Effects of Genetic Diversity on Thermoregulation in *Apis mellifera*

An honors thesis for the Department of Biology

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Abstract:

Honeybee queens mate with a large number of males. In our experiment, our honeybee queens mated with between 20-37 males. The evolution of hyperpolyandry in honeybees (*Apis mellifera*) is an interesting phenomenon, given that it will reduce the average relatedness amongst workers and potentially decrease the fitness benefits derived from cooperation. Past studies have suggested that mating with up to 10 males can be explained as a mechanism to decrease the production of genetic misfits (i.e. diploid males). The benefits of polyandry beyond 10 matings are poorly understood, although recent studies have shown some fitness benefits of hyperpolyandry on disease resistance, behavioral traits, and colonial thermoregulation.

We were interested in the benefits of genetic diversity on colony thermoregulation. We examined the relationship between thermoregulation and genetic diversity quantified as the patriline number of the colonies. We predicted that stability of thermoregulation, measured as temperature variance in the brood comb would correlate positively with patriline number. In addition, we explored the mechanism for the pathogenesis of behavioral fever in response to *Ascosphaera apis* infection. Based on previous experiments, we predicted that our experimental hives would increase brood comb temperature in response to *A. apis* spores.

Our data suggest that increasing genetic diversity is not a predictor of thermoregulatory stability. Although genetic diversity may have fitness benefits in hive development, stability of thermoregulation does not seem to be influenced by genetic diversity in naturally foraging, naturally mated colonies. Our results revealed that specific temperature conditions are relevant to the pathogenesis of behavioral fever against *A. apis* in honeybee colonies. During the experiment, our experimental colonies failed to mount a behavioral fever when they were exposed to *A. apis* spores at an ambient temperature of ~33^{o}C, which is above the germination

temperature of *A. apis*. This result suggests that there is a temperature requirement in the generation of group fever in honeybee.

Surprisingly, our results also showed a food-influenced temperature circadian rhythm in honeybees. When the colonies were given a sugar feed, hive temperatures were no longer correlated with ambient temperature, and comparison between the no-feed and feed periods showed a different thermoregulatory pattern. Although using food as an external cue for circadian patterns had been observed in organisms such as mice, a “food-related clock” in a superorganism has never been seen until now.

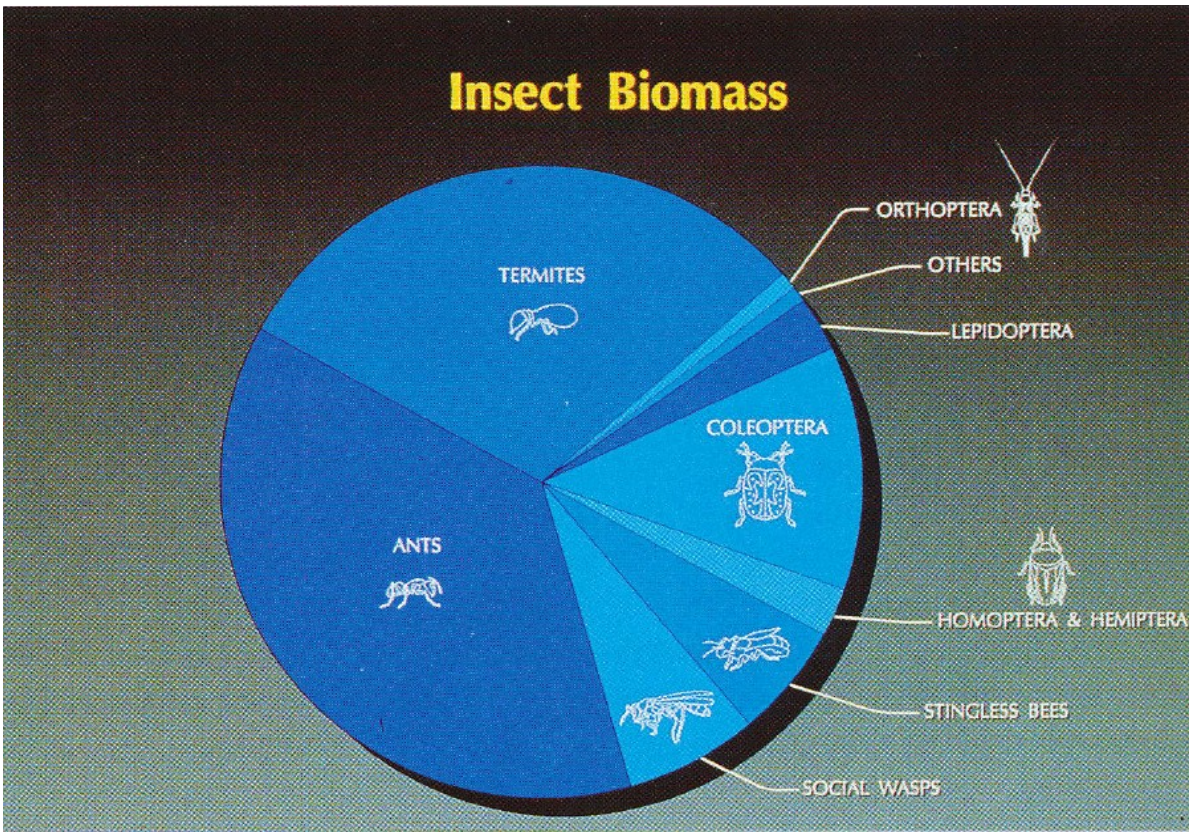
Introduction:

Background:

Apis mellifera, the honeybee, is perhaps the world's most economically important livestock due to its role as a pollinator for a wide range of agricultural crops. It is estimated that the honeybee is responsible for pollinating 35% of all agricultural products destined for human consumption (Klein *et al.*, 2007). In addition, bee products such as honey, beeswax, bee pollen, and royal jelly generate up to $70 million dollars a year. Furthermore, honeybees play a vital role in the maintenance of biodiversity by pollinating flowering plants and acting as a food source for different organisms . Due to its economic value and its importance in preserving biodiversity, the honeybee is one of the world's best-studied organisms.

Eusociality:

Studies of honeybees have not only given us a wealth of knowledge about its management, but have also provided us with an understanding of eusociality. Eusociality is a term coined to describe an evolutionarily advanced level of social living. Eusocial societies have three key characteristics: a reproductive division of labor, overlap of generations, and cooperative care of young (Wilson & Hollobler, 2005). Although all eusocial societies posses a worker caste that cares for the offspring of the queen(s), it is only in highly eusocial societies that the workers posses morphological alterations that cause them to be sterile. In these eusocial societies, the workers are specialized to perform specific tasks such as foraging and nest building. The division of labor and specialization in honeybees is carried out by age polyethism; the task of the colony is allocated by age. In *A. mellifera*, and nearly all other highly eusocial insects, the youngest adults are the brood care specialists while the older adults are forager.



