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Hygienic behavior is a mechanism of social immunity in which adult honey bees detect and remove unhealthy brood. Hygienic behavior can be exhibited by all workers, but some individuals are specialized in the trait and can perform it at exceptional levels. Studies have shown that task specialization scales with increasing group size. The current study investigates the performance of hygienic behavior by large and small groups of workers across three scales and whether task specialization promotes hygienic performance. Workers in groups of different sizes across small, intermediate, and large scales were subjected to standard hygienic assays and hygienic behavior was measured at various time intervals. Direct behavioral observations were made in the small and intermediate scale experiments to compare task specialization between groups. The number of cells uncapped was significantly greater by the large group at the large scale and disproportionately greater by the large group at the small scale. The result of the small scale experiment may have been more affected by worker density than by task specialization. Neither the number of uncapped cells nor the task specialization measured at the intermediate scale were statistically significant, which may be due to small sample size or deficiencies in experimental design. Overall, these results suggest an influence of group size on hygienic performance but the scaling of this effect and the role of task specialization could not be fully resolved. Worker density may be an important aspect of group size that needs to be addressed when assessing hygienic behavior performance.

Keywords: Honey bees, group size, task specialization, hygienic behavior, division of labor, social immunity, *Varroa destructor*

THE IMPACT OF HONEY BEE (*APIS MELLIFERA*) GROUP SIZE ON HYGIENIC
BEHAVIOR PERFORMANCE

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CHAPTER I

INTRODUCTION

Importance of Honey Bees and Health Decline

Honey bees are globally recognized for their role in modern agriculture through commercial pollination and contributions to food security. Despite challenges in estimating the impact of pollination on the global economy (Klein et al. 2007; Hein 2009), it is estimated that the value of crops dependent on honey bee pollination totaled \$11.68 billion in the US in 2009 (Calderone 2012). Honey bees pollinate approximately 100 crops globally with some, such as almonds and cherries, being almost totally reliant on their pollination for reproduction (McGregor 1976; Hein 2009; Ward et al. 2010).

A downward trend of honey bee health has been observed for several years (vanEngelsdorp et al. 2010; vanEngelsdorp et al. 2011; Lee et al. 2015; Bruckner et al. 2017; Bruckner et al. 2018). Poor nutrition, parasites, pathogens, and pesticides are regarded as the greatest threats endangering honey bee health (Aronstein and Murray 2010; Rosenkranz et al. 2010; Johnson and Percel 2013; Dolezal & Toth 2018). Synergism among these stressors have been shown to increase immune challenges in honey bees (Johnson and Percel 2013; Pettis et al. 2013; Degrandi-Hoffman et al. 2015; Johnson 2015). One of the threats that requires constant monitoring by beekeepers is the population of *Varroa destructor* in their hives. This obligate ectoparasitic mite is particularly difficult to manage as its reproductive cycle is dependent on the development

of the honey bee (reviewed in Rosenkranz et al. 2010). A female *Varroa* mite attached to a nurse bee enters an open brood cell prior to capping and feeds on the fat body of the developing honey bee pupae (Ramsey et al. 2019). The first egg develops into a male which then mates with the 2-5 female offspring. The female mites emerge from the cell with the adult bee and begin their phoretic stage by climbing onto another worker to complete the life cycle. During the feeding process, mites can transmit viruses such as Deformed Wing Virus and *Varroa destructor* virus-1 (Levin et al. 2016; Ramsey et al. 2019). These viruses can cause physical deformities, weaken the immune system, and can be easily transmitted vertically, from queen to offspring, and horizontally, from nestmate to nestmate (Chen et al. 2006 a; Chen et al. 2006 b).

Several chemical and non-chemical treatments are employed by beekeepers to manage mite populations. Non-chemical techniques, often used by small-scale beekeepers, require physical intervention and can be time consuming such as drone brood removal, artificial brood breaks, and requeening. Studies have shown these methods to be more effective when coupled with other techniques (Wilkinson and Smith 2002; Wagnitz and Ellis 2010). Chemical treatments have a toxin active ingredient such as amitraz, fluvalinate and coumaphos applied to a substrate which is installed in the hive. Some of these agents have been shown to reduce queen and worker lifespan (Dahlgren et al. 2012), interfere with detoxification pathways (Boncristiani et al. 2012), and synergistically increase toxicity of fungicides (Johnson et al. 2013). In addition to these risks posed to honey bees, beekeepers themselves, consumers, and the environment can also be affected by use of acaricides. Beekeepers must take precautions when

administering acaricides that require vaporization, such as oxalic acid, as the fumes can be toxic to the user. Other treatments, such as ApiVar strips (amitraz), can only be applied after the beekeeper has harvested the honey crop as the treatment will contaminate the honey and potentially make consumers sick. Some acaricide residues have been found in beeswax and other hive products (reviewed in Bogdanov 2006; Mullin et al. 2010) which has the potential to contaminate honey despite precautions taken by the beekeeper. An additional consequence of synthetic acaricide application is the potential of these chemicals leeching into the environment. While this idea has not been studied extensively, a few routes of transmission could be possible. Several organisms have been known to inhabit abandoned bee hives including mice, reptiles, and other insects which may ingest or encounter contaminated hive products and spread these chemicals outside the hive. Another possibility is beekeeping equipment being left outside which may get rained on and create run off to various waterways which may affect aquatic species. Due to these numerous effects, it becomes imperative to understand and augment natural defense mechanisms of honey bees.

Hygienic Behavior and Social Immunity

One promising method of *Varroa* control is breeding for hygienic behavior in honey bee populations. Hygienic behavior is the ability of specialized adult workers to sense and remove dead, diseased, or parasitized brood (Rothenbuhler 1964). This behavior involves a worker first removing the wax cap over the brood, known as uncapping, so that the exposed unhealthy brood may be evaluated and potentially

removed from its cell and transported out of the colony. Instances of natural hygienic behavior are seen in response to diseases like American foulbrood and chalkbrood, as well as *Varroa destructor* infestations (Milne 1983; Spivak & Reuter 2001; Cheruiyot et al. 2018). This rare trait (Arathi et al. 2000) was initially hypothesized to be operated by two recessive genes, one for uncapping and another for removing (Rothenbuhler 1964), but it is now thought to be the result of several interacting genes (Lapidge et al. 2002; Oxley et al. 2010; Behrens et al. 2011; Le Conte et al. 2011; Tsuruda et al. 2012; Boutin et al. 2015). Continued investigation into the genetic architecture of this trait will enhance marker-assisted selection of hygienic stocks. Even without marker-assisted selection, several hygienic lines of honey bees have already been selected based on phenotype, such as Minnesota Hygienics, POL Line, and *Varroa* Sensitive Hygienics (VSH). The Minnesota Hygienic line performs disease-associated hygienic behavior while the VSH and POL lines perform *Varroa* sensitive hygienic behavior. *Varroa* sensitive hygiene operates through uncapping parasitized cells which disrupts mite reproduction and can decrease overall mite population in the colony (Ibrahim and Spivak 2006). This trait can be beneficial to beekeepers as it can mitigate some use of harmful acaricides while having the potential to increase colony survival chances. Additionally, acaricide resistance in mites is a growing concern among beekeepers and researchers as mites will become increasingly harder to treat. By reducing the use of synthetic acaricides in place of hygienic stocks of bees, the rate of mite resistance to acaricides may also be reduced (Sammataro et al. 2005).

