandmother, Betty Anne Porch.

She knows a hundred tales of bees.
She would be delighted by a hundred more.
Here is another for her.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Optimal foraging theory predicts that individual behaviors maximize energy gain while minimizing energy expenditure (Pyke 1984). Organisms balance their foraging effort against other energy-consuming tasks including reproduction (Lu et al. 2011), territoriality (Loveridge et al. 2009), and nest defense (Ryttkonen et al. 1995). Dynamic ecological conditions, including food availability, predation risk, and resource competition, influence the costs and benefits of foraging beyond energy considerations (Carle and Rowe 2014, Chen et al. 2017). Solitary organisms weigh these energetic and ecological trade-offs to arrive at an optimal foraging strategy.

Groups of social animals with task specialization (i.e., division of labor (Wilson 1971)) also alter their foraging investment, but they can do so through multiple means. Individuals can change their effort (e.g., increasing the frequency or duration of foraging trips), or the group can dedicate more individuals to foraging (Page and Mitchell 1990). In some cases, social groups can respond rapidly to shifts in foraging demand by increasing workforce investment at low cost (Tenczar et al. 2014). However, task specialization can also limit a group's ability to respond to changes in foraging needs, particularly if individuals are costly to produce or are limited in their ability to switch tasks (Shingleton & Foster 2001, Charbonneau and Dornhaus 2015). Similar to solitary organisms, these costs can result in behavioral trade-offs between foraging and other traits that impact foraging strategy, e.g., nest defense.

Honey bees (*Apis mellifera*) live in large social groups, and workers are renowned for their ability to modify their behaviors in response to colony needs, particularly in the context of foraging effort (Seeley 1989, Seeley 1995). Workers are sterile females that perform most of the tasks required for colony function, including brood care, nest defense, and foraging for nectar,

pollen, and water (Winston 1987, Blom 1993). Adult workers shift tasks as they age, and individuals are able to accelerate, delay, or even reverse this process depending on social information and colony needs (Huang and Robinson 1992; Robinson 1992). Despite this flexibility, tasks have a physiological basis and high-energy tasks are performed by the same set of bees due to this basis. Foraging and defensive tasks require strong flight capabilities and high metabolic rate, thus both are presumably energy-limited behaviors (Vance et al. 2009). Trade-offs between foraging and nest defense are predicted to occur because only older workers are physiologically specialized with the metabolic capacity to perform these tasks (Breed et al. 2003, Margotta et al. 2014). Moreover, there is evidence that foraging and defensive behaviors are mutually exclusive for a given individual (Giray et al. 2000, Hunt et al. 2007), and that foraging experience alters the perception of and response to cues that induce defensive behavior (Finkelstein et al. 2019, Rittschof et al. 2019). Presumably as a result of these individual mechanistic constraints, foraging and nest defense are inversely correlated at the colony level, and investment in each behavior reflects colony needs and environmental conditions (Rivera-Marchand et al. 2008).

The relationship between nest defense and foraging effort is variable. During the colony growth phase in early summer, small colonies prioritize worker production and resource acquisition because they must achieve adequate worker numbers and store enough honey to survive the winter months (Winston 1987, Beauchamp 1992). As a result, these growing hives show reduced defensiveness (Page et al. 1995, Breed et al. 2004). Though high foraging effort may lead to reduced investment in nest defense due to the mutually exclusive nature of these behaviors (see above), colonies also have generally low defensive needs at this time of year because resource competition is low (Seeley 1995, Downs and Ratnieks 2000). Indeed, some

recent studies find positive relationships between foraging and nest defense for mature colonies (Wray et al. 2011) and colonies experiencing chronic disturbance (Rittschof and Robinson 2013), suggesting variation in foraging effort relative to defense is not always a result of mechanistic constraints such as sharing a physiologically-specialized workforce.

The varied relationships between foraging and nest defense in honey bees could reflect the methodology used to assess the presence of an energetic trade-off. Most studies to-date have measured foraging and nest defense behaviors across multiple colonies for a single point in time. The patterns that emerge from these types of measurements confound two sources of variation, within-hive plasticity in foraging and defensive investment, and trait variation among colonies or genotypes (Niemela and Dingemanse 2018). Genetic correlations between foraging effort and nest defense could give rise to positive relationships between these traits across hives (e.g., Wray et al. 2011), while for a single hive measured across different time points, energetic trade-offs may still manifest as negative trait relationships (e.g., Marchand-Rivera 2006). One way to address this confound is to measure foraging and nest defense behaviors repeatedly for multiple hives, specifically under ecological conditions that modulate the trade-offs associated with foraging and defense.

In the current study, we perform an ecologically relevant manipulation of foraging pay-off and measure temporal variation in foraging and defensive effort within and among honey bee hives. The context for our manipulation is honey robbing, in which a colony engages in opportunistic honey theft, typically from a weakened conspecific colony (Free 1954). Honey is unusually profitable due to its high sugar concentration relative to nectar (Southwick and Pimentel 1981), and this relative value increases in times of seasonal declines in floral abundance (Seeley 1995). Honey robbing is a particularly interesting ecological context to

evaluate temporal variation in the relationship between foraging and nest defense, because the environmental conditions that make robbing profitable also increase the likelihood a hive will become a robbing target: as floral resources decline, forager inspection of neighboring colonies, and thus invasion risk, increases (Downs and Ratnieks 2000). Honey bees have evolved a specialized type of defensive bee, the guard, to evaluate and reject foreign honey bees that may attempt to invade from neighboring hives (Moore et al. 1987). Colonies respond to conspecific intrusion by rapidly reducing guard permissiveness towards entering bees through heightened aggression (Couvillon et al. 2008). Prior to this study, no descriptions of the defensive responses of colonies participating in robbing were known. However, increased defensive effort is predicted as robbing indicates elevated risk of being robbed. We assess foraging and defensive effort before and during participation in robbing to determine if colonies dynamically balance these two traits that share a work force.

To complete this study, we first developed a methodology to induce robbing behaviors with uninhabited hives. We then evaluated shifts in both foraging and defensive behaviors for a hive engaged in a robbing event. Surprisingly, we found increased defensive behaviors during robbing directed towards returning nestmates. We then evaluate three possible explanations for increased guard aggression towards nestmates: increased rates of forager return (which may overstimulate guards and enhance aggression), altered nestmate recognition odor profiles (which may confuse guards leading to misplaced aggression), and increased robber aggression (which may provoke defensive behaviors from guards). The unique risks and benefits associated with honey robbing predict positive correlations between two colony-level phenotypes otherwise constrained by sharing a physiologically-specialized workforce. The cost of guards expressing increased defensiveness toward nestmates undermines robbing as an optimal foraging strategy

(Reeve 1989). However, the presence of multiple cues indicating to guards that their colony is participating in robbing (and thus requires increased nest defense) may suggest complex social feedback mechanisms allow a defensive response proportional to the relative risk indicated by various signals.

CHAPTER 2: MATERIALS & METHODS

Overview

Because robbing can lead to hive mortality (Free 1957), we first developed an artificial method to stage and study robbing, and verified this method produced behaviors typical of robbing (Study 1). Then, we used the artificial robbing method to compare colony foraging and nest defense behaviors for 8 hives during a robbing event and during typical foraging (Study 2). Last, we performed a series of experiments (Study 3) to evaluate three explanations for heightened aggression expressed by guards toward returning nestmate robbers in Study 2.

Honey bee sources and field sites

Honey bee colonies originated from a combination of sources, including commercial apiaries (Hosey Honey, Midway, KY, Guthrie's Naturals, Frankfort, KY, and Schoolhouse Bees, Covington, KY) and locally caught swarms. Bees represent a combination of outbred genotypes, commercially advertised to be derived primarily from *A. mellifera liguistica* and *carnica*. All colonies were headed by naturally-mated queens. We conducted Study 1 of from July to September 2016 at the C. Oran Little Research Center, a University of Kentucky agricultural research farm located in Versailles, KY, USA. We chose this site because it was outside the foraging radius of our working apiaries (>25km away). This was a precaution because robbing behaviors are known to spread among neighboring hives (Free 1954). We also did not observe any feral hives in range of this site that could interfere with our studies (see below for verification of this point). We conducted Study II and III from July to October 2017 at another University of Kentucky agricultural research farm (North Farm, Lexington, KY, USA)

STUDY I: DEVELOP AND VALIDATE ROBBING METHODOLOGY

During a robbing event, there are high levels of aggression displayed between intruder and resident bees. To minimize bee loss while studying the behavior of the robbing hive, we tested if colonies would exhibit behaviors characteristic of robbing in response to hive boxes stocked with honey frames but containing no bees (after Free, 1954). We could then use this artificial method to evaluate defensive and foraging behaviors associated with robbing.

We first tested the robbing response of a small focal colony housed in a five-frame hive box (Dadant & Sons Inc., Hamilton, IL, USA). Five meters away, we placed another five-frame hive box (hereafter the bait hive) stocked with two honeycomb frames filled with fully processed mature ("capped") honey. These frames were collected from an unrelated hive. We left the lid off of the bait hive box to encourage discovery by the focal hive. After one hour, we closed the lid on the bait hive, and observed the behavior of the focal colony over three hours from 14:00 until 17:00. Specifically, we observed how foragers approached and explored the bait hive. Outside of a victim hive, robbing foragers use distinct behavioral tactics to bypass guards (Free 1957). These include investigating the hive surface for alternative nest entrances, and flying in a side to side "casting" pattern, which is thought to be a way to surveil the victim hive for defensive bees and alternative entrances. Within a victim hive, foragers often cluster together with nestmates while collecting honey, which is thought to provide protection against defensive resident bees (Free 1954). After the observation period, we disassembled and removed the bait hive. This methodology was repeated with the same focal colony two additional times with a day between each observation. We then exchanged the focal colony for another with the same specifications and repeated our methodology. This pilot test showed distinct changes in focal

hive forager behavior in response to the artificial robbing stimulus, including increasing levels of foraging activity at the bait hive over time (indicative of recruitment by the focal hive), and forager alternative entrance seeking, casting behavior, and clustering on honey frames within the bait hive. We decided to proceed with a comparison of foraging and nest defense during robbing and normal foraging activity for a larger number of hives using this uninhabited bait hive methodology (Study II). During Study II, we quantified alternative entrance seeking, casting, and clustering on honey frames to provide evidence that our methodology initiates behaviors seen in natural robbing (See Study II: Observations of typical robbing behaviors).