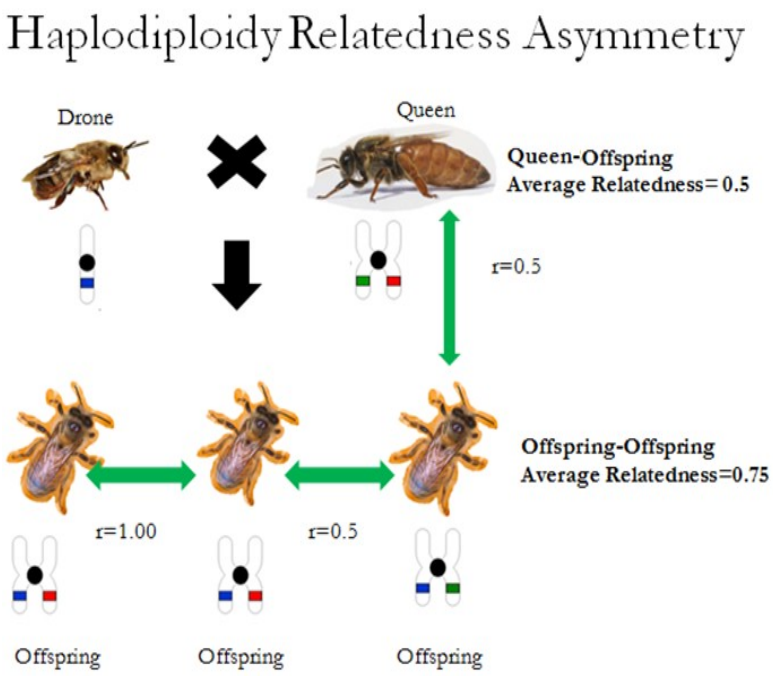
The survival of eusocial colonies is dependent both upon the complex group interaction within the worker caste and the relationship between workers and the reproductive queen(s). The interactions within the worker caste not only reduce the cost of various life activities by having multiple members of the colony address the same task, but also decrease the amount of time a task goes unattended, thereby increasing the overall efficiency of the colony. Group cooperation also allows eusocial society to mount better defenses against predators. The death of solitary insects translates to the end of its ability to contribute genetically to gene pool. In eusocial societies, the loss of workers incurred during nest and resource defense has a relatively low impact on its survival since it is composed of tens of thousands of workers. Eusociality also allows the colony to closely maintain homeostasis and cope with periods of harsh and highly varied climatic conditions (Wilson, 1990).

Eusocial organisms are found in a variety of environments and their distribution seems only limited by the coldest living conditions. The domainance of eusocial insects is apparent when approached from a quantitiative prespective. In a Brazilian tropical forest, the biomass of eusoical insects outweighed the combined biomass of all vertebrates by more than four times (Wilson, 1990). Of the 750,000 known insect species, roughly 2% are highly social insects. Suprisingly, it is that 2% of insects that comprise half of the world's biomass (Wilson, 1990). The domiance of eusocial insects is not limited to their numbers, cooperation between workers allows eusocial insects to monoplize high resource areas within their environment and exlude solitary insects to more transient locations . Gifted with the advantages of enhanced nest defense and increased efficiency in life activities such as foraging, nest construction, and the ability to modify environmental conditions eusocial insects are extremely successful in the wild .

Evolution of Eusociality:

Although rare in orders other than insects, such as *Hymenoptera*, *Isopteran*, and *Homoptera*, eusociality has also evolved in the naked mole rat, *Heterocephalus galbe*r, and a sponge-dwelling shrimp, *Synaplheus reaglis* . Given the advantages of eusociality, however, it seems odd that it has evolved in so few species. In order to understand why the evolution of eusociality is more common in insects, especially in the order *Hymenoptera*, we have to examine the selective forces leading to the emergence of eusocial behavior. Eusociality requires that some individuals give up their reproductive potential. Based on the cost associated with reproductive self-sacrifice, there must be some evolutionary payoff. In conjunction with other factors, such as resource limitations, and changes in colony life cycle, kin selective behavior may have helped pushed evolution of cooperative behavior to the "point of no return", characterized by the emergence of a sterile worker caste.

An allele can propagate through the gene pool in two ways: via direct reproduction by an individual or via indirect reproduction, specifically the reproduction of close relatives. The collective fitness benefit derived from direct and indirect reproduction is inclusive fitness. The fitness benefits of cooperative behavior can be evaluated using Hamilton's inequality $rB > C$. The evolution of a behavior such as eusociality becomes favorable when *B,* the fitness benefits (the increased reproductive success of close relatives) to the self-sacrificing individual, represented by rB, outweigh *C,* the cost (decreased reproductive potential) incurred for the behavior. Thus for advanced eusocial behavior to have evolved, maximum inclusive fitness must be derived from increasing the indirect reproduction of closely related relatives (Hamilton, 1964; Nicola, 2010).



The most important aspect of Hamilton's inequality is the coefficient of relatedness (r). The coefficient of relatedness will affect the fitness benefit derived from a eusocial behavior. For

instance, an elevated coefficient of relatedness translates to an increased in the indirect fitness benefits of the behavior. As a result of the Haplodiploidy of hymenoptera, siblings on average have a higher coefficient of relatedness (r=0.75) than a queen and her offspring (r=0.5). By applying Hamilton's inequality, r$B > C$, it is possible to explain why the offspring are more invested in helping the queen raise more daughters that could become future queens. (Hamilton, 1964; Holldobler & Wilson, 2009). Previous experiments have confirmed that workers have a 3:1 bias with respect to investment into workers over males. In contrast, the queen prefers a 1:1 investment.

The benefits derived from an elevated coefficient of relatedness are eroded, however, when the queen mates with multiple males. *A. mellifera* is a unique eusocial organism that carries polyandry to the extreme, performing one to five mating flights and mating with up to 44 males or more (Schluns *et al.*, 2005; Hayworth *et al.* , 2009). Given the time and energy demand of multiple mating flights, the increase risk of predation and exposure to disease, it is surprising that the honeybee queen would mate with multiple males (Sherman, *et al.*, 1988). Previous studies have suggested that polyandry evolved as a mechanism to reduce the costly production of sterile diploid drones (Page, 1980; Tarpy & Page, 2001).

In honeybees, males are hemizygous and females are heterozygous at the sex determination locus, however individuals that are homozygous at the sex determination locus end up as diploid males. Diploid males are genetic misfits and evolutionary dead ends that have no function (Woyke 1963; Page 1980). To prevent diploid males from consuming colony resources, they are recognized and eliminated by the workers early on in their development (Woyke, 1963). By mating with multiple males, the queen will decrease the probability of producing diploid males. The fitness benefits of diploid male avoidance through polyandry, however, rapidly diminished above 6-10 matings and therefore cannot explain extreme polyandrous behavior in honeybee queens.

Possible Benefits of Polyandry:

The reasons for the evolution and maintenance of extreme polyandry in *A. mellifera* are still being debated. Hypotheses include enhanced division of labor and decreased disease susceptibility as a result of increasing genetic diversity at disease resistance loci (Robinson & Page, 1989; Page, 1980 ;Sherman, *et al.* 1988) . The latter hypothesis is also known as the 'polyandry versus parasitism hypothesis' and proposed that polyandry evolved as a defense mechanism against pathogens and diseases. Since haploid males carry different alleles for disease resistance, a queen that mates with multiple males will decrease the probablity of her colony acquiring the same disease susceptible allele. By increasing allelic diversity, a multiply mated queen will increase the likelyhood of colony survival (Tarpy, 2003; Seeley and Tarpy 2007; Moritz & Southwick, 1992).