The act of sacrificing an individual for the colony is an important contribution to colony health and is a mechanism of social immunity (Shorter & Rueppell, 2012). Social immunity is a defense unique to social organisms that requires the collective cooperation of nestmates to manage pathogens and parasites. This process is particularly important for honey bees as the number of immune genes at the individual level is significantly reduced compared to other insects, such as flies and mosquitoes (Evans et al. 2006). It is thought that this decreased immunity at the individual level is due to the existence of social immunity reducing pathogens and parasites (Harpur and Zayed 2013). There are several modes of social immunity. Constitutive mechanisms are always expressed or inherent in the structure of the colony, while others are inducible, meaning they are activated in the presence of pathogens (reviewed in Simone-Finstrom 2017). Hygienic behavior is a type of inducible social immunity that occurs in response to the stimulus of unhealthy brood.

Division of Labor and Task Specialization

The honey bee caste system is primarily based on reproductive division of labor. As the reproductive caste, the female queens mate with male drones from other colonies and return to the colony to lay eggs in overlapping generations that maintain the colony's working population. This workforce is comprised of thousands of reproductively sterile females that perform a series of age-related tasks, known as temporal polyethism (reviewed in Beshers and Fewell 2001; Gautrais et al. 2002). The propensity of different individuals to perform these tasks is known as the division of labor and is a characteristic

measured at the colony level (reviewed in Beshers and Fewell 2001). Young workers begin with in-hive tasks such as nest-cleaning and brood nursing (Crailsheim 1992). As workers transition into middle-aged labor, they take on a variety of other tasks including entrance guarding, hive construction, and nectar processing (Johnson 2008). At this stage, workers engage in more risky tasks that involve increased contact with the environment outside of the hive. As the last age-related task transition, workers travel outside of the hive for pollen and nectar foraging.

Related to the division of labor is task specialization, the preference of an individual to perform one task over any number of tasks available (reviewed in Beshers and Fewell 2001). Several models have been proposed to address the concept of task specialization in social insects (Arathi et al. 2000; reviewed in Beshers and Fewell 2001). One proposed model, the response threshold model, poses that individuals have internal thresholds that can affect their likelihood to perform a particular task at an enhanced level (reviewed in Beshers and Fewell 2001; reviewed in Jeanson 2019). Some individuals have lower thresholds for certain stimuli than others which is thought to increase their likelihood for performing a task. Polyandry, the mating of a queen with multiple drones, is an important mechanism contributing to this variability (Frumhoff and Baker 1988). Some workers produced by these patriline are specialists that will perform a task a disproportionately greater rate compared to other workers (Hammel et al. 2015). These specialists have been studied little pertaining to hygienic behavior but evidence suggests that these specialists have the ability to engage in both uncapping and removal behaviors

(Scannapieco et al. 2016) and will also specialize in removal of dead adult bees from the colony, another form of social immunity (Perez and Johnson 2019).

Workers that will perform hygienic behavior are typically middle aged (Arathi and Spivak 2001; Scannapieco et al. 2016) and sensitive to specific odors produced by unhealthy or dying brood. According to the response threshold model, those workers that have lower internal thresholds for these stimuli will be more hygienically active. Studies have shown different odors associated with dead brood (McAfee et al. 2018) compared to parasitized or diseased brood (Nazzi et al. 2004; Swanson et al. 2009; Wagoner et al. 2019) which could affect which workers will perform hygienic behavior depending on the stimulus type. Some workers are highly sensitive to these odors and will initiate perforating or uncapping the targeted cell (Gramacho & Spivak 2003). Once a cell has been opened, removal of unhealthy brood can be completed by either the same worker or other workers through either cannibalization of brood or removal from the cell. Workers that engage more often in removal behaviors may have higher internal thresholds for sensing unhealthy brood odors than workers who initiate uncapping. This is thought to be due to recruitment of these workers specializing in removal or that removal activities may be easier to sense when unhealthy brood is already uncapped (Masterman et al. 2000).

Group Size

The effects of group size on task performance is a well-studied phenomenon both within and among social insect species (Jeanson et al. 2007; Su and Lee 2009; Meunier 2015; Tsvetkov et al. 2019; Wright et al. 2019). Pertaining to honey bees, the population

of either a feral colony or one managed by a beekeeper can range from about 10,000 to 80,000 workers. As group sizes increases, the division of labor in honey bees as well as other insect societies has been demonstrated to increase as well (Jeanson et al. 2007; Holbrook et al. 2011; Wright et al. 2019). Smaller colonies often prioritize tasks related directly to survival, such as foraging, and will transition into more maintenance related tasks as the colony grows (Holbrook et al. 2011). This shift allows increased task specialization (Gautrais et al. 2002; Johnson 2008) which becomes important as the number of available tasks to complete also increases (Fewell and Harrison 2016).

The effects of group size on hygienic behavior performance has not yet been studied. Investigation into this topic would elucidate task specialization and group size effects on task performance in general in honey bees as there have been difficulties in comparing task performance across insect species due to different colony organizations (Dornhaus et al. 2008; Su and Lee 2009; Dornhaus et al. 2012). From a practical standpoint, it becomes important to better understand the link between group size and hygienic behavior as colony size is not considered as a factor when assessing results of hygienic assays for selective breeding.

Hypothesis and Predictions

Based on the information above, I hypothesized that honey bee workers in larger group sizes would hygienically outperform workers in smaller group sizes across different scales. Specifically, I predicted that 1) the rate of hygienic performance is disproportionately greater in larger groups, 2) the increased rate of hygienic performance

is demonstrated across three different scales, and 3) the increased rate of hygienic performance is due to increased task specialization inherent to larger groups rather than more participating individuals.

These predictions were tested by subjecting two differently sized groups of workers to standardized hygienic assays and tracking their performance at various time points with a final evaluation at 24 hours. A 1:3 ratio was selected and three scales were chosen based on a logarithmic scale (Figure 1): small (1 versus 3 workers), intermediate (32 versus 96 workers), and large (1000 versus 3000 workers). The hygienic performance between groups and across scales was compared while also focusing on task specialization of workers in the small and intermediate scales. Therefore, the experimental aims are 1) to compare overall hygienic performance of two group sizes at three different scales, 2) to compare hygienic performance across all three scales, and 3) to compare how task specialization differs across these scales. Combined, these aims contribute to how hygienic performance is affected by group size at different scales and whether task specialization is an influencing factor on the rate of hygienic performance both within these groups and across all scales.

CHAPTER II

MATERIALS AND METHODS

Large Scale

Six replicates of the following trial were performed at the UNCG honey bee research facility: Starting with one randomly chosen established hive, one honey frame and three brood frames with bees were collected and placed into a 5-frame nucleus hive. Bees from two additional brood frames were brushed into the nucleus and the frames were returned to the mother colony. After sealing the entrance, the nucleus was then transported to a location several miles away from the mother colony for observation hive setup. This distance is required to prevent loss of foragers from the observation hive back to the mother colony on return foraging flights.