STUDY II: QUANTIFYING FORAGING AND DEFENSIVE EFFORT DURING ROBBING AND NORMAL FORAGING

Experimental set-up and impacts of robbing on foraging activity and defense

For each of 8 unique hives kept at four observation sites (see below), we performed two sets of observations of defensive effort and foraging activity in order to monitor how these behaviors changed during a robbing event. During one set of observations, the hive was engaged in robbing, and in the other, the hive was offered a sucrose feeder and otherwise allowed to forage normally. The sucrose feeder is a high-value food resource similar to honey, but it does not carry the same defensive risks as honey robbing. The order of the two sets of observations was assigned at random, and for a given hive, observations were performed 3 to 7 days apart. This latency period was chosen to allow typical colony behavior to resume as repeated disturbance can impact defensive response (Alaux and Robinson 2007) and to allow for the average single day guard replacement (Breed et al. 1992) to remove any effect of guard

experience (Shpigler et al. 2017). We opted to repeat treatments and observations for the same set of 8 hives across two rounds to better capture within-hive plasticity. All data collection occurred between Jul 29 and Aug 18. This relatively short time window encompasses a period of nectar dearth in our area of Kentucky, which is known to stimulate robbing (Seeley 1995).

We performed Study II observations on four sites at the University of Kentucky North Farm. Sites were approximately 1km apart and 1 km from our on-site apiaries (preliminary studies showed no evidence of robbing activity "spreading" among hives, and so we determined that this relatively close distance to other hives was permissible). Because interference from robbing foragers from feral colonies or our on-site apiaries could impact the results of this study, we first tested whether any honey bees in the area responded to exposed honeycomb frames before moving focal hives onto our sites. At each site, we place a single honeycomb frame (19" x 1 1/16" x 9 1/8", Dadant & Sons Inc.) filled with mature honey on the ground, first scraping off wax caps to expose honey which is known to attract robbing foragers. We monitored the frames for three hours on the same day from 11:00 to 14:00. This timeframe is similar to the timeframe for data collection during our bait hive robbing (see below). This test was performed on 23 July 2017, three days prior to introduction of the first set of four focal hives, and six days prior to the start of data collection. We repeated this test again in August 2017 one day before introduction of the second set of four focal hives (see below "Validating absence of interfering bees" for additional steps taken to validate the absence of interfering bees from other hives in our study). colonies used in the second round of observations (see also below "Validation of defensiveness towards nestmates" for additional validation of the lack of interfering bees from other hives).

After these preliminary measures to verify the absence of interfering foragers, we placed one small colony (identical to the focal colonies in Study I) on a wooden stand at each site. We

allowed the colonies to acclimate undisturbed for three days following placement prior to observations.

Each of the four focal hives was randomly assigned to either an artificial robbing treatment (see Study I for set-up) or sucrose feeder treatment. The sucrose feeder consisted of 400 mL 50% (m/v) sucrose solution scented with approximately 50 uL peppermint oil (LorAnn Oils, Lansing, MI, USA). This solution was spread in a thin layer over a 23 cm x 33 cm baking sheet that was ridged to allow places for bees to perch while feeding. To initiate treatments, we placed either a sucrose feeder or bait hive 5m from the focal colony, beginning between 11:00 and 14:00. We allowed 60 min following placement for foragers to discover food and begin to recruit nestmates prior to beginning data collection (foraging activity was noted in the bait hive at the first observation for all Study II robbing observations). Following this, we collected data on foraging activity and nest defense at the focal hive entrance over a 75 minute time period. For one minute every 15 minutes (5 timepoints over the 75 min period), one observer counted the number of foragers entering the focal hive. Simultaneously, a second observer tallied defensive behaviors displayed by guards toward returning foragers (see below), and counted the number of individual bees displaying guard-characteristic behaviors during this time. Over this timeframe, we also evaluated characteristic robbing behaviors at the bait hive (described in detail below, see "Observations of typical robbing behaviors"). In all cases, we performed observations between 11:00 and 16:00, and only on days without rain.

Foragers were defined as any bee entering the focal hive. We did not keep track of individual trip length, flight pattern, or resources carried (e.g., pollen, nectar, water, or honey). Guards were identified by their characteristic body posture, which includes raised forelimbs and lunging toward returning bees to smell them and determine if they are nestmates (Breed et al.

1992). Defensive behaviors of all visible guards were scored as a single tally, as it was prohibitively difficult to track behaviors of many individual guards simultaneously. Guard defensive behaviors towards bees entering the hive were identified following prior studies of guards and non-nestmate rejection (e.g., Couvillon et al. 2008, Li-Byarlay et al. 2014). In each case, we only tallied a behavior if the guard oriented towards a specific incoming bee either just before, during, or immediately after the defensive behavior was displayed. Each of the defensive behaviors can vary in duration, which could be interpreted as a measure of defensive effort (Preston et al. 2019). However, because measuring duration for multiple guards and behaviors simultaneously is difficult in the field setting of this experiment, we tallied each behavior as a discrete event. The different behaviors were (1) Antennation: antennal contact, (2) Mandibulation: antennation with open mandibles as if to threaten or bite, (3) Biting, (4) Flexion: the guard grabs the bee with her legs or mouth and flexes her abdomen in a sting-like motion without actual insertion of stinger, and (5) Stinging: the guard inserts her stinger into the forager. If a guard removed and re-inserted her stinger again, we counted this as two separate sting events. We calculated defensive scores by weighting behavior counts by a factor indicating severity (antennation by 1, mandibulation by 2, biting by 3, flexion by 4, and stinging by 5) then adding the resulting values (Li-Byarlay et al. 2014). After the first set of observations for each colony, we removed the bait hives and sucrose feeders, and left colonies undisturbed for three to seven days to allow undisturbed activity to resume (see above). We then repeated the above measurements with the other treatment (either the bait hive or the sucrose feeder, depending on the identity of the first treatment).

Each focal hive received the bait hive and sucrose feeder treatments twice (two replicates). We repeated the methodology for a set of four additional hives (8 unique hives in

total). The initial four hives were removed following completion of the second treatment. We allowed the field sites to remain empty for one week, then we performed the honeycomb test for interfering bees (see above), placed the second set of hives at the sites, and repeated the procedures above. We used linear mixed modeling (LMM) as the statistical test for a significant effect of treatment on forager activity and defensiveness.

Observations of typical robbing behaviors

For the 8 focal hives in Study II, we observed behaviors around the bait hive to verify the occurrence of robbing during treatments, and to further quantify the efficacy of our robbing methodology (see Study 1). We tallied instances of casting behaviors by foragers around the bait hive and sucrose feeder, the presence or absence of forager clustering behavior on honey frames inside the bait hive or on the sucrose feeder, and the presence or absence of alternative entrance-seeking behaviors at the bait hive during the honey robbing treatment. We performed these tallies over a 1 min period just after each 1 min foraging and defensive behavior observation period (above), resulting in 5 measurements per 75 min period. One observer made casting counts by tallying each discrete instance of the zig-zag flight pattern, which lasts for 1 to 3 seconds before normal flight resumes. The other observer noted the presence of alternative entrance-seeking during honey robbing treatments, defined by one or more bees landing on the sides or rear of the bait hive and inspecting the crevice between the hive body and the base board. The observers then counted the number of clusters on the sucrose feeder or opened the bait hive and counted the number of clusters on the frame. Clusters were defined as groups of five or more bees in physical contact, clearly separated from other bees by unoccupied space on the honeycomb or feeder. In some cases, the entire honeycomb frame was occupied preventing

cluster count, so we opted to treat clusters as present or absent (as in the Results). As none of the robbing-characteristic behaviors were ever observed in during any of the sucrose feeder treatment timepoints, we did not perform statistical analysis to compare treatments.

Validation of defensiveness toward nestmates

Guard defensive behaviors are known to increase when hives are under threat from invading bees from other colonies (Couvillion et al. 2008). Though defensive behaviors are typically directed towards non-nestmates, they are also displayed towards nestmates at a higher rate following conspecific intrusion as recognition cues overlap between colonies (Couvillon et al. 2008, Couvillon et al. 2009). Thus, validating that behaviors by guards at focal hives were directed toward returning forager nestmates and not interfering bees from nearby hives is import for interpreting the ecological meaning of our results. By monitoring honey-filled frames for foragers prior to the start of data collection (see above), we verified the absence of foragers from non-focal hives. Here we describe an additional experiment to assess the likelihood of the presence of interfering bees from nearby hives by marking foragers from our focal hives, and monitoring the presence of marked bees at bait hives.

Following the conclusion of all behavioral observations above, each of the four focal hives in the second set were equipped with an entrance "automarker". The automarker device (modified from Hagler et al. 2011, see Figure A1) was made out of a 50 mL, conical tube (Fisher Scientific, Northampton, New Hampshire, USA) that was 115 mm long. We removed approximately 1/3 of the plastic along the entire length of the tube. A piece of cheesecloth was glued over the portion with plastic removed. We filled the tube with pink or blue fluorescent powder (Day-Glo Color Corp., Cleveland, Ohio, USA). We built a small wooden frame that held

the tube in place at the hive entrance with the cheesecloth side down, leaving approximately 5mm space for a bee to walk between the cheesecloth and base. The rest of the entrance was blocked with duct tape so that exiting bees were forced to walk under the cheesecloth portion of the tube, and in doing so, were covered on their thoraces and wings with powder. This powder remained visible on the bees when they visited the bait hive. Immediately after applying the automarker, we then initiated robbing using our standard bait hive methodology (above), and tallied the proportion of bees marked while leaving the hive and the proportion of marked bees present at the bait hive for 1 min every 15 min over a 75 min robbing period (5 total tallies).

Even in the absence of interfering bees, not all bees at the bait hive are expected to have a paint mark.. Unmarked bees on the bait hive have three potential sources: (1) they are interfering bees from a nearby hive, (2) they are exiting bees from the focal hive that failed to be marked by the automarker as they left the hives, (3) they are foragers who left the hive prior to the installation of the automarker and thus arrived at the bait hive unmarked. This third possibility predicts that over time, a greater proportion of bees at the bait hive will show a paint mark (over repeated trips to and from the bait hive and focal hive, an increasing proportion of the work force will receive a marking). To account for automarker failure rate, we compared the proportion of successfully marked bees leaving the hive to the proportion marked at the bait hive at the final observing time point, expecting these proportions to be similar in the absence of interfering bees.