Genetic diversity seems to contribute to pathogen resistance in bubblebees and ants. In bubblebees, diverse colonies (four patriline) experienced a decrease in disease and parasite load in comparison colonies reared by a singly mated queen (Baer & Schmid-Hempel, 1999; Bear &

Schmid-Hempel, 2001). Bumblebee queens in nature are only able to mate once due to a copulatory plug inserted by the male. Tarpy (2003) showed that in comparison to colonies reared by queens inseminated by one drone, diverse honeybee colonies (24 patrilines) display less variance in their ability to resist *Ascophaera apis*, a common fungal pathogen in honeybee brood. This observation was confirmed by Tarpy and Seeley in 2006. Comparison of colonies with high genetic diversity (10 patrilines) against genetically uniform colonies (one patriline) show that colonies with higher genetic diversity are less susceptible to a range of brood diseases (chalk brood, sacbrood, Amercian foulbrood, and European foulbrood).

In addition to disease resistance, genetic diversity also seems to enhance behavioral traits that are essential to the growth of a colony. Mattila and Seeley 2007 found that genetically diverse colonies (15 patrilines) showed greater food storage, comb construction, and population growth. Jones *et al.* (2004) also found genetically diverse colonies showed fewer variances around the mean brood temperature, which suggests better thermoregulatory stability in the brood comb.

Maintaining a consistent hive temperature is an important function of a colony. Although brood rearing can occur at various ambient temperatures from 0ºC to 40ºC. The range of the temperature within the hive must be maintained between 33ºC to 36ºC, optimally at 35ºC, for proper brood development. If temperatures are below or aboves this range, it will result in shrivelled wings, brain damage, behavioral abnormality, and other developmental problems (Basile, 2009; Tautz *et. al.*, 2003; Groh *et al.*, 2004). Workers manipulate brood nest temperature by stationing themselves on brood comb where they generate heat via shivering. If the temperature of the brood comb gets too hot, workers will fan hot air out of the nest (Gates, 1914).

Thermoregultion of the hive can also play a role in disease resistance. Honeybees can mount a behavioral fever against heat-sensitive pathogens. Starks *et. al* (2000), found that honeybees will maintain elevated brood comb temperature in response to *Ascosphaera apis* exposure. *A. apis* is a fungus that will infect bee larvae. It germinates in the gut of susceptible larvae. Optimal temperature for *A. apis* is around ~30ºC, but it will germinate at tempertaure of ~32ºC. Bee colonies will induce a behavioral fever to reduce the susceptilbiy of the brood to *A. apis*.

To date, there has been a lack of studies examining how patrilines in naturally mated colonies correlate to thermoregulatory benefits. In this study we will be comparing thermoregulation stability, quantified as variance around the mean brood comb temperature during different environmental conditions (natural foraging, high nectar flow, exposure to *A. apis* a low virulent pathogen) against genetic diversity. In addition, we will be examining the pathogenesis of behavioral fever in response to *A. apis*. We predict that thermoregulation stability would correlate with patriline number and exposure to *A. apis* under non-germinating conditions would induce a behavioral fever.

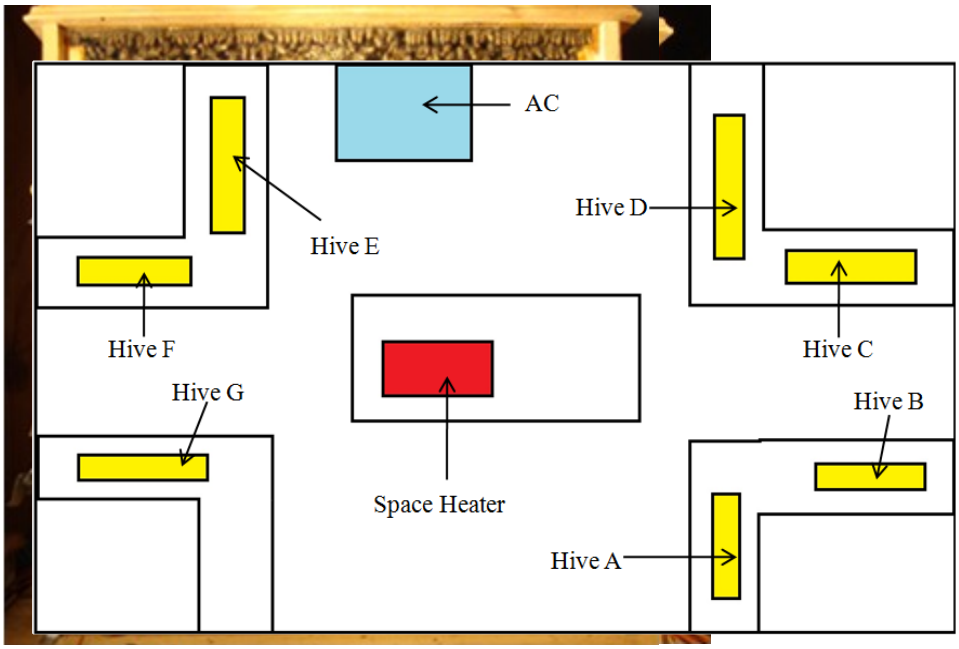
Material and Methods

Colony Establishment:

On June 21st, 2010, *A. mellifera L.* colonies, which consisted of a frame of brood and a queen cell, were obtained from Rick Reault, New England Beekeeping Supplies, Inc. The colonies included ~1200-2000 workers, and each of the colonies was placed in a two-frame observation hive (inner dimensions: 53 X 48 X 5cm) (figure 5). Observation hives were labeled A-H. The upper frame of each the observation hive was kept separated from the lower frame with a queen excluder and served as the foundation for honeycomb. Queens for the colonies were introduced in a wooden queen cell with a sugar plug. All eight queens were accepted by their workers. All observation hives were installed at the Tufts University Social Insect Research Facility. Their locations in relation to the heating and cooling source are given in figure 5.

Colony Care:

The colonies were allowed to forage naturally throughout the experiment, but starting on August 11, 2010 until August 29, 2010, their diets were supplemented with a 1:1 sucrose feed. The developmental progress of the hives was evaluated each week based on comb construction (brood and honey comb) and the number of worker present in the hive (estimated from photographs of the hive). The ambient temperature of the observation hut was gradually raised to 31-35°C prior to the experiment using a small space heater and a temperature controlled AC system.



Cultivation of *Ascosphaera apis*:

To determine the variance in mean brood comb temperature, and the level of fever response across patriline numbers, we artificially exposed our honeybee colonies to *A. apis*. Mycelia from a pure culture of *A. apis* (strain: A0015) was obtained from stock cultures at the Tufts University Social Insect Research Facility. The mycelia was grown on a Potato Dextrose Agar + 0.4% yeast extract plate at room temperature. There was furry growth after 2 days, and spores after 3-4 days. Twenty additional plates of *A. apis* were cultivated from our culture. Spores from the *A. apis* culture were isolated and the fungus was identified morphologically at 40x and 100x magnification using reference images from Chorbinski & Rypula (2003). The isolated spores were suspended in sterile water, and the concentrations of the spores were estimated using a hemocytometer.

