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Juvenile temperature regulation in *Apis mellifera* (Honey bee) and the impacts of brood temperature requirements on the colony

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Abstract

Little is known about the energetic costs to insects of raising young. Honey bees collectively raise young, or brood, through a series of complex behaviors that appear to accelerate and synchronize the timing of brood maturation. These include maintaining the brood nest at warmer and consistent temperatures and the exceptional activity of "heater bees." The temperature at which juvenile insects are raised can profoundly affect their development. *Apis mellifera* (Honey bees) cope with temperature-dependent development via social behavior that maintains the relatively high and constant temperatures within the nest where the brood are raised. Yet juvenile honey bee development is complex and can be categorized into egg, larvae, pupating juveniles, and pupae.

Honey bees use passive and active behaviors to maintain remarkably constant brood nest temperatures, from 33 to 35°C, across a wide range of ambient temperatures. In addition to these colony-scale behaviors, a small subset of nurse bees behaves as heater bees. Heater bees contract thoracic flight muscles to generate heat, but their thoraxes reach much higher temperatures than other bees responsible for brood care, ranging between 42 and 47°C. Heater bees focus their attention on incubating individual cells by moving among brood cells and regulating the temperatures of individual eggs, larvae, and pupae.

We constructed four sets of experimental hives to explore the developmental temperatures at which each juvenile stage is maintained, the energetic costs of raising juveniles, and the cost of heater bees. One set allowed us to record the temperatures of undisturbed young in the brood nest area established by the colony. The second set was designed to estimate the numerical allocation of individuals to the heater bee task. The third set was intended to contain only brood, which eliminated foraging and allowed us to quantify stored honey use when rearing

juveniles at 10 and 30°C. The final set was used to measure the respiration rates and energy expenditure of individual bees displaying resting, walking, heating, and agitated behavior. We first discovered that instead of simply maintaining brood nest areas at 33-35°C, honey bees provide extraordinarily precise but different temperatures for larvae and pupae. We found that the temperature at which heater bees regulate cells is above the overall average temperature range of the brood nest. Honey bees raised larvae at 36.38±0.02°C, substantially higher and with a narrower range than what has been reported for the brood nest, 33-35°C. Honey bees raised pupae at 35.18±0.04°C, also higher than the reported temperatures for the brood nest.

We further explored brood development by characterizing the developing juveniles' temperature profile throughout their entire 21-day developmental cycle. We found that eggs were maintained at 36.1 ± 0.03°C, larvae at 36.2 ± 0.02°C, pupating juveniles at 35.9 ± 0.03°C, and pupae at 35.8 ± 0.03°C. All stages were significantly different from all other stages, but importantly larvae were only 0.4°C different from pupae. We then conducted another experiment with brood frames without mature bees and in incubators at 34.5°C. Without nurse bees, the temperatures of eggs, larvae, and pupae were 34.4 ± 0.04°C, 34.7 ± 0.05°C, and 34.3 ± 0.04°C, with larvae different from all other stages, and a 0.3°C difference between larvae and pupae. When compared to the 1.2°C in Chapter 1, this 0.3°C difference suggests that heater bees may be a major driver of the differences between pupae and larvae. However, the 0.4°C difference between larvae and pupae in the second experiment reported in chapter 2, vs. the 0.3°C difference, suggests that the larvae themselves may be the major contributor to the temperature difference between the life stages. Either way, our results suggest honey bee development may involve far more precise temperature during the development of juveniles than previously known.

And finally, to determine the cost of maintaining juveniles at these warmer and more consistent temperatures, we compared the honey used by brood-only experimental colonies with whole-colony measurements of honey storage in the literature. We estimated that raising brood costs colonies half of their annual energy budgets stored as honey, or approximately 43.7±0.9 $kg \cdot yr^{-1}$. We estimated that roughly 2% of colony individuals perform the task of heater bee. Respiration rates of heater bees (19 mW) were more than those of resting bees (8 mW) but similar to those of walking bees (20 mW) and about half of those that were agitated (46 mW). The energetic cost of heating was more than an order of magnitude lower than reported values for the energetic cost of flying. By integrating data from our experimental hives, we estimate that the annual cost of raising brood is quite high; however, we estimate that heater bee behavior and physiology, though extreme, may require only about 7% of the annual honey stored by a colony.

Instead of simply maintaining brood nest areas at 33-35°C, honey bees provide extraordinarily precise but different temperatures for larvae and pupae. We do not know if these differences ultimately affect development, but they suggest that honey bees may exert far more precise control over the temperatures of their juveniles than previously known, which comes at a high cost at the colony level (macroeconomic), but a surprisingly low cost at the individual (microeconomic) heater bee level.