STUDY III: CAUSES OF INCREASED DEFENSIVE BEHAVIORS

We observed increased guard defensive behaviors directed towards returning nestmate robbers during Study II (see Results). We evaluated three hypotheses to explain this increase in

defensiveness: increased rate of forager return, altered nestmate odor profiles, and increased robber aggression.

Hypothesis 1: Increased rate of forager return

Colonies increase forager numbers in response to the discovery of high-value resources (Seeley 1995). In Study II, rapid recruitment to the bait hive and subsequent increase in returning nestmates may increase guard defensiveness through repeated exposure to defensive stimuli; guards who experience defensive stimuli become increasingly responsive to stimuli over time (Alaux and Robinson 2007, Shpigler et al 2017). To assess this possibility, we analyzed the relationship between foraging activity (forager rate of return) and guard defensive behaviors for each time point of data collection in Study II. If rate of return alone explains increased defensiveness during robbing, we predict a positive correlation between rate of return and guard defensiveness, regardless of treatment.

The eight focal hives in Study II were relatively small with low foraging activity compared to a mature hive. It is possible that the range of forager rate of return for those hives is below a threshold that induces a change in guard defensiveness. To account for this possibility, we collected additional data on forager rate of return and guard defensiveness from eight mature, full-sized hives during the course of normal foraging (data collection was identical to Study II). We used linear mixed modeling (LMM) as the statistical test for a significant effect of treatment and forager activity on defensiveness for the experimental hives of Study II. We used generalized linear modeling (GLM) to test for a significant association between forager activity and defensiveness for the large hives detailed above.

Hypothesis 2: Altered nestmate odor profiles

Odor profiles derived primarily from contact with wax honeycomb are used by guards to identify nestmates from non-nestmate intruding bees (Breed 1998). Robbing foragers contact foreign honeycomb when they visit victim hives and rip apart the wax caps that seal honey cells. Odor profiles can be altered in under five minutes of contact with honeycomb (Breed et al. 1995). We hypothesized that forager contact with foreign honeycomb during robbing may result in misidentification by guards upon return, and increased guard defensiveness.

To assess this hypothesis, we used a lab-based approach to manipulate an individual bee's honeycomb exposure and measured defensive behaviors displayed towards this bee by nestmates. A similar lab-based approach was originally developed by Breed (Breed et al. 1995) to assess the factors that impact nestmate recognition in honey bees. In this assay, bees are kept in small groups (in our study, 4 bees per group). A bee introduced to these groups will be attacked if it is identified as a non-nestmate; defensive behaviors towards the introduced bee (the "intruder") are tallied similar to the field methods described above (see "Impacts of robbing on foraging activity and nest defense").

We first performed a full-factorial experiment where we created four types of intruder bees relative to the 4-member groups, (1) nestmates exposed to honeycomb native to the group members, (2) nestmates exposed to honeycomb foreign to the group members, (3) non-nestmates exposed to the group members' native honeycomb, (4) non-nestmates exposed to honeycomb foreign to the group members. To generate these treatments, we collected frames of emerging one-day-old bees along with an additional frame of honeycomb (free of brood and containing mostly empty cells) from two different source hives. The additional frames of honeycomb served as a source of native honeycomb. Using two hives allowed us to replicate our experiment across

two distinct genotypes, while simultaneously generating both nestmates and non-nestmates to use as intruders. From a third hive, we collected two frames of honeycomb to serve as a source of comb foreign to group members (see below). Frames of emerging bees were placed in individual ventilated emergence boxes and stored overnight in a biological incubator (Percival Scientific, Perry, IA, USA.) at 34°C. The following morning, we collected emerged one-day-old adult bees and placed them in 10 cm x 10 cm x 8 cm ventilated plexiglass cages (~100 bees per cage) containing a 6 cm^3 piece of wax honeycomb cut from the second frame collected from the source hive. This honeycomb provides chemical compounds that impact bee cuticular odor profiles and the defensive response of nestmates (Breed 1998). We fed caged bees *ad libitum* 50% (m/v) sucrose solution and store-bought honey over the course of one week. This period allowed ample time for bees to acquire the odor profiles associated with their native honeycomb (Breed 1998). In previous studies, we have also demonstrated robust defensive behaviors for bees of this age (Rittschof et al. 2015). The remaining frames of honeycomb were stored separately in a 3°C refrigerator.

When caged bees were 8 days old, individuals from each source hive were haphazardly assigned to serve as group members or intruders in our assays. To tell group members and intruders apart during the behavioral assay (see below), we had to mark the bees. Because marking could impact the odor profile of the bees and thus the results of our experiment, we marked both the group members and intruders using a single paint color. We created 20 groups per source hive (four bees per group), marking each bee with a single dot of Testor's enamel paint (Testors, Vernon Hills, IL, US) which has no apparent behavioral effects (Breed 1988). We placed group members in 100 mm x 20 mm petri dishes with ~1.5 cm openings in the lids covered with tape (Harrison et al. 2019). Remaining bees from a given source hive were marked

with two dots of the same paint color (a similar total quantity of paint) and placed together in a plexiglass cage (above) to serve as nestmate or non-nestmate intruders.

To manipulate comb exposure and generate the four treatments listed above, we trapped either nestmate or non-nestmate intruder bees against frames of native or foreign honeycomb using a 100 mm diameter petri dish lid. We left adequate space for bees to walk on the comb while remaining in contact with the comb for the 9.5 min exposure period. The exposure period was selected in excess of the minimum exposure duration found to inhibit recognition in Breed et al. 1995 to account for the extent of contact typical in robbing. Robbers actively enter cells and tear apart comb into small particles (as opposed to exclusively resting upon it), and methods of simulating this contact such as shaking an intruder in dust may have behavioral effects due to disorientation. Comb was prepared for exposure treatments by crushing two ~50mm sections per frame with a gloved finger to simulate comb destroyed during robbing. After the 9.5 min exposure time to either foreign or native comb, each intruder was introduced to a group of four bees. Using this approach, we created four treatments in a full factorial design (see above, N=10 replicates per treatment per hive). An assistant managed exposure treatments to blind the behavioral observer, providing intruders in a randomized treatment order. The observer scored defensive behaviors of the group toward the intruder for 120 s following introduction. A total of 80 assays were held in two blocks on 8 and 11 September 2017 from 14:30 to 17:30 in a field laboratory on the University of Kentucky North Farm. One assay was excluded due to group mortality. We used linear mixed modeling (LMM) as the statistical test for a significant effect of treatment on groupmate defensiveness toward intruders.

Because the duration of exposure to honeycomb could influence the extent of the change in individual bee odor cues (Breed et al. 1995), we assessed whether exposure duration

influenced defensive behaviors towards nestmates in an additional experiment. We tested two exposure durations, 2.5 min, which corresponds to typical time spent filling the crop on an unlimited resource (Shackleton et al. 2016), and 9.5 min, as above. We used the same methodology as above to generate groups and intruders. Intruders were exposed for 2.5 min or 9.5 min to comb native or foreign to the group. We implemented a negative control treatment by placing intruders into an empty petri dish for 9.5min, as well as a positive control by introducing returning foragers collected by vacuum at the entrance of an unrelated hive immediately prior to holding the assays. Group defensiveness toward intruders was scored as above. We held a total of 108 assays in two blocks on 7 and 8 August 2017 from 11:30 to 15:00 in the same field laboratory. We used a nonparametric Kruskall-Wallis test to statistically compare groups, followed by a Wilcoxon Each Pair post-hoc test significant pairings. We then used a generalized linear model (GLM) to test for an effect of duration and comb source on groupmate defensiveness toward intruders.

Hypothesis 3: Returning robbers are more aggressive than typical foragers

Robbing foragers under typical circumstances will experience defensiveness from victim bees, and thus may elevate their level of aggression during robbing in preparation for defensive interactions. Elevated aggression could impact how these foragers are perceived by nestmate guards when they return to their hive. Because such a context-dependent and ephemeral behavioral shift can be difficult to measure observationally, we took a behavioral genomics approach, using brain gene expression measures that are predictive of aggressive behavior (Rittschof and Robinson 2013). We predicted that, relative to typical foragers, robbing foragers would show brain gene expression patterns characteristic of more aggressive bees. We used

quantitative PCR to measure the expression of a set of four biomarker genes that track variation in aggression that arises from many sources, including genotype, age, caste, experience of predator disturbance, exposure to alarm pheromone, and long-term exposure to aggressive nestmates (Alaux et al. 2009, Rittschof and Robinson 2013, Rittschof 2017). The four genes were *Inos*, *GB53860*, *Drat*, and *Cyp6g1/2* (Rittschof & Robinson 2013, Harrison et al. 2019).

We collected bees from one focal hive in Study II (above) on 29 September and 10 October 2017 at the University of Kentucky North Farm. Ten pollen foragers, identified by presence of pollen pellets attached to the corbiculae, were collected from each hive by bee vacuum and immediately flash-frozen in liquid nitrogen. Because honey bee foragers tend to temporarily specialize on pollen, nectar, or water collection (Riveros and Gronenberg 2010), pollen foragers were unlikely to have engaged in robbing immediately prior to collection. However, this procedure cannot eliminate the possibility that gene expression differences we observe result from specialization in nectar (or honey) versus pollen foraging. Robbing was initiated (see above) and hives were allowed to rob for one hour. After one hour, we collected ten returning foragers into liquid nitrogen. Heads were dissected in 95% ethanol over dry ice. We extracted RNA using E.Z.N.A. HP Total RNA kit with on-column DNase treatment (Omega Bio-Tek, Norcross, Georgia, USA). We synthesized cDNA using a Bionline SensiFAST cDNA Synthesis Kit (Bioline, Taunton, MA, USA). We performed qPCR using PerfeCTa SYBR green with low Rox (Quanta Bio, Beverly, MA, USA) with each 10µL reaction including 5µL of SYBR, 2µL of primers, and 3µL of cDNA in triplicate on 384-well plates on a Quanta Studio 6 (ThermoFisher Scientific, Waltham, MA, USA) using. We verified that three endogenous control genes, actin-1, rp55a, and elF3-S8 showed no significant variation across groups, and had a coefficient of variation equal to or less than 20% (Rittschof 2017, Preston et al. 2019). We

calculated these geometric mean of these controls and compared normalized expression for the four biomarker genes across treatment groups. See Harrison et al. (2019) and Preston et al. (2019) for primer sequences. We used one-tailed T-tests to test for significant expression differences between the pollen and robbing forager groups.

Data Analysis

We analyzed all behavioral data using JMP Pro version 14.3.0 (SAS Institute, Cary, NC, USA). We log-transformed forager return rates and square root-transformed behavioral scores for normality. We describe details of individual statistical tests in the relevant locations in the RESULTS section.