Treatment group selection:

The control hives for the experiment were selected based on hive developmental progress (comb construction and honey storage) and population size. Colony D was selected as a control

since its population was at the mid-range of the population size distribution for all hives. Colony E was also selected as a control group for the experiment. At the time of selection, hive E had the highest number of workers, therefore it was selected to control for population size's effect on disease resistance. The final treatment group consisted of hive A, B, C, F, and G, since hive H was excluded from the experiment due to a lack of developmental progress.

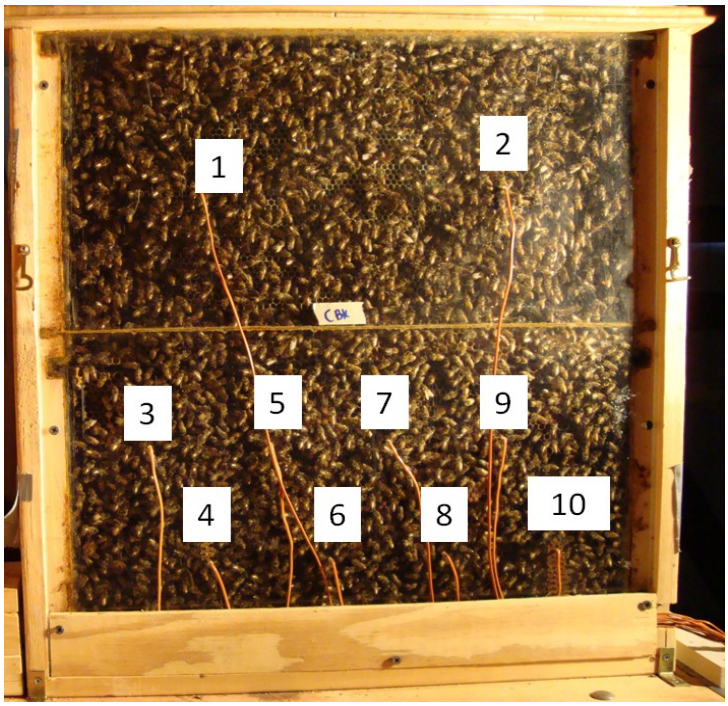
Treatment Periods:

Treatment Period	Treatment Conditions	Duration	Question Tested
No-feed	All colonies were allowed to forage naturally	Three days (days 1-3)	Influence of genetic diversity and thermoregulation stability in naturally foraging, naturally mated colonies
Feed	All colonies were given 300ml of 1:1 sucrose syrup feed each day	Five days (days 4-8)	Sucrose feed's effect on thermoregulation
Inoculation	Treatment hives were given approximately $4.2x10^5$ spores mixed with 100ml of 1:1 sucrose syrup feed each day (total: 1.25×10^6 spores/hive). Flores *et al.* 2004 showed that a concentration of 1.25×10^6 spores/hive given in glucose water induced a ~60% mummification of susceptible brood. The control hives received the same volume of 1:1 sucrose feed during this period, except *A. apis* spores were not	Three days (days 9-11)	Mechanism of the pathogenesis of behavioral fever

	mixed in with their feed		
Post inoculation-feed	All colonies were given 300ml of 1:1 sucrose syrup feed	Five days (days 12-16)	Mechanism of the pathogenesis of behavioral fever
Post inoculation no-feed	All colonies foraged naturally for the remainder of the experiment	Three days (days 17-19)	Mechanism of the pathogenesis of behavioral fever

Data Collection:

The hive temperature of each colony were measured using probes (sensitive to 0.1 ºC). Temperatures were recorded with an Omega OMB ChartScan-1400 Portable Data Recorder. Ten Thermistor probes were placed within each colony. Eight of the probes were placed in the lower frame of the observation hives, and two of the probes were positioned in the upper frame. The eight probes in the lower frame were separated into two categories, outer and inner brood comb, depending on the probes' approximate distance from the brood comb (Figure 6). An additional temperature probe was placed outside each of the observation hive to record the ambient temperature.



Genetic Analysis:

I. DNA extraction

On September 7th and September 8th, 96 foraging workers were collected outside of each colony and stored at -20°C until DNA extraction. DNA was extracted from the abdomen tissue of each specimen using a standard Chelex 100 DNA extraction method. Each sample was crushed using minipestles and incubated for 10-15 minutes at 95°C in 250 µl of 5% Chelex 100 solution. Three cycles of incubation were performed. After each period of incubation the samples were vortexed for 30 seconds. After the last incubation period, the samples were centrifuged at 13,000 rpm for five minutes. The supernatant of each sample was collected and diluted at a 1:1 ratio with ddH_2O. The extracted DNA was shipped to the Lake Wheeler Honey Bee Research Facility at North Carolina State University for further analysis.

II. Paternity Analyses:

The subfamily of each individual was determined using microsatellite alleles. Due to their high mutation rate, microsatellite markers are ideal for patriline analysis since they contain a large number of alleles. All samples were amplified at eight different microsatellite locus, Am043 (formerly A76; Estoup et al., 1994), Am098 (formerly Ap44; Estoup et al. 1994), Am125 (formerly A81; Stolignac et al., 2003), Am061 (formerly B124; Estoup et al. 1994), Am059 (formerly Ap113; Estoup et al., 1995), Am052 (formerly A88; Estoup et al., 1995), Am010 (formerly A24; Estoup et al., 1995), and Am553 (Delaney et al., 2010). The PCR products were visualized using fluorescently labeled primers. PCR amplification conditions are detailed in Tarpy *et al.* 2010. PCR products were separated using an ABI 3730 DNA analyzer at the Genomic Sciences Laboratory of North Carolina State University, USA.

Patriline of our workers can be identified due to the haplodiploid determination of sex in honeybees. We can determine the patriline of our workers based of their genetic variability at their respective microsatellite locus. Since all workers are diploid females, they received one set of alleles from their father and another set from their mother, the queen. Based on inheritance pattern of the workers, we can infer that any two alleles in equal proportion at a microsatellite loci are most likely derived from a heterozygous queen. By subtracting out the maternal alleles, we can then determine the number of patrilines amongst the workers . The software Genemapper 4.0 (ABI) was used to isolate the maternal allele and determine worker paternity.

Statistical Analysis of Temperature data:

Due to a temperature drop during the inoculation period that was correlated with a significant temperature drop outside of the observation hive (28^{o}C to 17^{o}C), we divided our temperature measurement into two data sets. Data set 1 is the no-feed period, feed period, and the first 26 hours of the inoculation period. Data set 2 is the remainder of the inoculation period, post-inoculation feed period and post inoculation no feed period.

1. Thermoregulatory Stability and Genetic Diversity

Hourly temperature of the inner and outer brood comb were calculated from the probes 5-8 and probes 3, 4, 9, and 10 respectively, and variance around the mean hourly temperature was compared against patriline line number. The inner and outer brood comb temperatures were studied because this area is most sensitive to temperature change and therefore it is a good indicator of thermoregulatory stability (Jones, *et al*., 2004). Given our small sample size (n=7), we used Spearman's rho, a non-parametric correlation test, to evaluate the relationship between variance and patriline number.