Bees from all frames in the nucleus were brushed into a bucket and mixed to randomize the workers. A wide mouth 32-ounce Mason jar was used to approximately measure the amount of workers for the two respective group sizes: One jar full of bees for the smaller size (~1000 workers), and three jars full of bees for the larger size (~3000 bees). To achieve equal bee density, the observation hive for the larger group had two brood frames and one honey frame while the smaller size had one brood/honey frame. This design was also intended to model small and large managed colonies in apiaries where lesser populated colonies tend to have resources in a more compact space. Both observation hives were established in the same room to maintain a similar external

environment between the two hives. Queen pheromone sticks were installed in both observation hives (stapled to the top of the middle frame in the large size and stapled to the top of the only frame in the small size) to simulate normal colony conditions without having to introduce a new queen into the hive after the experimental split. The entrances of both observation hives were opened to allow orientation and foraging throughout the acclimation and assessment periods. Upon complete observation hive setup, an acclimation period of 24 hours was allowed before performing the hygienic behavior assay.

A freeze-killed brood assay was performed on two areas of pink-eyed pupae on brood frames in both experimental groups. This assay involved exposing a section of brood to liquid nitrogen to kill the pupae in the cells and stimulate hygienic behavior. Complete removal of pupae was assessed after 24 hours. This assay is fairly labor intensive and impractical for the average beekeeper (Spivak and Downey 1998) but was selected for this experiment because treating multiple sections of comb can be done quickly in a standardized fashion with liquid nitrogen.

A PVC pipe sized with 5.08 cm diameter was used to create a seal around two selected areas of pink-eyed brood in each experimental hive. Liquid nitrogen was poured directly onto the brood in these sections and the PVC pipe was removed once the liquid nitrogen had evaporated. The frames were then returned to the respective observation hives. Progress of complete pupae removal was assessed after 2, 4, 8, and 12 hours and a final evaluation was performed at 24 hours. After this first treatment, a period of 24 hours

was allowed before repeating the freeze-killed brood assay and assessment process once in each colony before returning frames and bees back to the mother colony.

A multi-level survival analysis was conducted in the R statistical environment (R Core Team 2020) using the “survminer” package on the number of pupae removed. This approach was conducted by pooling both assays per each trial and treating every cell in the assay as an individual that survived until it was removed. Pupae that were not removed by the end of the assay were censored. A t-test was used to separately analyze the percentage of pupae removed at each timepoint in the assay.

Intermediate Scale

Eight replicates of the following experiment at the intermediate scale were performed at the UNCG honey bee research facility: Frames of emerging brood from multiple unselected colonies were collected to emerge in an incubator over 24 hours. Approximately 500 age-matched workers were color-marked on the thorax using Testors® enamel paint and placed into one of three fostering colonies. After 14 days, the color-marked workers were retrieved and randomly assigned to either of the two group sizes, 32 workers or 96 workers. Workers were anesthetized in glass vials at 4°C for 3.5 minutes. The two group sizes were distinguished by two different colored number tags that were glued to each thorax of the workers with Gorilla Glue®. Workers that lost their tag, escaped, or did not recover were replaced with other color-marked and number-tagged bees. The workers were then released by their respective group into one of two observation hives constructed to the dimensions of two mating nucleus frames containing

one mating nucleus frame of brood. A 2:1 solution of sugar water was given *ab libitum* if no honey or nectar was present on the frame. The hive entrance was sealed to prevent loss of workers and guarantee the specific number of workers needed for the group size. Both observation hives were placed in the same room. A queen pheromone stick was omitted from this observation hive setup to not overwhelm the relatively small number of workers with queen pheromone. Workers were left to acclimate to the new conditions for 18 hours before the hygienic assay was performed.

A pin-killed brood assay was performed on one rhombus shaped section of 40 brood cells aged pre-pupae to white eyed brood in both groups. This pin-killed brood assay, as described in Newton and Ostasiewski 1986, involved piercing each cell once through the center of the wax capping over the brood to the base of the cell using a #5 entomological pin (38 x 0.60 mm). While the assay is comparatively simple and can be completed anywhere, it has been criticized for overstimulating hygienic behavior because hemolymph of the pupae can leak through the hole left by the pin which facilitates hygienic detection by workers (Spivak and Downey 1998; Gramacho et al. 1999). This assay was selected for this experiment because it was necessary to perform the assay through a wire mesh screen to contain the workers in the observation hive, which made a freeze-killed brood assay was not feasible.

A video recorder (Akaso EK7000) was set up ~12.7 cm in front of the section of pin-killed brood to record all behavior continuously for 24 hours. To assess the hygienic effort contributed by individuals and compare it between the two groups, all individuals contributing to hygienic behavior of several, randomly selected cells was manually

determined from these videos. Two to five cells were chosen based on the number of uncapped cells in the 32 worker group after 24 hours. The 32 worker group tended to uncapped fewer cells than the 96 worker group and thus served as the basis for cell selection. For example, if the 32 worker group uncapped three cells, then three cells were also selected at random in the 96 worker group for behavioral observation. Only cells that were entirely uncapped to the edge of the cell were considered for behavioral observations. No more than five entirely uncapped cells were observed per group per trial. Removal of pupae was not considered as a criterion because the event was too rare for meaningful inclusion. An individual bee had to be uncapping a cell for at least three continuous seconds to be counted. The entire length of time in seconds that an individual was uncapping a cell was recorded. This uncapping behavior was recognized by the swiveling motion of the individual's head while the mandibles were chewing away the wax capping and rotating of the body around the cell.

A paired t-test was used to compare the overall number of uncapped cells between the 32 and 96 worker groups. For this comparison, all cells that were manipulated by the workers visibly opening their cap were counted. To assess the degree of hygienic task specialization, Morisita's index of dispersion I_P (Morisita 1962) was used in this

experiment because it is scale-independent and often used as a measure of behavioral skew (Tsuji and Tsuji 1998). $I_P = N \frac{\sum x_i^2 - \sum x_i}{(\sum x_i)^2 - \sum x_i}$, where x_i denotes the total time in seconds spent performing hygienic behavior on the selected cells by the i^{th} individual. A paired t-test was used to compare the Morisita's index values between the 32 group and 96 group. The Morisita's index values for both groups were further correlated to the

number of uncapped cells regardless of group size to test whether it showed any relationship to hygienic efficiency.

Small Scale

289 replicates of one worker and 119 replicates of three workers were performed at the Zrifin Apiculture Breeding Station in Rishon LeTsiyon, Israel. For the trials investigating the hygienic performance of one worker, frames of emerging workers from two colonies were collected and painted on their thorax (Uni Posca ® paint) using one of two colors. The workers were returned to their original colony and retrieved on the day of the experiment 11-16 days later based on their thorax color.