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Juvenile temperature regulation in Apis mellifera (Honey bee) and the impacts of brood temperature requirements on the colony

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Chapter 1: Dissertation Introduction

Insects are ectotherms, but many have a remarkable ability to thermoregulate. Insects employ two general methods to keep their body temperatures within optimal ranges: basking and shivering (Heinrich 1995). Basking is behavior that utilizes solar energy. By positioning themselves in the sun, insects can regulate heat gain and loss. For example, marsh fritillary larvae (*Euphydryas aurinia*) congregate on vegetation in the sun, where they can raise their body temperature as much as 22°C above ambient (Porter 1982). Shivering is the rapid contraction of flight muscles which generates metabolic heat. Shivering can produce very high body temperatures, allowing some species to occupy wide ranges of diurnal and annual temperatures. Many dragonfly species (*Leucorrhinia hudsonica, Sympetrum obtrusum, S. vicunum, Ladona julia,* and *L. libellula*), which have a limited ability to shiver, incorporate both shivering and basking behavior. These dragonflies require thoracic temperatures between 27 and 39°C to fly and cannot produce spontaneous flight, even when threatened, outside this range. For them, increases in thoracic temperatures generated through shivering are related to ambient temperatures averaging 2.0°C/min at 10°C to 5.3°C/min at 25 (Vogt and Heinrich 1983). In contrast, Bumblebees can generate thoracic temperatures as high as 37.5°C even when ambient temperatures are as low as 3°C (Jones and Oldroyd 2006), and sphinx moths (*Sphingidae*) can maintain thoracic temperatures as high as 42°C in ambient temperatures as low as 16°C (Heinrich 1970).

Social insect species can extend this heating ability to the nest environment. Wood ants (*Formica polyctena*) bask on the surface of their nest and then cluster within the nest, transferring the heat acquired through basking to the internal environment of their mounds (Frouz 2000). Many stingless bee species (*Meliponini*) encapsulate the brood in an involucrum made from wax and propolis. The involucrum traps the heat generated from brood metabolism, which helps to maintain temperatures suitable for brood development (Roubik 1983).

Most species of hymenopterans maintain strikingly consistent brood temperatures. European paper wasps (*Polistes dominula*) design nests to passively heat their brood. When nest temperatures exceed 35°C, adults use evaporative cooling to decrease single-cell temperatures by as much as 8°C (Höcherl *et al*. 2016). The tropical stingless bee, *Melipona colimana*, experiences high brood mortality when juveniles are outside the 25 to 33°C temperature range. Nest heating is seldom necessary in the tropics; nevertheless, *M. colimana* workers increased sugar syrup consumption and thoracic temperature when experimentally exposed to a cold environment (7°C). When exposed to 40°C temperatures, the workers reduced nest temperatures through evaporative cooling (Macías-Macías *et al.* 2011). Bumblebee foundress queens press tightly to brood cells, warm their abdomen with thoracic contractions, and transfer this heat to the brood. Once the offspring emerge, they continue this incubation behavior keeping the developing juveniles within 28 to 32°C (Heinrich 1972).

To my knowledge, no social insect species actively maintains a constant brood temperature environment as precisely as honey bees (*Apis mellifera*). Honey bees incorporate passive behavior to control temperature through nest habitat choice and active shivering to regulate brood temperature. They construct nests in closed cavities and place developing juveniles in the center of those cavities, surrounded by honeycombs, which provide insulation from external environmental conditions. They also incorporate active brood heating activities that are initiated via flight muscle contractions and then behaviors to distribute heat to the developing juveniles. These include resting superheated thoraces on top of capped brood cells, entering empty brood cells among occupied ones, and then superheating their thoraces to transfer heat to the occupied cells (Kleinhenz *et al.* 2003).

Honey bees also exhibit cooling behaviors. Adults collect water when the brood becomes too hot and regurgitate it into the brood area, where it evaporates and cools the brood (Seeley 1985). Bonoan *et al*. (2014) reported a behavior called heat shielding. When the brood area is too hot, adult bees press their bodies against the heated wax in the brood nest to absorb heat into their bodies and then move to peripheral regions of the nest to dissipate the heat, which cools the brood. These behaviors maintain a remarkably consistent temperature in the brood area, which remains between 33-36°C (average of 34.5°C) regardless of outside temperatures.

In addition to these behaviors that are prominent during periods of brood rearing, honey bees also display very unusual winter behavior. Unlike the vast majority of other insects in temperate and colder environments, which overwinter as juveniles or in torpor, honey bees overwinter as a fully functional social unit of active adults. Even related taxa, bumblebees, overwinter as single reproductive individuals in torpor. Honey bees remain in their hive and generate heat through metabolic heat production that maintains the temperature of the colony cluster throughout the winter months. Colonies become more compact when temperatures drop below 5°C to prevent heat loss by decreasing ventilation passages. Their plumose thoracic hairs interlock readily, creating a compact, insulated mass that can conserve metabolic heat (Southwick 1985). When clustered in this way, honey bees can maintain core cluster temperatures between 25 and 35°C even when ambient temperatures are -20°C (Owens 1971). Southwick (1987) found that a colony of 16,000 individuals can survive at -80°C for 12 hours. Therefore, clustering with metabolic heat production, combined with the insulative value of the nest structure, is a sufficient mechanism for overwinter survival…if the colony has access to an energy source to fuel metabolic heat production.