2. Pathogenesis of Behavioral Fever Against *A. apis*

Previous experiment have shown that exposure to chalkboard (a fungal disease) can trigger a preventive behavioral fever in honeybees. In the experiment, we were interested in changes in mean temperature and variance change of our experimental hives after they were exposed to *A. apis* spores under non germination conditions (above 32^{o}C). We include the no-feed period in our comparison to control for variance change due to the sucrose feed. One sample KS test of normality revealed that temperature distribution across all hives was not normally distributed, therefore we compared the mean hourly temperature of the inner brood comb from each treatment periods against each other using Kruskal-Wallis H tests, followed by a non-parametric post-hoc pair wise comparisons.

3. Effect of Nectar Flow and Thermoregulation:

To compare the effect of high nectar flow we compared the brood temperature distribution between the no-feed and feed period using the Kolmogorov-Smirov (KS) test. To determine whether there was a change in the colony temperature circadian rhythm, we compared the temperature trend of the no feed and feed period graphically. We also compared inner brood temperature distribution between the post-inoculation feed and post inoculation no feed period using the KS test to determine if the temperature regulation would return to the no-feed base line after the colonies were taken off the sugar feed.

All statistics were calculated using SPSS for windows (v. 17).

Results:
Genetic Diversity:

Across our seven colonies, we found a range of 20-37 patrilines per colony (Mean ± SD: 27.14 ± 6.31). The mean temperature at the brood area across the four day unfed period ranged from 34.61-35.19ºC (Mean ± SD: 34.89ºC ±0.19ºC). In comparison to previous studies, we saw an increase in the number of patrilines in our colonies, from 12 ± 6.41(Tarpy and Nielson 2002) and 20.8± 5.2 (Wilson-Rich, 2011) to 27.14±6.31 presented here. The range of mating number also shifted upwards to 20-37 from 1-28 (Tarpy and Nielson 2002), 8-27 (Palmer and Oldryod 2001), and 8-29 (Wilson-Rich, 2011).

Table 1. Estimated number of patriline of colonies A-G. 82-92 worker bees from each colony were genotyped at eight microsatellite loci. Observed mating number (N_o), effective mating frequency (M_e), and the 95% CI around M_e were also calculated for each colony.

Colony	Treatment	Number of Loci Used	Sample Size	Mating number (N_o)	Effective mating frequency (M_e)	95% C.I. of M_e
A	*A. apis* spore exposure	6	86	28	19.34	4.50365
B	*A. apis* spore exposure	6	92	27	14.05	3.99414
C	*A. apis* spore exposure	8	85	33	10.06	5.84371
D	Control	5	82	37	37.75	7.19305
E	Control	7	85	25	10.23	3.83394
F	*A. apis* spore exposure	5	84	20	14.06	2.76017
G	*A. apis* spore exposure	6	82	20	8.72	2.82466

Thermoregulation and Genetic Diversity

We found no significant correlation between genetic diversity and thermoregulation stability in the inner or outer brood comb (Spearman's rho, N=7, correlation coefficient=-0.018,

P =0.969; N=7, correlation coefficient=0.505, P=0.248, respectively). Variance of the inner comb across all hives was lower than the variance of the outer brood comb, except in hive G.

Figure 7. Genetic diversity does not seem to promote thermoregulation stability in naturally mated honey bee colonies. There are no correlation between variance of the inner brood comb during the no-feed period and the number of patriline of the colony (Spearman's rho, N=7, correlation coefficient=-0.018, P=.969). Brood comb variance of the outer brood comb was also not correlated with the number of patriline of the colony (Spearman's rho, N=7, correlation coefficient=.505, P=.248). In comparison to variance around the outer brood comb, variance of the inner comb was lower across all hives except for hive G.

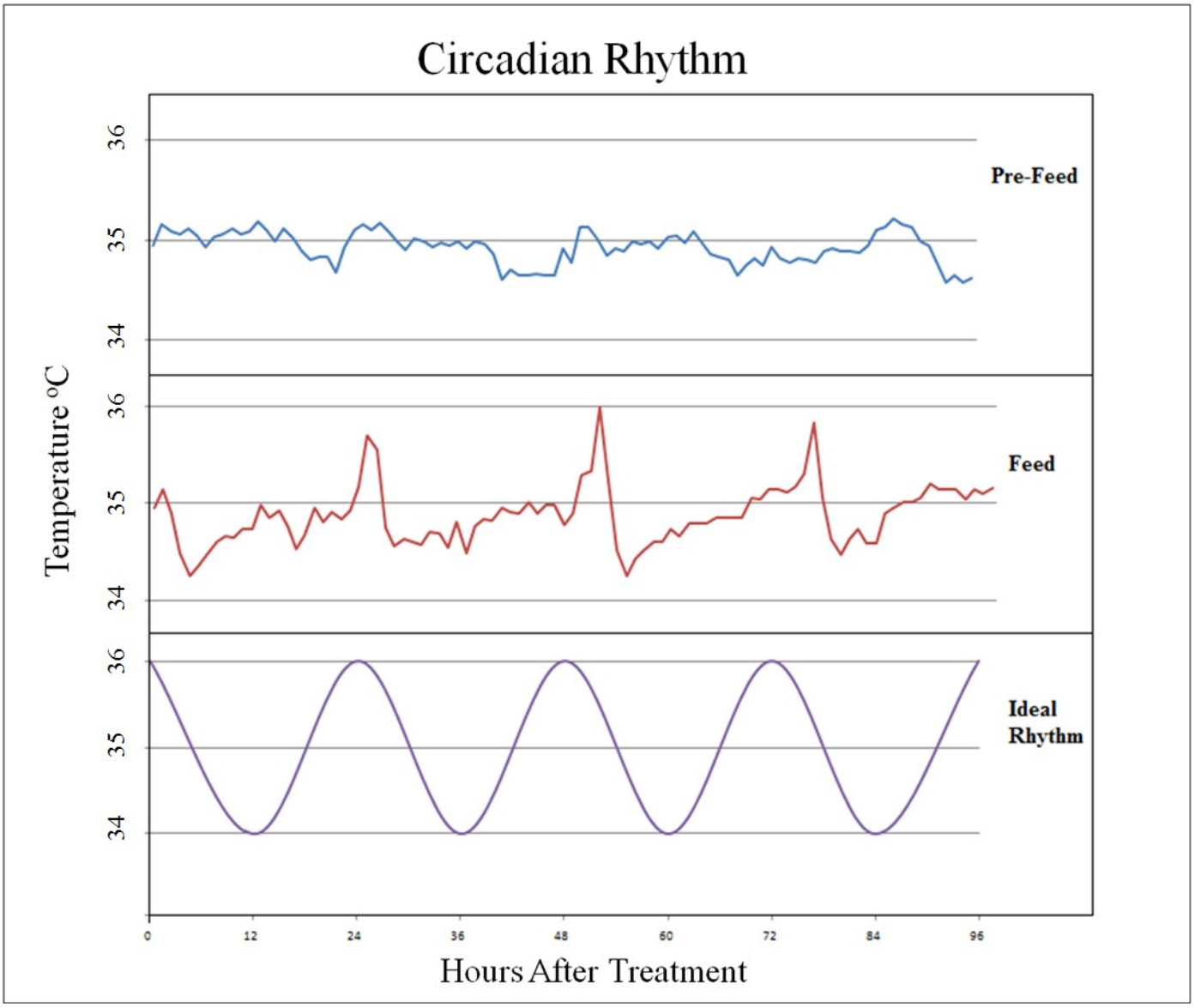
Pathogenesis of Behavioral Fever against *A. apis*

Kruskal-Wallis H comparison of mean temperature during different treatment period of the inner brood comb data set 1 revealed a significant difference between the treatment periods across all hive (see supplementary table 2 for test statistics). Post-hoc pair wise comparison using Tamhane's test revealed that there was a significant difference between the no-feed and feed period across all hives (all P<0.0001; supplementary table 3), however the feed and inoculation period across all hives were not significantly different (all P>.062; supplementary table 3).