For the trials investigating the group of three workers, bees that were on frames of brood and appeared to be nursing were collected the day before the experiment. Due to the higher volume of workers required for these trials and having been conducted later in the season, there were not enough emerging workers able to be obtained. These workers were anesthetized at 4 °C until they were unconscious then painted on their thorax (Uni Posca ® paint) using one of three colors. All workers were returned to their original colony to be retrieved on the following day for the experiment.

Large Petri dishes were used as experimental arenas to accommodate one or three workers of two colony origins. The Petri dishes measuring 100 mm in diameter were lined with wax foundation with a Petri dish measuring 60 mm in diameter glued to the center. Each dish contained one ball of pollen approximately 2 mm in diameter rolled in

confectioner's sugar and one-1.5 mL microcentrifuge tube containing 1:1 sugar-water solution with a hole punctured at the tip.

Frames of white-eye to pink-eye stage brood were collected from several colonies two days prior to the day of the experiment. The back of these frames was intentionally damaged using a hive tool so that the bees would clean out the brood and prevent additional killed brood stimulus in the experiment. These frames were retrieved on the day of the experiment and circular sections of brood were cut out to suit the size of the smaller Petri dish (Figure 1). For the hygienic assessment of a single worker, thirty individuals from both colony origins were collected and a single bee was placed into each Petri dish. For the hygienic assessment of three workers, 90 individuals from both colony origins were collected and three workers with the different color marked thoraxes were placed into each Petri dish. Bees were left to acclimate for one hour until performing the hygienic assay.

A pin-killed brood assay was performed on each section of brood. This assay was selected for this experiment for its ability to be performed simply on the small cut out sections of brood. The hygienic performance of single workers ($n = 289$) was assessed at 24 hours by counting the number of uncapped cells. Any cell caps that were manipulated by the workers were counted as an uncapped cell. The hygienic behavior of the three worker groups was monitored using a scan sampling method for every 10 minutes of the first 6 hours of the assay. The definition of uncapping behavior in the intermediate scale was also adopted for this experiment. Hygienic removal was characterized by cannibalization of an uncapped pupae or holding the brood with the mandibles and

pulling the pupae out of the cell. This method of tracking worker behavior generated a behavioral profile for each individual to quantify task specialization. A final assessment of overall hygienic performance of the three worker groups was made after 24 hours to compare the amount of hygiene performed by single workers compared to groups of three workers.

A Mann-Whitney U test was used to compare the number of uncapped cells between the individual workers and three workers. A Mann-Whitney U test was used to compare the relative number of uncapped cells between the two groups by adjusting the final score of the group of three workers to divide the work evenly to all three workers. A chi-square test was used to compare the number of hygienic and non-hygienic workers across all trials of the individual and three worker groups to assess the proportion of hygienic individuals in these groups. Individuals that were deceased by the end of the assay were disregarded in the analysis.

CHAPTER III

RESULTS

Large Scale

Removal of pupae over the 24 hour assay period was significantly greater in the 3000 workers than in the 1000 worker groups (hazard ratio = 0.56, s.e. = 0.028, $z = 0.028$, $p = 0.035$, Figure 3). However, when analyzed separately, the percentage of pupae removed at any specific timepoint was not significantly different between the two groups (2 hrs: $n = 6$, $t = 2.30$, $p = 0.07$; 4 hrs: $n = 6$, $t = 1.55$, $p = 0.18$; 8 hrs: $n = 6$, $t = 1.84$, $p = 0.12$; 12 hrs: $n = 6$, $t = 1.95$, $p = 0.11$; 24 hrs: $n = 6$, $t = 1.20$, $p = 0.29$, Figure 4).

Intermediate Scale

The proportion of cells uncapped by the groups of 32 workers and 96 workers across all trials was not significantly different ($n = 8$, $t = -0.83$, $p = 0.42$) despite the median number of cells uncapped by the 96 worker groups being 150% greater than that of the 32 worker groups (Figure 4). The degree of specialization, calculated as Morisita's index, was not statistically different between groups ($n = 8$, $t = -1.55$, $p = 0.17$, Table 1). N/A is recorded in place of Morisita's index values in Table 1 for trials that had no sufficiently uncapped cells. Morisita's index was not significantly correlated to the number of uncapped cells in either the 32 or 96 worker groups alone, and the two

variables also showed overall only a moderate, non-significant relation ($n = 8$, $t = 1.1$, $R = 0.35$, $p = 0.31$, Figure 5).

Small Scale

Instances of complete removal in both groups were rare, therefore uncapping was used as a basis to compare hygienic response. In comparing the hygienic response of workers as individuals and in small groups of three workers, final uncapping scores were significantly greater ($n = 408$, $z = 10.7$, $p < 0.001$) in the small group size of three workers compared to individual worker performance (Figure 8). When the final uncapping score performed by three bees was adjusted by dividing the score by three (to account for the increased density of bees in the experimental arena), uncapping done by the small group was still greater ($n = 408$, $z = 9.9$, $p < 0.001$) than individual performance despite the highest number of cells uncapped across all trials in both groups not being very different (12 cells uncapped by the individuals versus 14 cells in the three worker group). (Figure 9). The difference in the number of workers who uncapped cells versus the number of workers who did not uncap cells was significantly different between the individual workers and the three worker group sizes ($\chi^2 = 9.018$, d.f. = 1, $p = 0.0027$).

CHAPTER IV

DISCUSSION

In social insect societies, the rate of disease transmission is potentially much higher than in solitary insects due to the degree of relatedness of nestmates and the amount of interactions between nestmates in a densely populated environment (Traniello et al. 2002). Hygienic behavior is one means of social immunity employed to remove unhealthy individuals from the colony before they become infectious to other nestmates (Arathi et al. 2006). Hygienic behavior is a task that can be performed by all bees but, when performed at exceptional levels by individuals, involves increased task specialization (Arathi et al. 2000; Arathi and Spivak 2001; Arathi et al. 2006). Task specialization has been shown in other studies to scale with increasing group size (Gautrais et al. 2002; Jeanson et al. 2007). It has been evidenced by several studies that task specialization increases colony efficiency (reviewed in Beshers and Fewell 2001; Dornhaus et al. 2008; Fewell and Harrison 2016) and that hygienic task specialization can greatly impact the amount of cells uncapped and removed (Arathi et al. 2000; Arathi et al. 2006; Scannapieco et al. 2016). My study provides the first systematic investigation on how group size affects hygienic behavior and it supports my predictions of increased hygienic performance by larger groups although I found little evidence that this result is due to task specialization. I will first discuss the results of the small and large scales as these experiments were thought to be successfully executed and characterized by

consistent worker behavior across trials. I will then discuss the intermediate scale experiments and speculate on inconsistencies between trials as potential explanation for its inconclusiveness. Finally, I will discuss all three scales in a broader context and draw final conclusions.