CHAPTER 3: RESULTS

Study I: Develop and assess honey robbing methodology

We described the qualitative observations of small colonies presented with bait hives containing frames of honeycomb in our pilot study to determine if our paradigm would induce responses similar to natural robbing. The effects of bait hive introduction on the focal colony were characterized by general increases in forager activity, specifically, increased visits to the bait hive and increased traffic at the focal colony entrance. We also observed increased guard defensiveness. Discovery of and recruitment to the bait hive typically occurred within a 30min to 1 hour period following establishment of the bait hive. Following this, large numbers of bees were continuously noted in the air around the bait hive and the entrance of the focal colony, with an associated increase of traffic at the entrances of both. Foragers at the bait hive formed large congregations on the honeycomb, destroying the comb as honey was collected. Aggressive interactions between guards and returning bees at the focal colony entrance were commonplace, with guards threatening and biting some, but not all, entrance-seekers. This response persisted until removal of the bait hive, after which activity dwindled and ceased over a period of approximately 30 minutes.

In Study II, we supplemented the above pilot study observations by quantifying robbing-associated behaviors in the presence of a bait hive or a sucrose feeder control (see table A2). Casting flights per minute were counted at five timepoints for each of 8 hives per treatment across two rounds. Casting flights were observed during the bait hive treatment (round 1: n=40, mean=7.75, SE=1.79; round 2: n=40, mean=10.5, SE=1.78), and no casting flights were ever observed at the sucrose feeder with the same level of sampling. Casting flights were observed during at least one of the bait hive observation timepoints in 6 of 8 hives in the first round and 6

of 8 hives in the second round. Only one hive did not exhibit casting behaviors in either round. We observed clustering during at least one of the bait hive observation timepoints in 6 of 7 hives in the first round (an additional hive omitted as the surface of the honeycomb frames were completely occupied by the first observation point) and five of eight hives in the second round. All hives exhibited clustering behavior during at least one of the trials. No cluster was ever observed on the sucrose feeder with the same level of sampling. We observed alternative entrance-seeking behaviors at the bait hive for one round, and 6 of 8 hives demonstrated alternative entrance-seeking during at least one of the time points; there is no corresponding measure for alternative entrance-seeking at the sucrose feeder (see methods).

Study II: Quantifying foraging and defensive effort during robbing and normal foraging

We evaluated shifts in both foraging and defensive behaviors for a hive engaged in a robbing event compared to normal foraging at a sucrose feeder. We used rate of forager return as a measure of foraging effort. We assessed the effect of treatment (robbing versus normal foraging) on rate of forager return using a linear mixed model. Because rate of forager return varied over the observation time frame, we selected the highest observed rate of forager return from amongst the five observations for a given hive as the response variable in this analysis (log-transformed for normality). We included treatment, round, and round by treatment interaction terms as fixed effects, and included hive identity as a random effect. We found a significant effect of treatment on forager rate of return, with no other significant fixed effects (LMM, treatment: $F_{1,21}=6.78$, $p=0.017$; round: $F_{1,21}=0.12$, $p=0.738$; treatment*round: $F_{1,21}=0.26$, $p=0.618$). Rate of forager return was higher in the honey robbing treatment (N=16, mean=240.81, SE=45.86) than in the sucrose control (N=16, mean=139.94, SE=22.48; Figure 1).

Colony defensive effort is regulated by adjusting the frequency of defensive behaviors displayed by individual guards, and by adjusting the number of guards present at the entrance (Couvillon et al. 2008, Couvillon et al. 2010). We accounted for potential variation in individual guard behavior and in guard number when assessing colony defensive effort by treating total defensiveness of all guards, guard number, and defensiveness per guard separately in the analysis (Figure 1). Defensive behaviors varied over the 75 min observation period, but not in a pattern that was consistent across all hives. Thus, we selected the maximum value for each of the three defensive metrics amongst the five observation points for a given hive. We used linear mixed models with treatment, round, and their interaction as fixed effects, and hive identity as a random effect to test for an effect of treatment on each metric.

We used a LMM with treatment, round, and their interaction as fixed effects, hive identity as a random effect, and the square root transformed maximum defensiveness score (see METHODS for calculation; transformed for normality) as the response variable. Treatment significantly predicted total guard defensiveness, with no other significant fixed effects. (LMM, treatment: $F_{1,21}=42.44$, $p<.0001$; round: $F_{1,21}=0.01$, $p=0.925$; treatment*round: $F_{1,21}=0.82$, $p=0.375$). Total guard defensiveness across both rounds was higher in the honey robbing treatment (N=16, mean=98.44, SE=17.08) than in the sucrose control (N=16, mean=12.25, SE=3.19).

We assessed the effect of treatment on the number of guards present using a linear mixed model with untransformed maximum guard count as the response variable. We included treatment, round, and an interaction effect of treatment and round as fixed effects, and included hive identity as a random effect. Treatment did not significantly predict the number of guards observed, and we found no significant effect of round or an interaction effect between treatment

and round. (LMM, treatment: $F_{1,21}$=0.54, p=.471; round: $F_{1,21}$=0.54, p=0.471; treatment*round: $F_{1,21}$=3.65, p=0.0.70). The number of guards present across both rounds was not significantly different in the honey robbing treatment (N=16, mean=2.75, STE=0.21) compared to the sucrose control (N=16, mean=2.44, SE=0.38).

We assessed the effect of treatment on per-guard defensiveness by dividing the total defensiveness score by the number of guards present at that time period. We used a LMM with the square root transformed (transformed for normality) maximum per-guard defensiveness score as the response variable. We included treatment, round, and an interaction effect of treatment and round as fixed effects, and included hive identity as a random effect. Treatment significantly predicted per-guard defensiveness, but we found no significant effect of round or an interaction effect between treatment and round. (LMM, treatment: df1=1, df2=21, F=33.84, p<.0001*; round: df1=1, df2=21, F=0.38, p=0.547; treatment*round: df1=1, df2=21, F=0.27, p=0.606). Per-guard defensiveness across both rounds was higher in the honey robbing treatment (N=16, mean=42.59, STE=7.37) than in the sucrose control (N=16, mean=7.82, SE=2.04).

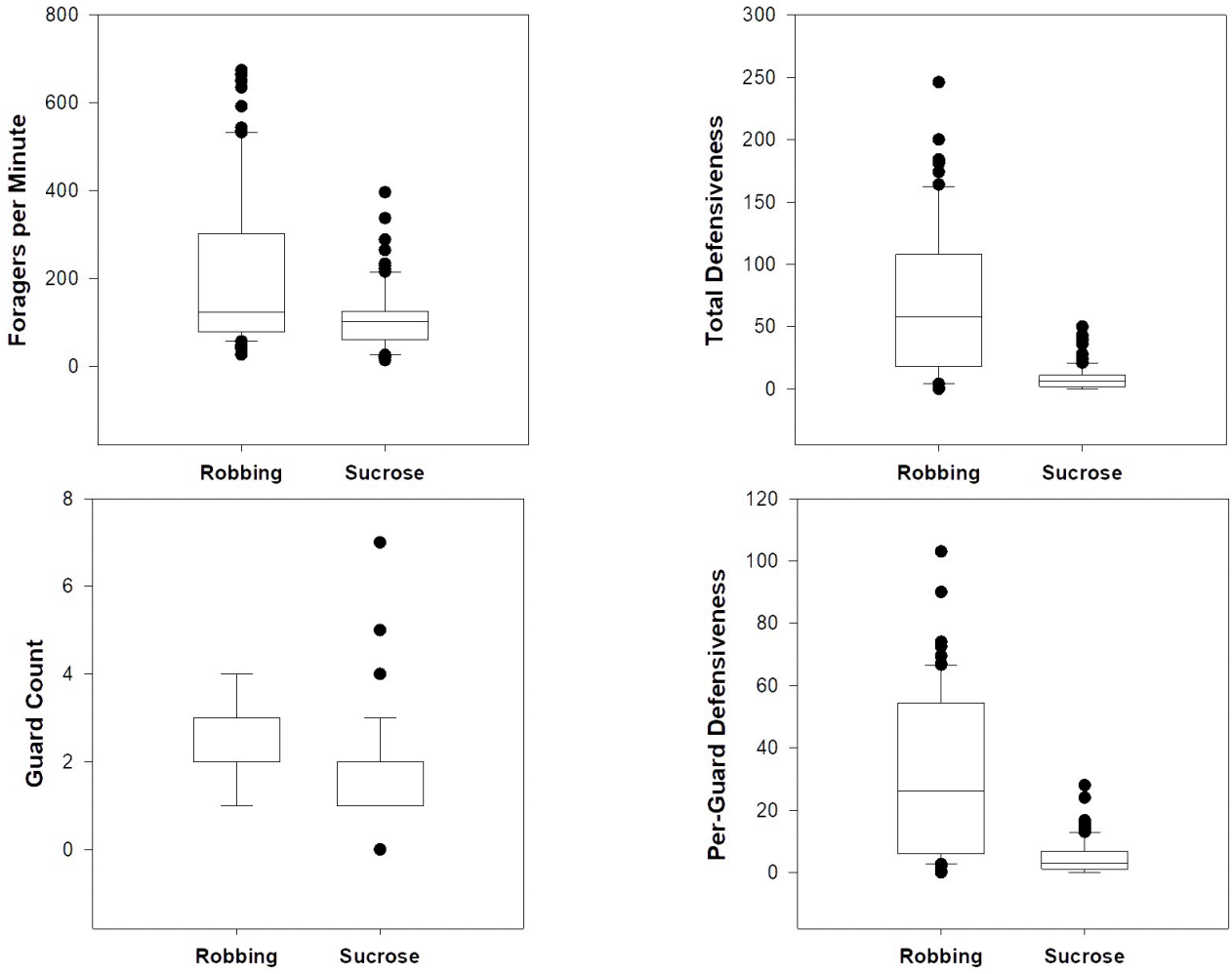


Figure 1 Foraging effort and guard defensiveness increased during the bait hive (robbing) treatment across all hives and both rounds. Treatment was a significant predictor of rate of foraging return (LMM, $p=0.017$), total guard defensiveness (LMM, $p<.0001$), and per-guard defensiveness (LMM, $p<.0001$), but not the number of guards present (LMM, $p=0.471$).