Nectar Flow and thermoregulation:

KS comparison of the no-feed and feed period of the inner brood comb revealed a significant change in the thermoregulation pattern of our honeybee colonies (all P<0.0001). Variance of the inner and outer brood comb increased significantly across all hives between the two period (Wilcoxon Sign Ranked Test, N=7, Z=-2.366, P=0.018; t, N=7, Z=-2.371, P=0.018, respectively). Graphic comparison of temperature regulation patterns showed the emergence of ~24 hour cyclic patterns across all seven hives, which suggest that high nectar flow can influence the thermoregulation pattern of a colony (supplementary figures 8-10). KS comparison between the post inoculation feed and post inoculation no-feed period revealed a statistically significant

difference in temperature distribution in the inner brood comb across all colonies (all $P < 0.0001$; supplementary table 5).



Discussion:

Polyandry vs. Thermoregulation:

Since consistent brood temperature is essential for the development of larvae, if the temperature of the colony is not maintained between 33-36ºC, it can result in development abnormality in the larvae. Therefore, the ability to maintain stable temperature within the hive is an important colony-level behavior that can provide a selective advantage. In superorganism, many different workers work together to regulate hive temperature. The efficiency and stability by which the workers thermoregulate follows the threshold model of allocation.

In this model, temperature regulation is related to the worker's sensitivity to task stimulus. Colony-level thermoregulatory stability is derived from the interaction of workers with different temperature threshold. Given that task threshold sensitivity is patriline specific, we would predict that diverse colonies will show less variance in hive temperature.

The results from Jones *et al.* (2004) are consistent with these predictions. In their experiment, they compare the thermoregulatory stability of open mated colonies and genetically uniform colonies (one patriline). They found that variance in temperature maintained by the uniform colonies was significantly higher in comparison to diverse colonies. In addition, they also showed that workers derived from different patrilines respond to change in ambient temperature differently.

Although Jones *et al.* (2004) were able to show a difference in thermoregulation ability of genetically diverse and uniform colonies, the genetic uniform colonies (one patriline) they used in the experiment are extremely unusual in nature. The lowest experimentally determined patriline number in naturally mated honeybee queen is eight (Tarpy and Nielson 2002; Wilson-Rich, 2011), and in our studies, the mating number of our colony ranged from 20-37. Therefore their results cannot fully support the hypothesis of enhanced thermoregulatory stability driving the evolution of hyperpolyandry in honeybees.

To strengthen Jones *et al.* (2004)'s results, we compared the variance around mean brood temperature across colonies with different patrilines. Surprisingly, we did not find a positive correlation between stability of thermoregulation and patriline number. Based on these results, we speculate that there is a plateau to the thermoregulatory benefits that can be derived from genetic diversity.

Extreme polyandry may have other colony-level fitness benefits such as disease resistance and enhance colony performance that drove its evolution. We know that colonies reared by hyperpolyandrous queens develop faster, and this might be due to their enhanced recognition of stimulus that is unrelated to thermoregulation (Mattila and Seeley 2007). In addition, multiple experiments have showed that in comparison to genetically uniform colonies, diverse colonies show enhanced disease resistance (Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007)

Future studies should examine the relationship between disease resistance and genetic diversity in naturally mated colonies. In addition, genome comparison between workers of different patrilines could be useful in determining the threshold response of patriline specific alleles.

Pathogenesis of group fever response to *A. apis*

In honey bees, exposure to chalkbrood (a fungal disease) can trigger a behavioral fever. Chalkbrood is caused by *Ascosphaera apis*, which is a common fungal pathogen to bee larvae. *A. apis* germinates in the gut of the larvas, upon maturity the mycelium of the fungus will penetrate the larva's body causing the appearance of chalk-like mummies. *A. apis*' optimal temperature for growth is around 30^{o}C, however, it will start to germinate when the hive temperature drop below 32^{o}C for more than two hours (Hornitzky 2001; Bailey & Ball 1991). When the colony is exposed to *A. apis*, members of the colony induce a behavioral fever by increasing the hive temperature beyond the optimal germination temperature of *A. apis*. (Starks *et al.* 2000). The process of chalkbrood recognition and the process for the activation of temperature upregulation is unknown.

Starks *et al.* (2000) showed the colonies will mount a preventive fever response before symptoms are visible, therefore there must be some sort of rapid recognition system for *A. apis* infection by adult workers. Therefore we speculate that it is the recognition of the spores that can trigger a behavioral fever reponse.

In our experiment, we attempted to artifically induce a behavioral fever resposne by introducing *A. apis* spores to the experimental colonies under non-germination condition. Our colonies fail to show any significant changes in their thermoregulation patterns after they were

exposed. Based on these results, we speculate that in addiition to spore recognition, there is a temperature check point before a behavioral fever response can be activated.

Future experiment could expose non-viable spores to the colonies under germinating condition (below 32^{o}C). If a behavioral fever is not generated under these condition, there might be chemical signal produced by non- symptomatically infected larve.

High Nectar Flow and Circadian Rhythms:

Circadian rhythms are daily rhythmic change in physiological and biological function. Many organisms have evolved circadian clocks that are sensitive to external time cues derived from the daily rotation of the earth. By synchronizing physiological functions to specific changes in light and temperature, organisms can anticipate environmental change such as seasonal transitions. The ability to predict change and response appropriately will increase the likelihood of survival. . Fuller *et al.* (2008) have observed that a "food-related clock" can take precedence over the "light-based" circadian pattern. It appears that a "food-related clock" can help animals switch their sleep and wake cycles to maximize their access to food related resources.

There are a few studies documenting circadian pattern of O_2 consumption in wintering bees and diurnal rhythms of metabolic rate and locomotor activity in honeybees. These circadian rhythms seem to be associated with day and night cycles. Metabolic rates and locomotor activity both peaked during the day and were lowest at night (Southwick, 1982; Kronenberg and Heller, 1982). The mechanism by which colony-level circadian rhythm is regulated remains unclear. There have also been no studies documenting food induced colony-level circadian rhythms in *A. mellifera*.

This study has shown that the availability of food can act as circadian rhythm cue, and it can induce a new cycle of thermoregulation. During feed periods, the colony-level circadian

rhythms of our colonies were adjusted to feeding times. Hive temperature typically peaks after sucrose feeding and reach the nadir ~12 hours after the feed (figure 7). Graphical comparison of the thermoregulation pattern between the no feed and feed period also show a drastic difference.