Small and Large Scales

Overall performance of hygienic behavior increased with group size in all three scales, although the difference was only significant at the large and small scales, and performance by the larger group was only disproportionately greater at the small scale. This latter result is evidenced by the increased hygienic performance by the group of three workers even when the final uncapping score was adjusted to a per-capita basis by dividing the score by three. This disproportionately increased amount of hygienic behavior in the groups of three workers compared to single workers was, however, likely not due to task specialization because of the significant difference in the total number of bees performing hygienic behavior in the two groups. A meaningful comparison of Morista's index to assess specialization was not possible at this scale. Despite more statistical outliers among the individuals tested alone (denoted by asterisks in figures 6 and 7), their increased hygienic behavior, compared to their non-hygienic counterparts, did not significantly impact the overall performance by the group as the median number of cells uncapped was still zero. The disproportionately greater hygienic performance by the groups of three workers may be more contributed to by increased worker density rather than task specialization. The area of brood between the individuals and groups of

three workers was held constant which constitutes a three-fold increase in worker density in the groups of three workers. While this three-fold increase likely increased the likelihood of testing a hygienic individual, worker density might also contribute to synergistically increasing hygienic behavior. Increased group density overall has been correlated to greater rates of information flow (King and Cowlshaw 2007; Dornhaus et al. 2012) and as hygienic behavior largely relies on brood odors for information, the communication among nestmates may vastly improve accuracy in regard to assessing location and status of killed brood (King and Cowlshaw 2007). Few studies have investigated the effects of worker density on task performance (Pacala et al. 1996) in social insects. However, one study suggests that rates of foraging and scouting in the ant species *Temnothorax rugatulus* are significantly increased when worker density is increased (Cao 2013). Further investigation into hygienic behavior and how it is affected by worker density may improve our understanding of hygienic behavior and elucidate general principles of communication and task performance in social insects.

The result of disproportionately increased hygienic performance at the small scale is not reflected in the large scale experiment. Although the 3000 worker group performed a significantly greater amount of hygienic behavior than the 1000 worker group, the hygienic performance was only more than three times greater at the 2-hour timepoint where there was a 14.1-fold difference in pupae removal. The difference is lower at all other timepoints in the assay decreasing with time (4 hrs = 2.7-fold difference, 8 hrs = 2-fold difference, 12 hrs = 1.7-fold difference, 24 hrs = 1.3-fold difference). While task specialization was not directly measured at the large scale, the major difference in pupae

removal at the 2-hour timepoint and the significantly increased hygienic performance overall might be due to increased task specialization in the 3000 worker group. In this large scale experiment, density was held constant between the 1000 and 3000 worker groups because the number of brood frames was proportioned to the number of workers in both groups. Thus, overall density was not a factor, but individual specialization might have contributed. Specialized workers that have lower internal thresholds for hygienic stimuli may become active sooner than others with higher internal thresholds (reviewed in Beshers and Fewell 2001), which suggests that the most opportune time to sample hygienic specialization in a FKB assay (Spivak and Downey 1998) may be early in the assay.

Differences in the experimental designs between the small and large scale experiments allowed for different conclusions from both investigations. Firstly, the two different hygienic assays used may have affected the way in which the workers communicated at those scales. The pin-killed brood assay, used for the small scale, elicits an immediate hygienic response as brood odors emit quickly through the leakage of hemolymph via the hole left in the cell cap (Spivak and Downey 1998; Gramacho et al. 1999). Contrarily, the odors produced by dead brood in a FKB assay, used for the large scale, tend to build up over time as brood decomposes. This major difference may create different levels of brood odors and alter communication routes within groups which may also have contributed to the disproportionately increased hygienic performance at the small scale. To sufficiently test this theory, both scales would need to be tested using the two hygienic assays.

The second difference concerns the area of brood which differed dramatically between the scales. The large scale experiment was designed to model traditional colony sizes in apiaries where resources in the colony tend to be proportionate to population. The large scale models a typical hygienic selection performed by beekeepers and breeders where a hygienic assay, usually a FKB assay, is performed on a single section of brood and the frame is placed back into the colony for 24 hours. Workers thereby are thought to encounter the section of killed brood either 1) by chance of being in proximity to the section of killed brood, or 2) being recruited to the area for hygienic behavior via communication by nestmates. By proportioning brood frames to workers at the large scale, this concept can be modeled and may be another reason why hygienic performance was not disproportionately increased overall in the 3000 worker group. In the small scale experiments, the workers were restricted to the experimental arena in direct contact to the compromised cells for the entirety of the assay. This set up eliminates the factor that workers would not have the chance to encounter the killed brood, which may have led to disproportionately increased hygienic performance in the groups of three workers. Differences in hygienic performance between the scales should not be statistically evaluated due to the different experimental designs and further speculation will follow in the last section of the discussion.

Intermediate Scale

The comparisons of overall hygienic performance and task specialization skew between groups of different size at the intermediate scale yielded non-significant results.

There was no significant difference in hygienic performance overall despite the median number of cells uncapped by the 96 worker group being 150% greater than that of the 32 worker group. The difference in Morisita's index values between groups was also not significant and weakly only non-significantly correlated with the number of uncapped cells. Despite the non-significance of our measure of task specialization in the intermediate scale, this study presents a viable method of measuring task specialization skew regarding hygienic behavior. The specific metric was selected because it allows for size-independent quantification of the degree of task specialization in different group sizes (Tsuji and Tsuji 1998). Although the results were not significant in this study due to factors discussed below, Morisita's index of dispersion can be used as a viable measurement in future studies investigating hygienic task specialization and group size.

The inconclusive results of the intermediate scale experiment may be attributed to several factors. Namely, the sample size (= number of independent replicates) was impacted by disregarding several replicates due to one or a combination of the following observations. Firstly, the organization of the experimental arena may have not been conducive for allowing normal behavior. The mating nucleus sized observation hive, with only one mating nucleus frame of brood in it, may have created excess empty space that was not present in our small and large scales. While temperature was not measured, the excess space in the observation hive may have caused workers to allocate more time to thermoregulation of the brood nest over hygienic behavior performance (Abou-Shaara et al. 2017). Secondly, the state of queenlessness in the observation hive could have led the workers to perceive the hive conditions as abnormal. Workers in the small scale were

also subjected to queenless conditions though it was determined that they were relatively unaffected as they appeared to behave normally, i.e. walking around the experimental arena, cleaning cells, removing debris. In the intermediate scale, workers would sometimes behave sporadically i.e. clustering in another part of the observation hive away from brood or remaining on the glass walls for long periods of time. While absence of queen pheromone was not believed to affect the workers in the small scale, the addition of more workers in the intermediate scale may have required a certain amount of queen pheromone stick to assist in simulating normal hive conditions. An entire pheromone stick was found to be too disruptive in early trials because workers had clustered around the pheromone stick and remained there throughout the assay. No other studies investigating hygienic task specialization have reported significant behavioral inconsistencies across trials (Arathi et al. 2000; Arathi et al. 2006; Scannapieco et al. 2016; Perez and Johnson 2019) so making adjustments to the experimental arena, such as limiting excess space and introducing some queen pheromone, may improve the assay to study hygienic behavior performance at this scale more effectively.