Validation of defensiveness toward nestmates

We took two steps to verify that the increased defensiveness observed by robbing hives was directed towards nestmates and not intruding bees from nearby colonies attracted to the bait

hives. Prior to moving our focal hives, we performed pre-experimental observations of bee visitation to exposed honeycomb frames (see METHODS). No bees were observed to visit the honey frames at any of the field sites for the duration of the pre-experimental test for interfering bee presence prior to the first or second trials. As a second step, we performed an automarker experiment to confirm that bees visiting the bait hives during a robbing event originated from the source colony. For the follow-up experiment of the presence of bees from non-focal hives using the automarker, we found that for the three hives in which robbing was initiated, the proportion of marked to unmarked bees in the bait hive increased over time (See Table A4) as predicted and that by the end of the observation period, the marking rate of the automarker was similar to the proportion of marked bees in the bait hive. The colony at the fourth field site failed to recruit any bees to the bait hive. Statistical analysis was not performed on this data given the low sample size.

Study III: Causes of increased defensive behaviors towards returning nestmates

Hypothesis 1: Increased rate of forager return

We evaluated whether the high rates of forager return during robbing explain increased colony defensiveness. To do this, we re-analyzed data from Study II using a linear mixed model (Table 1A; Figure 2). The response variable was total guard defensiveness (square root transformed for normality) score as the response, with round, treatment, rate of forager return (log transformed for normality), and an interaction effect between treatment and rate of forager return as fixed effects, and hive identity as a random effect. Treatment, rate of forager return, and their interaction effect were significant predictors of guard defensiveness, but round was not

(LMM, treatment: $F_{1,152}$=104.46, p<.0001*; rate of forager return: $F_{1,149}$=6.34, p<.0001*; treatment*rate of forager return: $F_{1,151}$=4.90, p=0.036*; round: $F_{1,149}$=1.02, p=0.32*).

We repeated this analysis to assess the effect of rate of return and two additional metrics of defensiveness, guard count and per-guard aggression. We used a linear mixed model with guard number (untransformed) as the response variable, including round, treatment, log-transformed rate of forager return, and the interaction effect between treatment and rate of forager return as fixed effects, and with hive identity as a random effects. Round, rate of forager return, and the interaction between rate of forager return and treatment were significant predictors of guard number (LMM, treatment: $F_{1,155}$=3.60, p=0.060; rate of forager return: $F_{1,90}$=7.30, p=0.008*; treatment*rate of forager return: $F_{1,154}$=23.46, p<.0001*; round: $F_{1,150}$=13.29, p=0.0004*). We used a linear mixed model with guard count (square root transformed for normalcy) as the response variable, with round, treatment, log-transformed rate of forager return, and the interaction between treatment and rate of forager return as fixed effects, with hive identity as a random effect. Treatment and rate of forager return were significant predictors of per-guard defensiveness, with no other significant fixed effects (LMM, treatment: $F_{1,151}$=82.93, p<.0001*; rate of forager return: $F_{1,153}$=13.74, p=0.0003*; treatment*rate of forager return: $F_{1,150}$=0.50, p=0.482; round: $F_{1,149}$=0.07, p=0.794).

Because the robbing treatment caused a general increase in rate of forager return to a level not observed in the sucrose control, it is possible that the relationship between rate of return and aggression only occurs at higher levels of foraging activity, and is not a function of robbing specifically. We used observations of defensive and foraging behavior from large, unmanipulated hives that have overall higher rates of return than our small focal hives to test for a correlation between guard defensiveness and rate of return outside of the robbing context

(Table 1B; Figure 2). We used a linear mixed model with total guard defensiveness score (square root transformed for normality) as the response variable, with hive as a random effect. We found a significant effect of rate of return (LMM, rate of forager return: $F_{1,17}$=6.82, p=0.018*). We repeated the above model for the other two metrics of defensiveness: guard count and per-guard defensiveness. We used a linear mixed model with guard count (untransformed) as the response variable, with rate of forager return (log transformed for normality), with hive as a random effect. We found a significant effect rate of return (LMM, rate of forager return: $F_{1,24}$=5.50, p=0.028*). We used a linear mixed model with per-guard defensiveness score (square root transformed for normality) as the response variable, with hive as a random effect. We did not find a significant effect of rate of return (LMM, rate of forager return: $F_{1,23}$=0.38, p=0.544).

Figure 2 Simple Regression of Forager Rate of Return and Total Guard Defensiveness

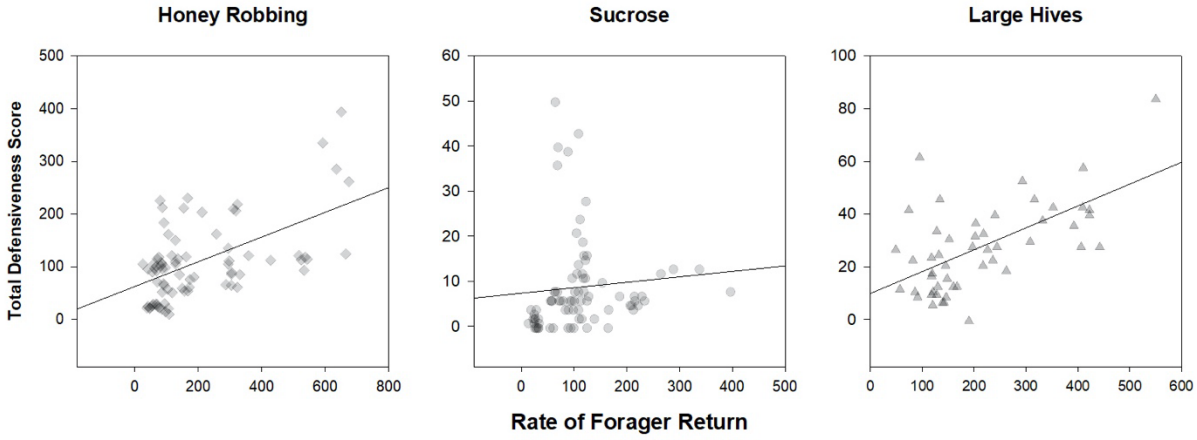


Figure 2 Simple regression of forager rate of return and total guard defensiveness indicates a positive relationship between foraging and defensive effort in the context of robbing and for unmanipulated large hives. The positive relationship for large hives may be a function of total

guard number (LMM, p=0.028), whereas individual guard behavior drives the correlation for robbing hives (LMM, p<.0001).

Table 1 Summary of modeling results for the effect of rate of return on defensiveness

A. Experimental Hives Linear Mixed Modeling Results Summary

Defensive Metric	Effect	*F*	*p*
Total Defensiveness	Treatment	104.46	<.0001
	Rate of Return	6.34	<.0001
	Treatment*Rate	4.9	0.036
	Round	1.02	0.32
Guard Number	Treatment	3.6	0.06
	Rate of Return	7.3	0.008
	Treatment*Rate	23.46	<.0001
	Round	13.29	0.0004
Per-Guard Defensiveness	Treatment	82.93	<.0001
	Rate of Return	13.74	0.0003
	Treatment*Rate	0.5	0.482
	Round	0.07	0.794

B. Large Unmanipulated Hives Linear Mixed Modeling Results Summary

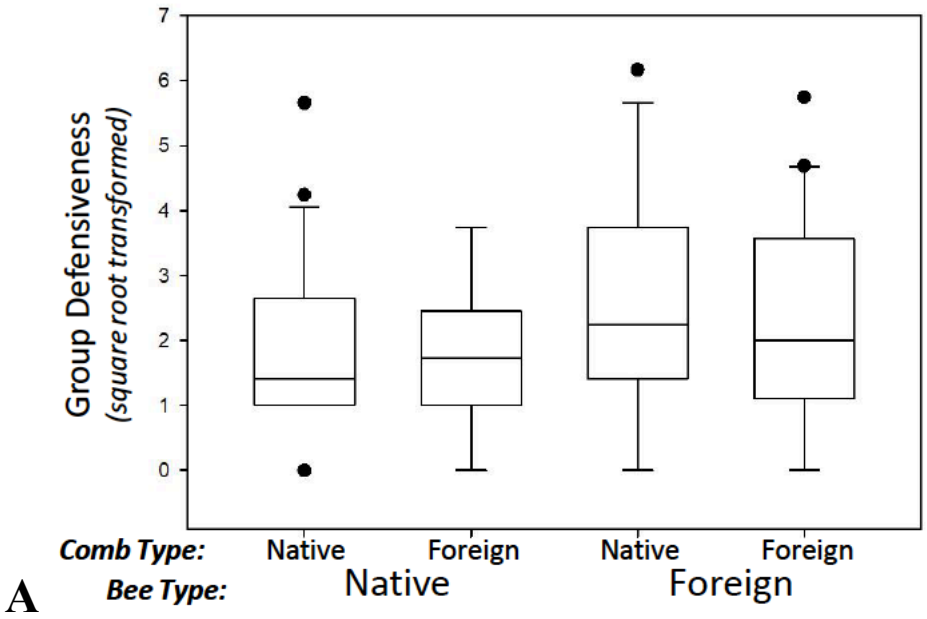
Defensive Metric	Effect	*F*	*p*
Total Defensiveness	Rate of Return	6.82	0.012
Guard Number	Rate of Return	5.5	0.028
Per-Guard Defensiveness	Rate of Return	0.38	0.543

Hypothesis 2: Altered nestmate odor profiles

We determined how honeycomb exposure typical of what occurs during a robbing event impacted aggressive behaviors displayed towards nestmates in a lab-based assay. We used a linear mixed model to test for the effect of intruder comb exposure (native versus foreign) and intruder identity (nestmate versus non-nestmate) on defensive behaviors displayed by small groups of four bees. We used the group aggression score (square root transformed for normality; see calculation in METHODS) as the response variable, including intruder identity, intruder comb exposure, and an interaction effect between identity and exposure as fixed effects, and included the hive origin of the group as a random effect. We found a significant effect of intruder identity on group aggression, but no effect of comb exposure or the interaction between identity and exposure (LMM, identity: $F_{1,75}$=4.01, p=0.048; exposure: $F_{1,75}$=0.08, p=0.779; identity*exposure: $F_{1,74}$=0.00, p=0.995; Figure 3).