Previous studies have demonstrated that superorganisms follow many of the same energetic constraints as eukaryotic organisms. In most animals, the metabolic rate is proportional to their mass to the power of 0.75. The metabolic rate of honeybees and other superorganisms are in proportional to their mass to the power 0.81, which is statically indistinguishable from 0.75 (Hou, *et al.*, 2010). The lifespan and growth rate of honeybee colonies also follow the same mathematic models seen in other eukaryotic organisms (Hou, *et al.*, 2010). Given that superorganisms such as the honeybee follow other metabolic models seen in eukaryotic animal and the fitness benefits derived from a circadian rhythm that can be used to predict food access, it is not surprising that superorganisms can posses a food-related circadian rhythm.

Future tests may include examining average colonial hormone level and how behavioral and physiological effectors are integrated into thermoregulation cycles. Differences in gene expression between period of high food availability and normal foraging can also be studied using microarrays.

Conclusion:

Our study showed that thermoregulatory stability is not correlated positively with patriline number in naturally foraging, naturally mated colonies. Based on the lack of response to *A. apis* exposure, we speculate that there is a temperature checkpoint before the activation of a behavioral fever against *A. apis* in honeybee. We have also introduced novel evidence for a food-based circadian rhythm in honeybees.

Supplementary figures and table:

Table 2. Kruskal Wallis Comparison of inner brood comb temperature during the no feed, feed, and inoculation period.

Hive and Hive Region	Chi Square	df	P value
Hive A inner brood comb	79.288	2	P < 0.0001
Hive B inner brood comb	89.385	2	P < 0.0001
Hive C inner brood comb	58.673	2	P < 0.0001
Hive D inner brood comb	91.675	2	P < 0.0001
Hive E inner brood comb	47.987	2	P < 0.0001
Hive F inner brood comb	52.977	2	P < 0.0001
Hive G inner brood comb	82.185	2	P < 0.0001

Table 3. Tamhane's post-hoc pairwise comparison, period of comparison and P value are shown in the table.

Hive	Period of Comparison	P value
A Inner Brood Comb	No feed and feed	P < 0.0001
	Feed and inoculation	P=0.941
B Inner Brood Comb	No feed and feed	P <0.0001
	Feed and inoculation	P= 0.832
C Inner Brood Comb	No feed and feed	P < 0.0001
	Feed and inoculation	P= 0.110
D Inner Brood Comb	No feed and feed	P < 0.0001
	Feed and inoculation	P= 0.999

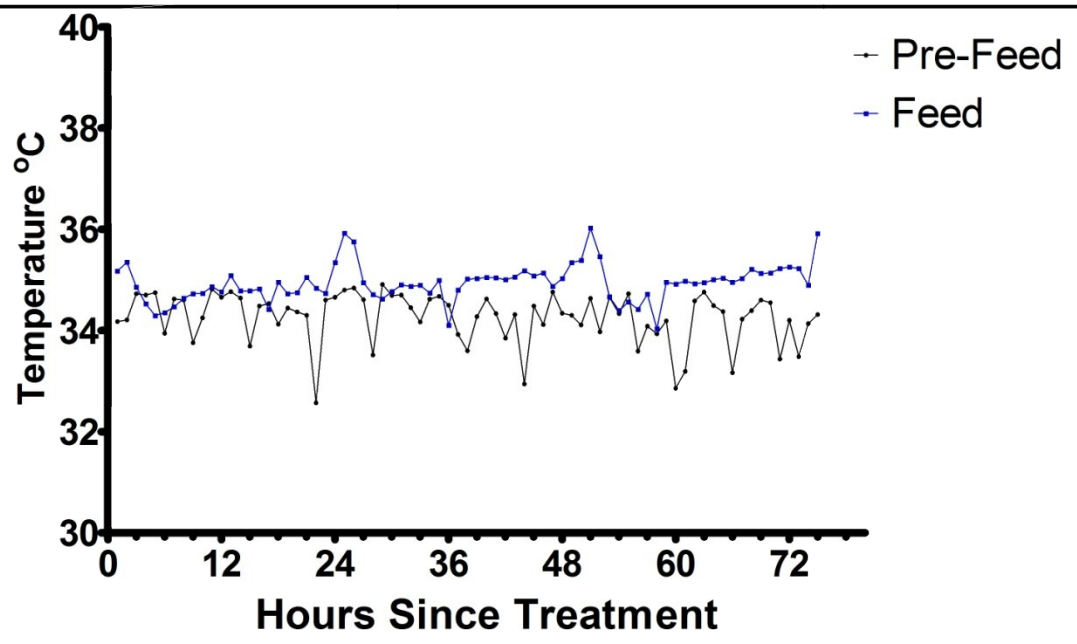
E Inner Brood Comb	No feed and feed	P < 0.0001
	Feed and inoculation	P =0.788
F Inner Brood Comb	No feed and feed	P < 0.0001
	Feed and inoculation	P = 0.440
G Inner Brood Comb	No feed and feed	P < 0.0001
	Feed and inoculation	P =0.062

Table 4. Kolmogorov-Smirnov comparison of the equality of temperature distribution during the no feed and feed period. Kolmogorov-Smirnov Z test statistic and P value is provide for each hive.

Hive and Hive Region	Kolmogorov-Smirnov Z	P value
Hive A inner brood comb	3.627	P < 0.0001
Hive B inner brood comb	3.772	P < 0.0001
Hive C inner brood comb	3.192	P < 0.0001
Hive D inner brood comb	4.571	P < 0.0001
Hive E inner brood comb	3.047	P < 0.0001
Hive F inner brood comb	2.902	P < 0.0001
Hive G inner brood comb	4.208	P < 0.0001

Table 5. Kolmogorov-Smirnov comparison of the equality of temperature distribution during the post inoculation feed and post inoculation no feed period. Kolmogorov-Smirnov Z test statistic and P value is provide for each hive.

Hive and Hive Region	Kolmogorov-Smirnov Z	P value
Hive A inner brood comb	3.916	P < 0.0001
Hive B inner brood comb	3.323	P < 0.0001
Hive C inner brood comb	3.962	P < 0.0001
Hive D inner brood comb	3.504	P < 0.0001
Hive E inner brood comb	3.277	P < 0.0001
Hive F inner brood comb	3.732	P < 0.0001
Hive G inner brood comb	3.962	P < 0.0001



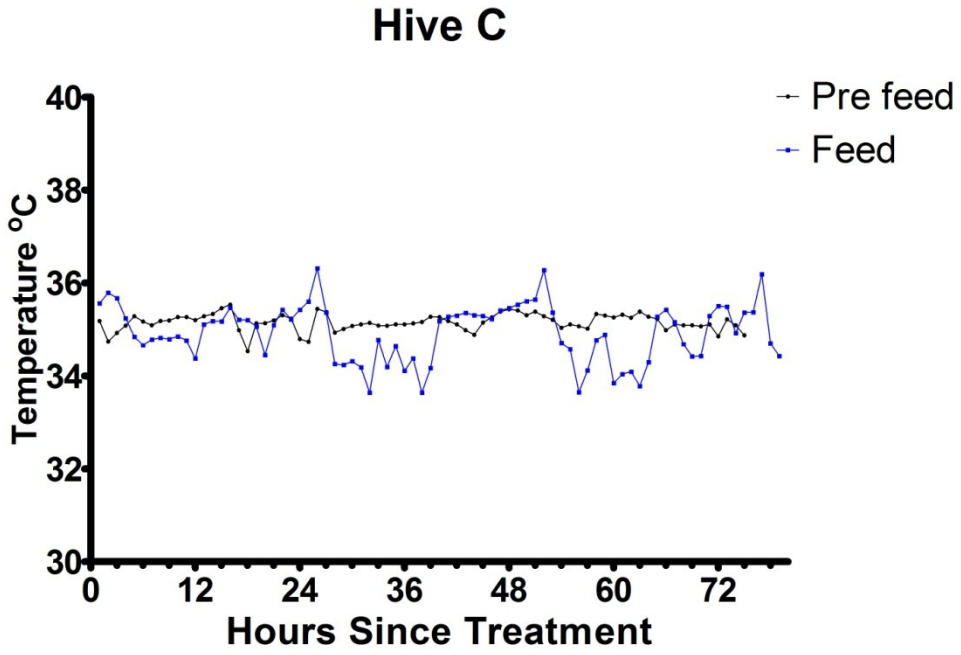


Figure 8. Comparison of thermoregulation pattern during the pre-feed and feed period in hive B and C. Average hourly temperature during the pre-feed and feed period from the inner brood comb are shown.