Comparison Across All Scales

There were several differences in experimental arenas, sampling and data collection methods, and worker collection methods that invalidate formal statistical analysis among the three scales. Primarily, all experiments were performed in different locations and in different experimental arenas which may have altered worker behavior, as discussed previously. Secondly, the definition for identifying hygienically manipulated

cells varied across scales based on the extent of hygienic behavior performed at each scale. For example, partial uncapping of a cell was counted in the small scale experiment because complete uncapping and pupae removal were very rare, in contrast to the large scale experiment where removal was readily observed and consequently only fully uncapped cells were included in the hygienic performance score. Using the same definition for hygienic behavior to compare across scales may have simplified statistical analysis but would not have yielded meaningful comparison between group sizes at all scales, which was the primary objective. Thirdly, workers were collected and prepared differently for experiments at the three scales. Collection methods of workers were similar between group sizes within scales but differences in these methods differed among experiments. This inconsistency did not impact behavior majorly the comparisons within scales but compromised a comparison across scales. For these reasons, any interpretation of results across scales can only be speculative.

As basis for speculative comparison across scales, the proportion of cells uncapped among all experimental cells and the proportion of cells uncapped per bee were compared (Figures 8 & 9). The former results follow a diminishing return curve where the proportion of cells uncapped increases less drastically with increasing group size. This result is practically relevant for beekeepers performing hygienic assays for selective breeding because it indicates that there may not be significant group size effects on hygienic performance among their traditional colony sizes. However, there would likely be drastic differences in hygienic performance in comparing colony sizes of 300 workers and 3000 workers, for example. The FKB used in our large scale experiments yielded a

high proportion of uncapped cells. In order to accurately distinguish hygienic qualities at the large scales of realistic beekeeping conditions, it is therefore important to ensure that the assays are designed to avoid ceiling effects. Comparing the number of cells uncapped per each worker across scales revealed a declining function (Figure 9), meaning that the hygienic performance per capita actually declined with group size. Overall, this downward trend can be explained by the different scoring metrics between scales and the exclusion of unsuccessful trials at the intermediate scale.

Overall Conclusions

Honey bees are a great model species to study group size effects on behavioral interactions, especially for behaviors that are complex and focus on social immunity. Our understanding of hygienic behavior is particularly important because selective breeding for hygienic behavior can greatly mitigate synthetic acaricide use and acaricide resistance in mites. The current study emphasizes the need for further investigation into task specialization and group size effects on hygienic behavior performance. Overall, increasing group size did have some increasing effect on hygienic behavior performance though only disproportionately so at the small scale. Based on the results of this study, I cannot conclusively assess the effect of task specialization on overall hygienic behavior performance and more studies will be necessary.

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APPENDIX A.

LIST OF TABLES

Table 1.

Comparison of Morisita's Indices for the 32 and 96 Group of Workers at the Intermediate Scale. The difference in Morisita's indices between the 32 group and 96 group over four trials in the intermediate scale was not significantly different.

Group size	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
32	11.45	15.58	N/A	22.07	9.55
96	38.83	19.85	10.76	N/A	31.35

APPENDIX B.

LIST OF FIGURES

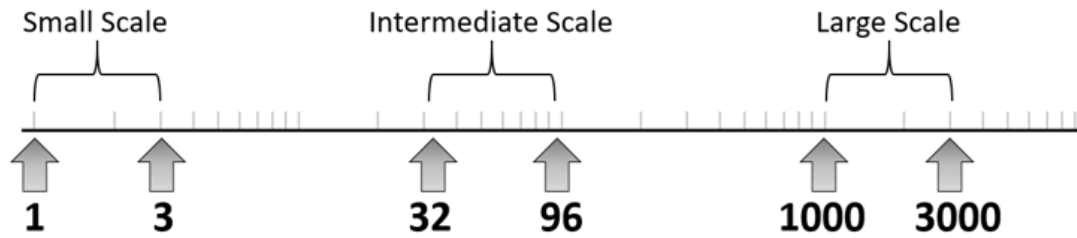


Figure 1. Logarithmic Scale Determining Group Sizes at Three Different Scales. A logarithmic scale was used to determine 1:3 group size ratios to investigate hygienic behavior performance across three scales.



Figure 2. Example of a Petri Dish Experimental Arena to Observe Hygienic Response at the Small Scale. A cut-out section of pin-killed brood is at the center of the large Petri dish suspended within a smaller Petri dish. The large dish is lined with wax foundation and includes a pollen ball rolled in confectioner's sugar with a microcentrifuge tube of 1:1 sugar-water solution for food.

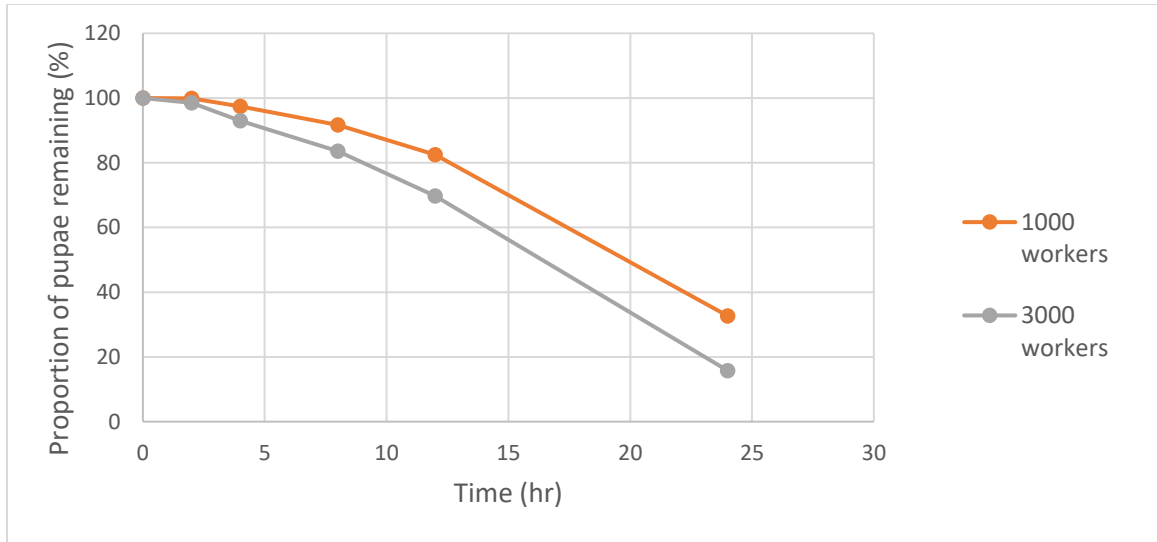


Figure 3. Proportion of Pupae Remaining in Response to a Freeze-Killed Brood Assay Over 24 Hours at the Large Scale Experiments. The difference in brood removal between the 1000 workers and 3000 workers was significantly different (hazard ratio = 0.56, s.e. = 0.028, $z = 0.028$, $p = 0.028$). However, the pooled and averaged number of brood removed was not significantly different at any timepoint (2 hrs: $n = 6$, $t = 2.30$, $p = 0.07$; 4 hrs: $n = 6$, $t = 1.55$, $p = 0.18$; 8 hrs: $n = 6$, $t = 1.84$, $p = 0.12$; 12 hrs: $n = 6$, $t = 1.95$, $p = 0.11$; 24 hrs: $n = 6$, $t = 1.20$, $p = 0.29$). While the large group size removed brood at a faster rate, the lack of difference between groups may be due to a diminishing effect of group size at this scale compared to small and intermediate scales.