We assessed how exposure duration to foreign comb impacted defensiveness toward nestmates. We conducted a non-parametric Kruskall-Wallis test because the data did not fit assumptions of normality, using aggression scores (see METHODS for calculation) to examine differences in aggressive behaviors displayed by small groups of four bees toward groupmates exposed to the group's comb (native) for either 3 or 10 min, or unrelated comb (foreign) at either 3 or 10 min durations. We included a positive control of foragers collected from unrelated hives and a negative control of untreated groupmates for a total of 6 groups with n=18 per group. We found significant differences in expressed aggression by treatment (Kruskall-Wallis, df=5, X^2=36.05, p<0.0001*). We re-analyzed the data after omitting the positive (unrelated forager) and negative (unexposed groupmate) controls as high levels of aggression were expressed only toward the positive control (Table 2). We used a linear mixed model with defensiveness score (square root transformed for normality) as the response variable, including exposure duration,

comb type (native or foreign), and an interaction between duration and comb origin as fixed effects, with hive identity as a random effect. We found no evidence of a significant effect of exposure duration, comb type, or an interaction between duration and comb type (LMM, duration: F_{66}=2.17, p=0.146; comb type: F_{66}=0.01, p=0.944; duration*comb type: F_{66}=0.45, p=0.517; Figure 3B).



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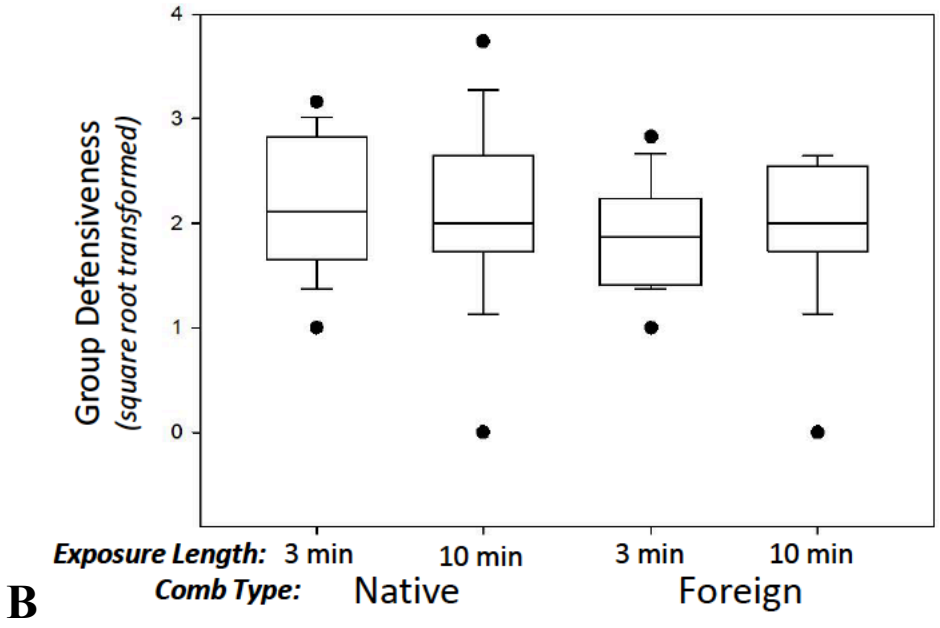

Figure 3 Groups of bees exhibited greater defensiveness toward unrelated (foreign) intruders regardless of comb treatment (A: LMM, p=0.048) and did not show increased aggression toward related (native) intruders exposed to foreign comb (LMM, p=0.779). Duration of comb exposure did significantly predict groupmate defensiveness toward treated groupmates (B: LMM, p=0.146)

Table 2 Wilcoxon Each Pair Results for Comb Duration Aggression

Pair		Z-score
FOR	F3	**4.68*** **
FOR	F10	**4.52*** **
FOR	CTL	**4.27*** **
N3	F3	1.59
N10	F3	1.14
N3	F10	0.97
F10	F3	0.87
N3	CTL	0.37
N10	F10	0.38
N10	CTL	0.14
F10	CTL	-0.18

N10	N3	-0.38
F3	CTL	-0.94
N3	FOR	**-4.20*** **
N10	FOR	**-4.26*** **

Significant tests indicated in bold; ***, $p \leq 0.001$. FOR, returning forager; F3, foreign 3 minute; F10, foreign 10 minute; CTL, unexposed control; N3, native 3 minute; N10, native 10 minute

Hypothesis 3: Returning robbers are more aggressive than typical foragers

We compared brain expression patterns of four aggression biomarker genes (Rittschof & Robinson 2013) to assess whether robbing foragers show evidence of elevated aggression compared to normal returning foragers. In one-tailed tests of the hypothesis that robbing bees show higher aggression than typical foragers, we found that all four genes were differentially expressed as a function of robbing in a pattern identical to the differences in expression comparing soldiers (bees specialized for defense) and foraging bees (Table 3; Figure 4): *GB53860*: t_{38}=5.83, p<0.0001 (up in robbing, up in soldier in Rittschof & Robinson 2013); *inos*: t_{38}=-1.68, p=0.05 (down in robbing, down in soldiers); *drat*: t_{38}=1.78, p=0.04 (up in robbing, up in soldiers); *Cyp6g1/2*: t_{38}=2.58, p=0.007 (up in robbing, up in soldiers).

Table 3 Aggression Biomarker Gene Expression

				Expression compared to foraging	
Name	BeeBase ID	Description	t_{38}	Robbing	Soldier[†]
unknown	GB53860	none	5.83***	up	up
inos	GB55016	Inositol-3-phosphate synthase 1B	1.68*	down	down
drat	GB51125	Death resistor ADH domain containing target	1.78*	up	up
cyp6g1/2	GB52023	Cytochrome P450	2.58**	up	up

[†]Rittschof & Robinson 2013; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$

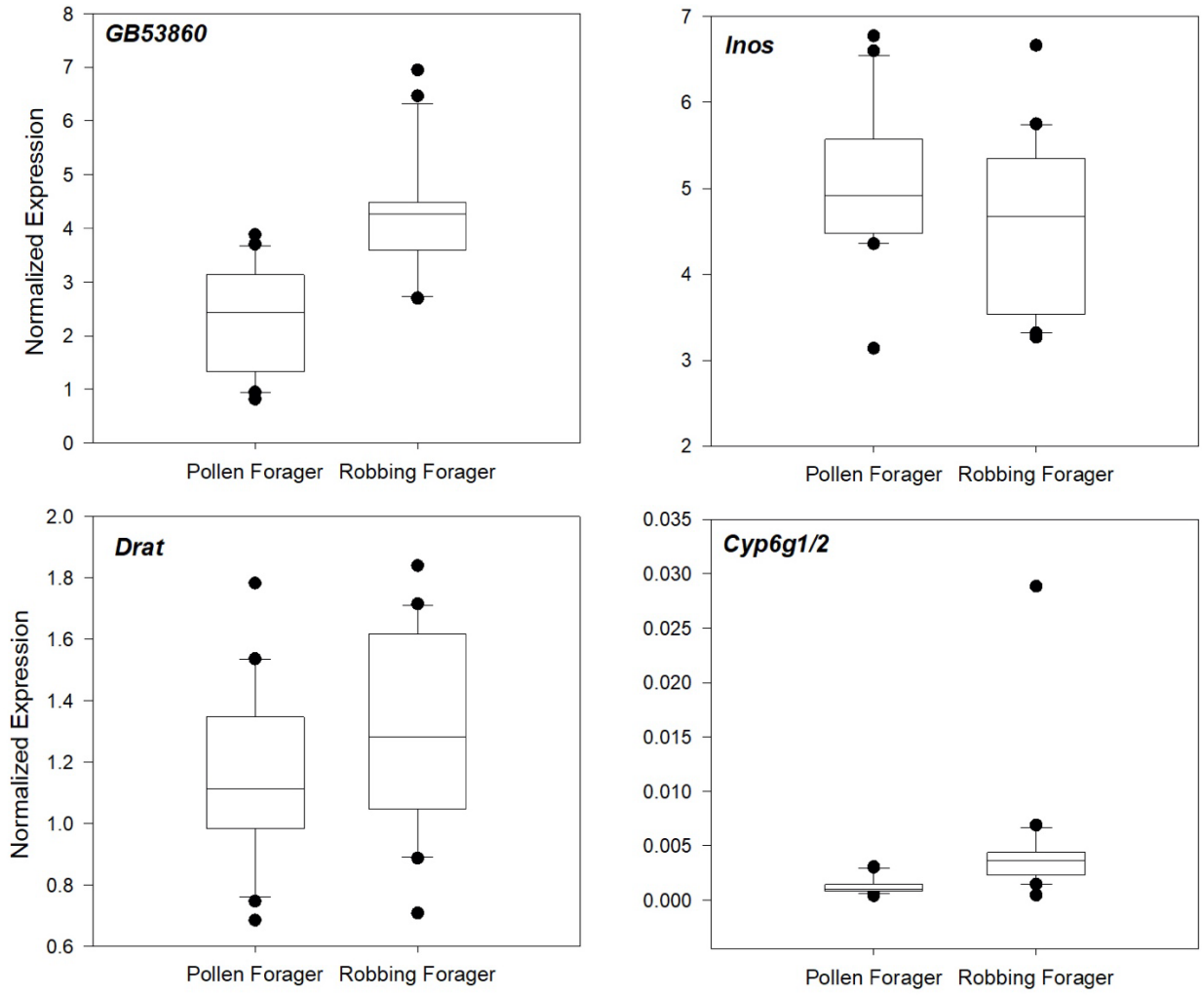

Figure 4 The four aggression biomarker genes assessed were differentially expressed between pollen and robbing foragers in one-tailed tests: *GB53860* (T-Test, p<.0001), *inos* (p=0.05), *drat* (p=0.04), and *cyp6g1/2* (p=0.007). These patterns are consistent with differences between aggressive soldiers and typical foragers.

CHAPTER 4: DISCUSSION

The honey robbing strategies of *A. mellifera* provide a useful context for the demonstration of predictions under optimal foraging theory. Seasonal floral phenology is associated with shifts in defensive effort against intraspecific threat, corresponding long-held beekeeper wisdom about the prevalence of late-season robbing (Downs and Ratnieks 2000). Similarly, acute increases in defensive effort as a response to perceived conspecific intrusion occur independent of floral resource availability, as weak colonies provide attractive opportunistic targets (Couvillon et al. 2008). Defensive effort is inextricably linked to patterns of honey robbing, further supported by the evolution of the guard sub-caste principally as a response to robbing pressure (Breed et al. 2012). The positive relationship between robbing effort and defensive effort is evident in the antagonistic interaction between the aggressor and defender colonies. An increase in robbing effort and investment by the aggressor is required to overcome corresponding increases in defensive effort and investment by the defender. These increases constitute costs to both aggressor and defender, influencing the relative optimality of a robbing strategy and subsequently the relative value of defensive investment (Pyke 1984). We propose this relationship between robbing and defensive effort holds not only *between* colonies in the context of aggressor and defender interactions, but extends to the *within* colony context of trade-offs between defensive and foraging (robbing) effort.