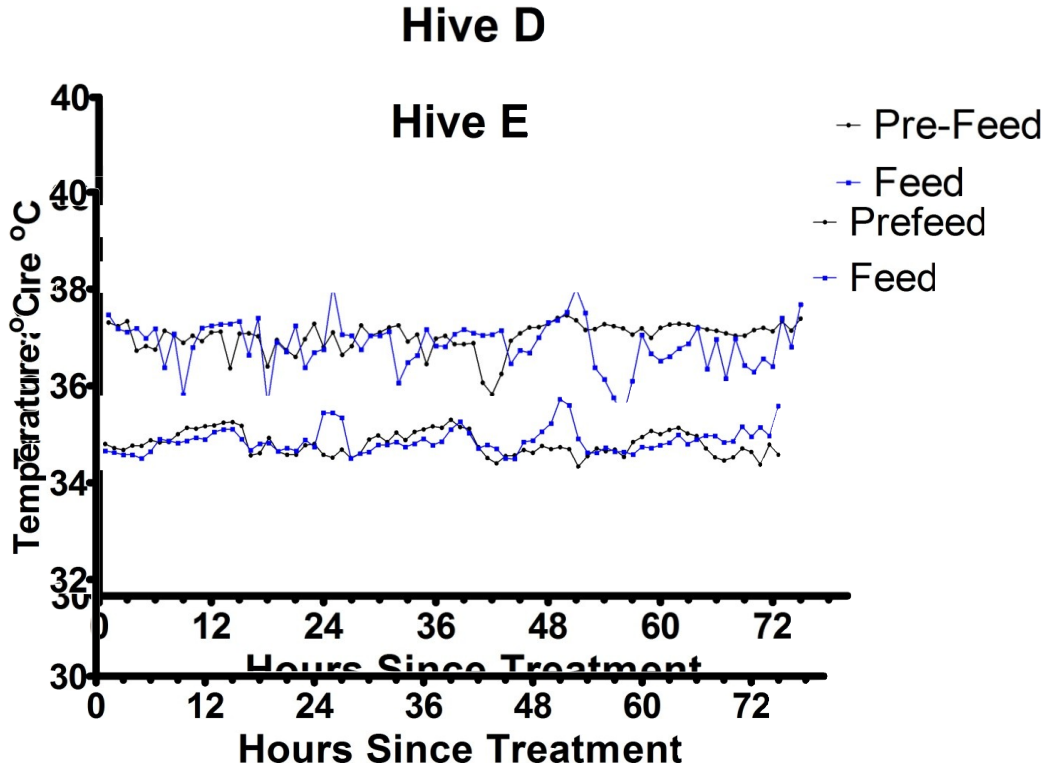


Figure 9. Comparison of thermoregulation pattern during the pre-feed and feed period in hive D and E. Average hourly temperature during the pre-feed and feed period from the inner brood comb are shown.

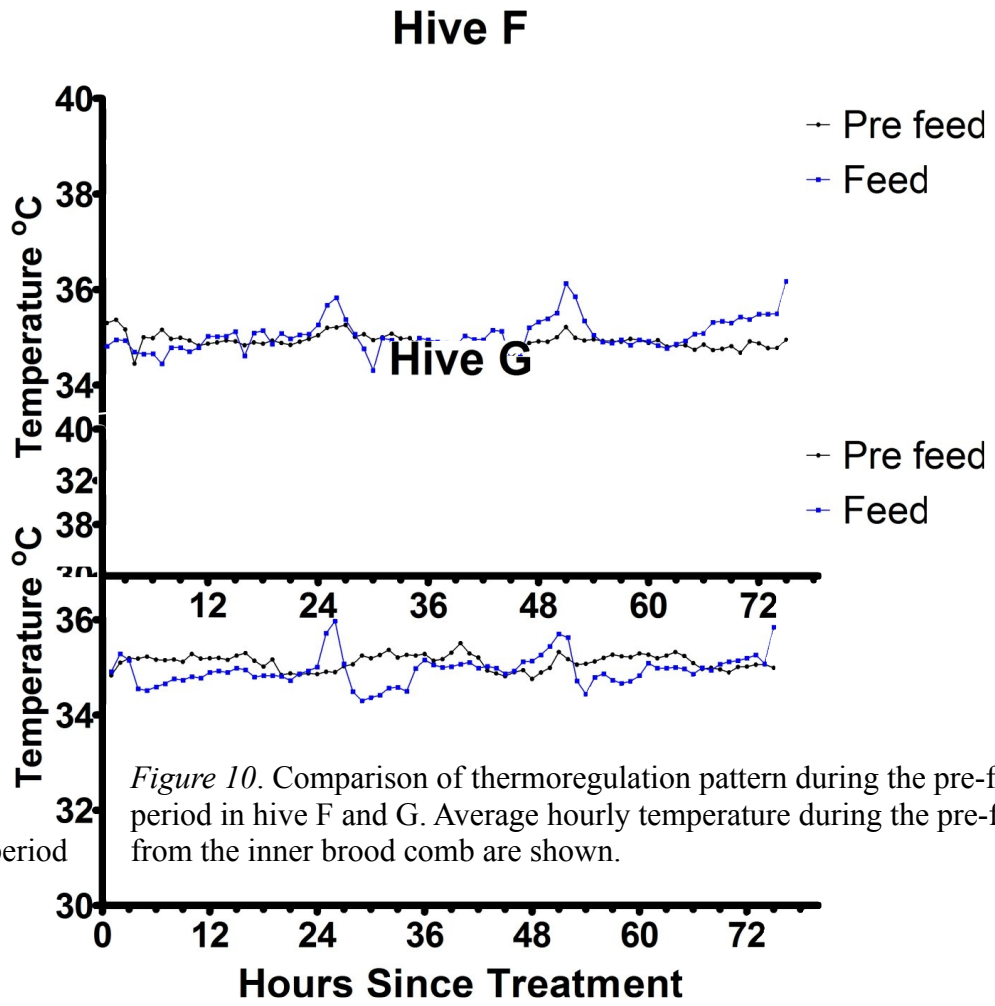


Figure 10. Comparison of thermoregulation pattern during the pre-feed and feed period in hive F and G. Average hourly temperature during the pre-feed and feed period from the inner brood comb are shown.