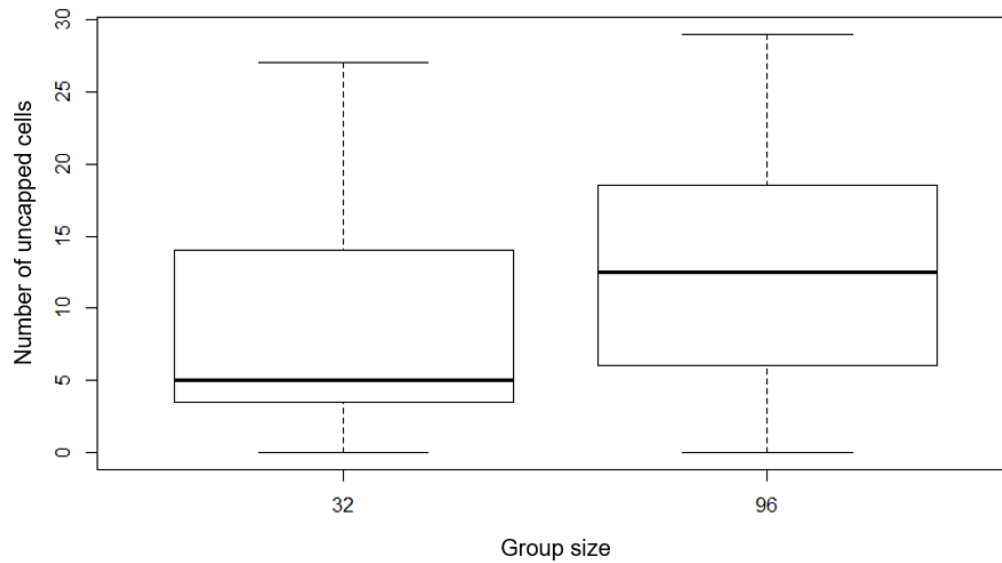


Figure 4. Number of Cells Uncapped by the 32 Group and 96 Group Across All Trials at the Intermediate Scale. Despite the average of uncapped cells in the 96 group being greater than that of the 32 group, the difference between both groups was not significant ($n = 8$, $t = -0.83$, $p = 0.42$) which is speculated to be contributed to by flaws in the experimental arenas. The black bar within the boxes represents the median number of cells uncapped in both groups.

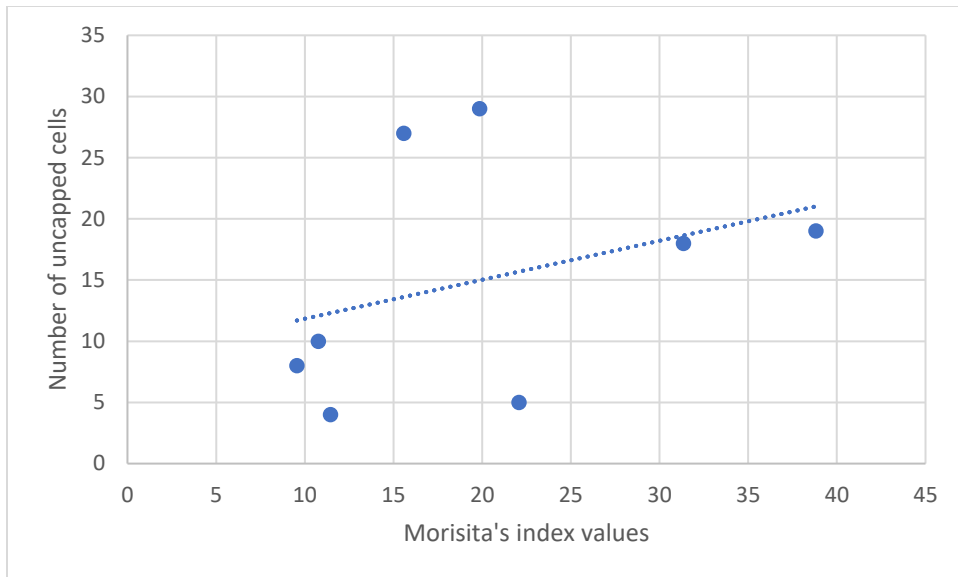


Figure 5. Correlation of Morisita's Index Values and the Number of Cells Uncapped at the Intermediate Scale. No significant differences in the correlation of the number of cells uncapped to Morisita's index values were observed between the 32 and 96 worker groups. The above graph models this correlation irrespective of group size. The correlation, while not significant ($n = 8$, $t = 1.1$, $R = 0.35$, $p = 0.31$), provides some evidence that task specialization can increase hygienic behavior overall

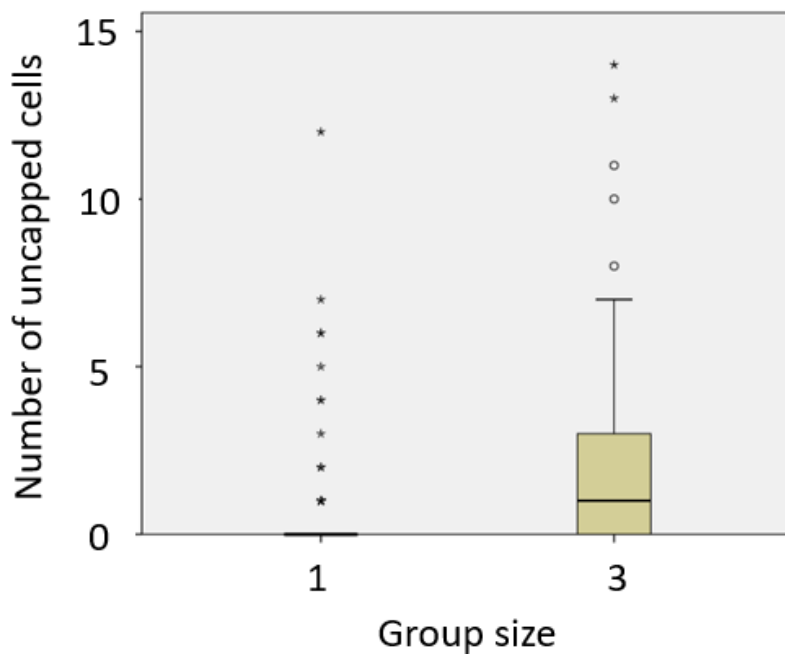


Figure 6. The Number of Cells Uncapped by Individual Workers and Three Workers at the Small Scale. In response to a pin-killed brood assay ($n = 408$, $z = 10.7$, $p < 0.001$), most individual workers did not open any cells while the median (black line in the bar) is at one for groups of three workers. Open circles represent individuals and asterisks represent outlier individuals. Despite the overall pronounced difference, the maximum number of cells opened was not very different (12 vs 14) between experimental groups. The black bar within the box represents the median number of cells uncapped, the open circles represent mild outliers, and the black asterisks represent extreme outliers.