The within-colony relationship between robbing and defensive effort is an extension of the plastic defensive responses associated with the perception of relative risk in between-colony interactions. Immediate increases in defensive effort against opportunistic robbing is a result of guard perception of intrusion (Couvillon et al. 2008). The relative risk is

perceived as high and defensiveness is accordingly increased as intruder presence may indicate nearby colonies are scouting for robbing opportunities. Similarly, the seasonal wane of floral resources indicates heightened risk as the relative value and thus the prevalence of robbing increases, though the mechanism by which colonies perceive this risk is unclear (Downs and Ratnieks 2000). Within the colony, engaging in robbing could provide a reliable indicator of elevated risk to the colony when thusly engaged. Relative risk is increased as the presence of an opportunistic resource to rob may also draw other robbing colonies to the vicinity, or as ecological conditions promoting the optimality of the colony engaging in robbing also promote the strategy for nearby colonies. We found increases in defensive effort occur as a colony engages in robbing with repeated measures of multiple colonies accounting for in-colony plasticity and genetic differences between hives. These observations occurred within a short period of time removing the effect of seasonality, and occurred without evidence of conspecific intrusion.

The increased defensiveness of colonies engaged in robbing suggests a colony's defensive needs may constrain robbing optimality. Robbing hives may in turn be robbed, resulting in colony death and necessitating some minimum defensive capacity (Seeley 1995). Foraging (robbing) and defensive effort should trade off both acutely due to a shared worker pool, and long-term by differential energetic investment in worker production (Rivera-Marchand et al. 2008). The increased risk of being robbed due to reduced defensive investment may present a cost associated with the robbing strategy. However, we demonstrate the ability of colonies to simultaneously and rapidly increase both foraging and defensive effort while participating in robbing. Task allocation decisions may function through satisficing, such that robbing does not occur unless defensive needs are sufficiently met (Ward 1992). Satisficed nest defense could be

assessed through colony size, similar to how colony-level reproduction is initiated through reaching demographic thresholds (Smith et al. 2014), Another potential explanation is colonies maintaining an excess of inactive workers in either role beyond what is needed. A small proportion of foragers are shown to perform disproportionately large amounts of work, leaving a reserve which could be activated if the need arises, i.e. robbing (Tenczar et al. 2014). Additionally, the trade-off may never be effected if foraging effort has an upper limit fixed by the availability of receiver bees to accept honey (Anderson et al. 1999) Alternatively, increases in effort for foraging and defensiveness may occur at different scales. Significant increases in defensiveness did not correspond to significant increases in individuals performing guard tasks, while increases in foraging effort corresponded to the addition of approximately 72% more individuals. Though our study did not assess metrics of individual foraging effort, robbing colonies may be able to dynamically balance defensive and foraging needs by modulating foraging effort primarily at the colony level and defensive effort primarily at the individual level. A trade-off between foraging and defensive effort may exist, but the low cost of increased intraspecific defensiveness relative to foraging effort may result in a negligible penalty to the optimality of a robbing strategy.

A colony's defensive needs may also constrain the value of robbing by increasing the amount of foraging effort needed. The defensive behaviors of guards observed in this study were expressed toward returning nestmates. Defensive behaviors during robbing were seldom injurious (see Table A#) and lethal behaviors rarer still, but even delay for antennation may represent an additive cost. This may provide an explanation for the lack of observed increase in guard number during robbing, as opposed to studies which found guard number increased in response to actual intrusion by conspecifics or predators (Nouvian et al. 2016). A response of

elevated guard number and elevated guard defensiveness could unsustainably impede robbing effort, thus our observations may indicate a state of heightened alert rather than a misperception of intrusion. However, this proposition requires consideration of the mechanism by which guards determine the colony is participating in robbing, as misidentification of returning robber nestmates presents a strong explanatory candidate. We assessed three potential cues guards could use to perceive the robbing state of the colony: transfer of the identifying odors of the robbed hive to robbers, persistent aggressive behaviors of nestmate robbers, and elevated foraging activity.

Robbing foragers make extensive contact with the comb inside other hives as they chew apart the wax cells containing honey, which could result in the acquisition of non-nestmate odors (Breed 1998). We found no effect of robbing-analogous comb exposure on the aggression expressed by groups of young adult bees toward intruders. We also found no effect of comb exposure or duration of exposure in a second assay of similar design. Our results do provide evidence that the groups of young bees used in these assays were capable of discrimination, as nestmate status was a significant effector of aggression in the first assay, and groups responded to an unrelated forager positive control as expected in the variable duration trial. We used a well-established assay of aggression (e.g., Rittschof & Robinson 2013) however conclusions relevant to the robbing context may be limited by our use of young bees, which have a reduced odor profile compared to forager-aged bees (Vernier et al. 2019) and the use of a laboratory setting (Couvillon et al. 2013) despite evidence of robust aggression in similar methodologies (Rittschof et al. 2015). These findings were surprising as studies of honey bee nestmate recognition place wax comb in a mediating role (Breed 1998). Conversely, consistent transfer of foreign odor cues to robbers sufficient as to prevent nestmate recognition would be a heavy constraint on the

optimality of the robbing strategy. The rarity of injurious behaviors in the field experiment may also indicate guards are not mistaking nestmate robbers as actual intruders as would be expected if odor identity was wholly obscured. Robbers may have some method of mitigating odor transfer, or perhaps acquisition in general occurs through a more complex process than comb contact alone.

Direct assays of guard response to nestmate robber behavior are complicated by the transience of the robbing behavioral syndrome, the dependency of guard function on a nest entrance context, and the large colony-level response (Free 1954, Couvillon et al. 2013). We instead opted for an indirect assessment of robbing nestmate aggression as a potential guard cue. The differential expression of aggression biomarker genes between robbing and typical foragers provide a strong indicator that robbers are in a state of heightened aggression when returning to the home colony. The four aggression biomarker genes used robustly track variation in behavior, supporting the hypothesis that nestmate robbers persist in exhibiting aggressive behaviors which guards can perceive (Alaux et al. 2009, Rittschof 2018). Persistent aggression by robbers may be expected if robbers are increasing foraging effort through multiple trips to the robbed colony. The state of elevated aggression in robbing foragers occurred despite the absence of defenders at the robbed hive, interestingly contrasting with Free's (1954) hypothesis that guards at the robbed hive initiated the robber behavioral syndrome. Robbers may attain this state through interactions with guards of their own colony expressing increased defensiveness. This may provide preliminary evidence for the presence of a positive feedback mechanism where both guards and robbers are provide excitatory signals for the other role, generating a colony-level robbing state until the resource is exhausted. Alternatively, other aspects unique to the robbing experience such as destroying comb or entering a foreign nest could provide a cue. Future studies assessing

expression patterns of robbers foraging at bait hives in various states of deconstruction could elaborate.

We found foraging effort as a function of rate of returning foragers significantly increased during robbing, explained primarily by the mobilization of large numbers of additional foragers. We hypothesized defensive activity may increase proportional to foraging activity independent of context. This could occur as a long-term trend if the number of guards relative to foragers remains at a fixed ratio as a colony grows. Similarly, short-term increases in forager activity when a valuable resource is discovered would also be associated with elevated defensiveness, as we observed during robbing. Forager return rate was generally a significant predictor of the metrics of defensiveness in the robbing study, as was the interaction between rate of return and treatment. However, the correlation was evident only at the very high rates of return observed during the robbing treatment. Large hives with unmanipulated forager activity similar to the level of forager activity in the robbing treatment showed a weaker correlation, with rate of return only significantly predicting total defensiveness and guard number, but not per-guard defensiveness. The high levels of defensiveness and rate of return in large hives appear to be a function of population level, and not individual guards responding to high levels of returning foragers. Forager activity may contribute in part to guard defensiveness, but does not appear to exclusively account for the defensive response in the robbing context. Our study assessed the relationship between a rate of forager return corresponding to a simultaneous defensiveness measurement. In the robbing treatment, large increases in rates of return were occurring over the hour between presentation of the bait hive and the beginning of measurement. Guards could be responding to this acute change over time, as opposed to a proportionate response. We do not address how guards may perceive rates of forager activity. Guards could

track rate of interactions, similar to how physical contact rates inform colony-level reproduction (Smith et al. 2017). Alternatively, the gestalt of a sudden influx of foragers entering the nest may be indistinguishable from large-scale intrusion to guards.

Interspecific defensiveness in honey bees presents a complex intersection of environment, genetics, and social information, and it is expected that this complexity extends to intraspecific threats as well (Nouvian et al. 2016). We examined three potential cues which could provoke increases in guard defensiveness, and found at least some association with forager activity and robber behavior. The initiation of increased defensiveness by guards when the colony participates in robbing is likely a result of multiple cues. The incorporation of information from multiple cues may enable dynamic balancing of the defensive response. If one cue or experience initiates a guard defensive response, an additional cue could be used to target or moderate the level of response relative to risk. An analogous process occurs in foraging decisions, where foragers differentially respond to social cues indicating resource value in light of other cues indicating risk resulting in a social feedback mechanism which balances benefit and risk (Wray et al. 2012, Jack-Mccollough and Nieh 2015). Interactions between guards and robbing nestmates mediated by multiple cues could represent a social feedback mechanism resulting in optimized defensive responses which minimize impediment of nestmate robbers while still providing sufficient defense.

Predictions of such a feedback mechanism would be supported if the robbing defensive state is elevated relative to typical foraging, but restrained relative to actual conspecific intrusion or disturbance by interspecific predators. Indeed, our study found lethal behaviors were rare and increases in guard number were minimal. Increases in defensiveness during robbing were also insufficient as to significantly reduce the volume of foragers entering the hive, instead

demonstrating a positive relationship in contrast to the negative relationship predicted for actual intrusion. The elevated but restrained defensive state observed during robbing is also consistent with defensive and foraging effort modulated at different organizational levels, explained by only individual guards increasing defensiveness without subsequent activation of colony-level defenses such as soldiers (Breed 1990).