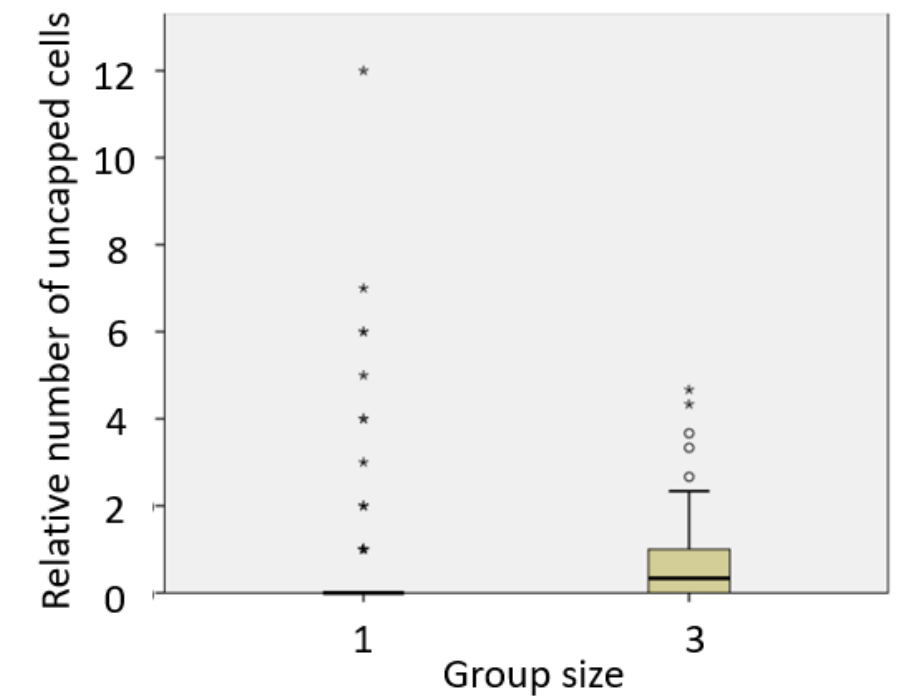


Figure 7. The Relative Number of Cells Uncapped by Individual Workers and Three Workers at the Small Scale. Uncapping of cells was also significantly ($n = 408$, $z = 9.9$, $p < 0.001$) higher for the groups of three bees compared to individual workers. The black bar within the box represents the median number of cells uncapped, the open circles represent mild outliers, and the black asterisks represent extreme outliers.

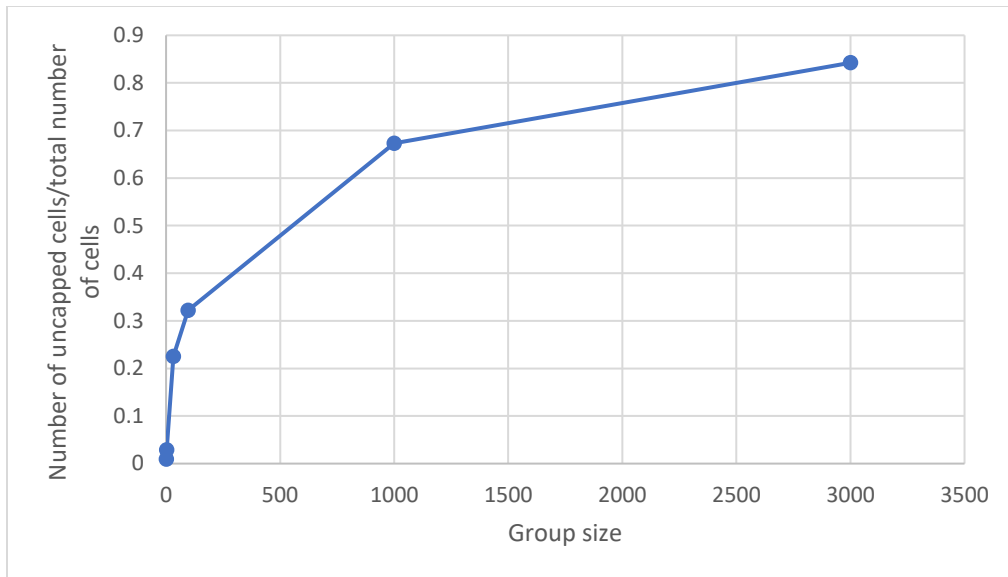


Figure 8. The Proportion of Uncapped Cells to Total Number of Cells Subjected to a Hygienic Assay Across All Three Scales. While not statistically evaluated due to experimental differences among scales, the proportion of hygienically manipulated cells increases with group size overall. This graph represents a typical diminishing return curve where hygienic performance is less dramatic as group size increases.

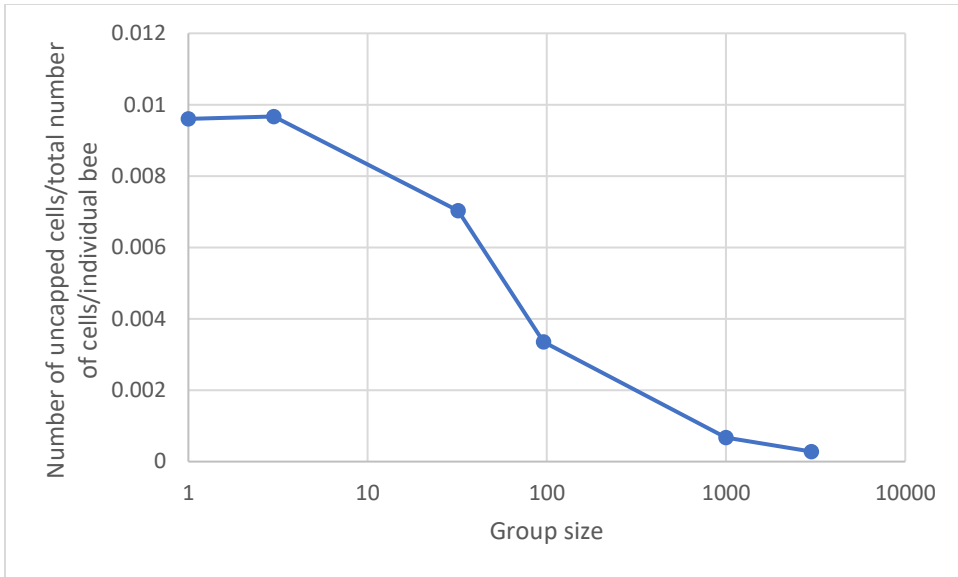


Figure 9. The Proportion of Uncapped Cells and Total Number of Cells Subjected to a Hygienic Assay per Individual Bee in Group Sizes Across All Three Scales. While not statistically evaluated due to experimental differences among scales, the number of cells uncapped per bee decreases as group size increases. Differences between group sizes at the small and large scale are negligible but there is a marked difference between the 32 and 96 worker group. This may be due to inconsistencies within the experiment at this scale.