Notably, shifts in intraspecific defense are not mediated by alarm pheromone, which guards release to initiate colony-level defensive responses to predator disturbance (Couvillon et al. 2010). Otherwise, a colony-level response to guard misperception would represent a severe devaluation of robbing optimality or restrict cues to only very reliable signals. Restricting variable intraspecific defensiveness to the level of individual guard may manage the foraging and defensive effort trade-off by reducing a colony's necessary investment in producing defensive individuals, allowing greater investment in forager production. Doing so may also allow efficient moderation of defensive effort and may capitalize on guard recognition errors, increasing defensiveness during periods of elevated risk associated with robbing without the excess cost of erroneously activating defensive elements. Lastly, regulation at the level of individual guard may facilitate robbing as an optimal foraging strategy by allowing a greater number of workers available to rob instead of guard, and minimizing the cost increased guard number would incur through impeding nestmate robbers.

Honey robbing is both a useful tactic and pervasive threat to colonies. The optimality of robbing is apparent in the widespread and repeated emergence of conspecific resource theft across social hymenopteran taxa, which includes obligate kleptoparasites of congeners (Breed et al. 2012). Robbing spurs not only myriad defensive adaptations, but is also implicated as a driver of social complexity (Gruter et al. 2017). Our study explored one such driver by assessing if and

how colonies manage the trade-off between foraging and defensive effort when participating in robbing. A dynamic, calibrated balance may be necessary to achieve simultaneous increases in both and to maximize the energetic value of robbing without risking intrusion. We found colonies were capable of elevated foraging and defensive effort when robbing, and determined defensive effort is likely a result of guards responding to multiple cues. We additionally presented a novel methodology for initiating honey robbing without the associated destruction of colonies, enabling repeatable testing without incurring heavy logistical loss. Future studies should assess for socially-regulated feedback mechanisms governing the link between guard defensiveness and nestmate robbers. Identifying the cues initiating heightened states of aggression in robbers and assessing for robbing-related shifts in other task roles such as receivers could clarify to what extent robbing provokes a colony-level response. Determining if defensive shifts during robbing are isolated to individual guards could support shifts at different levels of organization as strategy for managing trade-offs.

APPENDIX

A1: *Cuticular Hydrocarbon Preliminary Study*

We predicted guard defensiveness towards returning nestmate robbers was a result of robbers acquiring recognition odor cues through contact with the wax comb of the robbed hive (See above, *Study III, hypothesis 2: Altered nestmate odor profiles*). In addition to laboratory assays of nestmate recognition, we assessed for differences in odor profiles between foragers and nestmate returning robbers. Due to late-season nectar flow, we were unable to initiate robbing following initial collection resulting in an insufficient sample size. We present the results below.

METHODS

We placed a single small, queenright colony (as those used in Study II, see above) at a field site used in Study II on 02 July 2019. We prepared ten 3mL glass reactions vials with 20mm open-top screw caps and PTFE/rubber discs (ThermoFisher Scientific, Waltham, MA, USA), autoclaved and cleaned with alternating hexane and acetone washes. Each vial was filled with 2.5mL of hexane and sealed with the lid before transport to the field site. We held collections on 03 September 2019. We collected a single returning forager into each vial using forceps cleaned with alternating hexane and acetone washes before and between collections, for a total of five samples. Samples were agitated gently by hand for 10 minutes to facilitate extraction, then the bee was removed with a metal loop, also cleaned as the forceps. We then initiated as described in Study II (above), allowing one hour between placement of the bait hive and collection. We then repeated the collection technique for five returning robbers. We reduced the sample volume for all vials to 1mL under a nitrogen stream. We prepared 1.5mL autosampler vials for analysis by including 20µL of 100ng/µL C_{20} (icosane) standard and 100µL of sample. Analyses were conducted using an Agilent 6890 Gas Chromatograph interfaced with an Agilent 5975 Mass Selective Detector (Agilent, Santa Clara, CA, USA). The GC was equipped with a 30 m DB5 column (250 µm internal diameter, 25 µm film thickness). A temperature ramp from 60°C (2 minute hold) to 320°C (2 minute hold) at 10°C per minute was used. The MSD was operated in EI mode with mass scan from 40 to 550 m/z. Data were normalized to the known quantity (ng) of icosane internal standard.

RESULTS

We log-transformed ng/bee values for normality. We first assessed for a difference in total cuticular hydrocarbons between foraging and robbing bees. We used a two-tailed t-test of the log-transformed ng/bee mass of all hydrocarbons. We found no significant difference between foragers and robbers (t-test, df=7, t=0.10, p=0.921). We then compared individual compounds between treatments using two-tailed t-tests of log-transformed ng/bee. Only 11-+13 methyl nonacosane significantly differed between the 28 compounds assessed in this study (t_7=3.10, p=0.017). We noted trends in Henicosane and 11-+13 methyl Heptacosane being elevated in robbers, with Triacontane, Dotriacontane, and Tetratriacontane being elevated in foragers.

Table A1 Cuticular Compounds (ng/bee)

Name	Forager Mean	Forager SE	Robber Mean	Robber SE	t_7
Icosane (Standard)	2000		2000		n/a
Henicosane	234.19	96.33	665.08	139.88	2.04

Docosane	399.05	73.10	623.11	237.99	0.87
Eicosen-1-ol	6171.42	3419.89	14342.42	4891.99	1.81
Tricosene	653.61	137.07	987.83	195.28	1.36
Tricosane	3767.61	763.83	6722.35	2295.81	0.85
Tetracosane	310.27	68.41	412.41	140.08	1.5
Pentacosene	1534.36	434.18	1858.60	624.53	0.18
Pentacosane	8546.28	3357.19	15125.10	5398.55	1.03
Hexacosene	4336.21	3889.84	422.73	118.18	-0.62
Hexacosane	936.03	162.34	1356.85	380.65	0.66
Heptacosene	2061.96	954.75	1596.54	705.03	-0.27
Heptacosane	25585.46	8494.19	35592.43	11470.20	0.48
11-+13 methyl Heptacosane	595.12	145.85	1002.71	157.24	2.3
Octacosane	3040.15	343.43	2273.06	347.73	-1.6
Nonacosene	3262.62	1537.21	2661.95	367.42	0.2
Nonacosane	25331.24	6178.13	25919.03	6261.39	0.06
11-+13 methyl Nonacosane	366.36	82.09	700.89	94.33	**3.10***
Triacontane	4203.62	660.49	2737.17	480.88	-1.9
Hentriacontene	1259.35	297.37	727.60	212.81	-1.41
z-(7)-Hentriacontene	8516.80	2022.57	9179.90	1366.55	0.47
z-(9)-Hentriacontene	7795.91	1551.16	7634.48	1573.32	-0.05
Hentriacontane	19808.59	3931.97	17427.75	4440.43	-0.49
Dotriacontane	7911.19	4111.27	2190.49	511.10	-1.92
Tritriacontadiene	3695.54	734.68	2176.06	407.23	-1.32
Tritriacotene	15394.00	2730.86	20815.04	5844.62	0.45
Tritriacotane	4903.86	745.42	3858.88	628.86	-1.11
Tetratriacontane	3039.23	557.98	1657.72	407.93	-2.05

Significant tests indicated in bold; *, $p \leq 0.05$. N=5 per treatment.

DISCUSSION

Successful defense of the nest requires guards to discriminate between nestmates and interspecific or intraspecific intruders. Guards use visual cues to identify wasps and other predatory insects, while non-nestmate conspecifics are identified primarily through odor cues (Nouvian et al. 2016). These odor cues are colony-specific arrays of hydrocarbons, fatty acids, and esters embedded in the wax cuticle (Breed et al. 1995). Transference of odor during contact with the wax comb of the nest is proposed as a mechanism for both acquisition and homogenization within a hive. These odor cues may be modified through contact with the environment, as both contact with comb from unrelated nests and treatment with floral oils can increase rates of rejection by nestmate guards (Breed 1998). Cuticular hydrocarbon arrays also differ within a colony between individuals of different ages, performing different tasks, or those infected with pathogens (Vernier et al. 2019). The amount of variation in total mass of specific chemicals and between-chemical ratios required to elicit a rejection response from guards is unclear.

We hypothesized changes in odor profile from contact with foreign comb during the course of robbing could result in the increased level of guard defensiveness observed in Study II. However, we found typical foragers and returning robbers showed little variation in cuticular hydrocarbon arrays. This finding provides some evidence for guards responding to robber behavioral cues when increasing defensiveness as a response to the colony engaging in robbing. Our study also only evaluated hydrocarbons, while other compounds such as fatty acids are also implicated as odor cues used in recognition. Lastly, returning foragers sampled at the hive entrance during robbing may not necessarily have been engaged in robbing or been in sufficient contact with foreign comb. Our findings provide further evidence that CHC array acquisition is the result of complex social and environmental interactions which do not occur in the process of robbing, and that the observed increase in guard defensiveness when a colony engages in robbing is not exclusively a function of odor transference.

TABLE A2: *Study I, Validation of Robbing Methodology.*

Table A2 Characteristic Robbing Behaviors by Treatment

	Behavior	Sucrose	Robbing
Round 1			
	Mean Casts/min	0	7.75
	SE Casts/min	0	1.79
	Clusters Observed	0	0.857
	AES Observed	n/a	
Round 1			
	Mean Casts/min	0	10.5
	SE Casts/min	0	1.78
	Clusters Observed	0	0.625
	AES Observed	n/a	0.625

Casting: N=40 observations across 8 hives; Clusters and AES are proportions of N=8 hives displaying the behavior at any timepoint.

TABLE A3: *Study II, Observation of Guard Defensive Effort*

Table A3 Observed Guard Behaviors by Injury Category

		Non-injurious	Injurious	Lethal
Robbing				
	Count	3018	300	7
	Proportion	0.908	0.09	0.002
Sucrose				
	Count	403	36	0
	Proportion	0.918	0.082	0

Pooled across 80 one-minute timepoints across 8 hives and two rounds per treatment.

TABLE A4: *Study II, Validation of Defensiveness Toward Nestmate*s

Table A4 Proportion Marked by Automarker and on Honeycomb Frame by Time

		Time 1	Time 2	Time 3	Time 4	Time 5
Hive A						
	Automarker Success	0.90	0.82	0.72	0.57	0.86
	Marked on Frame	0.57	0.55	0.56	0.67	0.71
Hive B						
	Automarker Success	0.67	1.00	0.33	0.72	0.50
	Marked on Frame	0.67	0.67	0.71	0.67	0.83
Hive C						
	Automarker Success	0.69	0.54	0.88	0.84	0.83
	Marked on Frame	0.67	0.67	0.63	0.70	0.82

FIGURE A1: The Automarker used in Study II: Validation of Defensiveness Towards Nestmates.

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