For honey bees, honey is that energy source. Colonies consume honey for many reasons, and not all are directly related to temperature regulation. For example, wax is a significant expenditure in the construction of hives, and honey is consumed at a rate from 8:1 to 24:1 ratio of honey to wax weight. Hepburn *et al*. (2014) estimates that a typical hive requires 57.6 kg of honey to build, and honey bees only expand their nest structures when nectar is abundant, perhaps ameliorating the consumption of stored honey. Still, nectar foraging for nest building may conflict with nectar collection for honey storage for brood rearing and overwinter survival (Seeley 2009). Honey is also vital to fuel the workforce as the consumption of honey fuels every activity from foraging to brood temperature regulation.

The complex behaviors and the expensive infrastructure produced by honey bees described above target two critical outcomes: the collection and storage of resources for winter survival and colony reproduction in the form of swarming. Achieving these outcomes requires more than real-time energy consumption; they require energy storage, and honey bees store energy in the form of honey. Storage allows honey bees to maintain highly consistent temperatures for very large numbers of developing brood, to survive winters without torpor, and to fuel building nest structures.

Honey is derived primarily from flower nectar that is collected by bees, sequestered in their ingluvies (honey crop), and transported to the hive to be processed into honey. The honey crop is an expandable section of the esophagus. This expandable section allows honey bee foragers to transport up to 60 µl of nectar weighing as much as 70 mg (close to 90% of the bees' body weight, Snodgrass 1956). Nectar is comprised primarily of water and a 10-30% sucrose solution, with trace amounts of amino acids, fats, organic acids, vitamins, and minerals (Crane 1975). On the other hand, honey is primarily carbohydrates (nearly 80%). To convert nectar into honey, the colony must remove the water found in nectar and convert the sucrose into glucose and fructose (White and Doner 1980).

When foragers return to the nest with a nectar load, they do not deposit the nectar into cells. Instead, they transfer their load to a house bee, which, in turn, transfers the nectar to one or several other house bees until the nectar is placed in a cell for ripening. The number of house bees included in the chain depends on colony size and nectar influx rate (Crane 1975). Each time the nectar is transferred to another bee, enzymes (invertase, amylase, and glucose oxidase) are added to the nectar. These enzymes transform the sucrose in the nectar into simple sugars, primarily glucose and fructose, though trace amounts of other simple sugars are present in honey (White *et al*. 1962).

Once the nectar/enzyme mixture is deposited into cells, it becomes subject to active and passive ripening processes from the colony. House bees actively engage with the mixture by drawing it into their honey stomachs and then emptying it back onto the walls of the cells as a thin film. A single bee might repeat this for 20 minutes. The film is exposed to the hive environment's warm air, which can reduce the water content to as low as 40% (Ruiz-Argueso and Rodriguez-Navarro 1975). The speed of the passive curing process depends on the colony's size, ventilation effort, and the hives' temperature.

House bees deposit the half-cured honey produced from the active processes into cells. These cells are only 1/4 to 1/3 filled and left uncapped, where they remain for up to 4 days. This reduces the water content by up to another 25%. Once the water content is between 17 and 20%, the honey is moved and combined with other ripened honey into cells that are then capped (Crane 1975).

By transforming nectar into calory-dense honey, storage space requirements are significantly reduced. Honey is also highly resistant to microorganisms that cause spoilage due to its higher osmotic pressure.

The efficient use of honey is crucial for honey bee survival and reproduction, and efficiency has been studied frequently in the context of foraging behavior. For example, honey bees and most other pollinating insects selectively forage on the plants with the highest sugar concentrations in their nectar. This behavior allows maximum honey acquisition into the colony while minimizing the foraging cost (Goulson 1999). They also visit familiar flowers, thereby reducing handling time and increasing nectar income (Goulson 1999). Honey bees, due in part to their large society and division of labor among the colony members, recruit to highly rewarding foraging locations, which allows for focused foraging efforts and minimal foraging costs while maximizing the acquisition of resources (Von Frisk et al. 1955, Seeley 1989, Camazine and Sneyd 1991, Goulson et al. 2007).

In North America, honey bee colonies spend most of their annual cycle consuming honey. Colonies have constant weight loss from September through April (Seeley 1985); during this time, a single colony consumes about 25 kg of honey (Seeley 2009). Between late April and early September, colonies amass resources for the rest of the year (Seeley and Visscher 1985), and colony size can triple during this time (Seeley 1985). During this window of high resource influx, colonies consume roughly another 35 kg of honey. Another 25 kg is needed to survive the rest of the year, bringing a typical colony's total annual honey requirement between 60 and 80 kg (Seeley and Visscher 1985). Whether or not colonies store enough honey for annual consumption is crucial. About 76% of founder colonies established after swarming perished from starvation over the winter months (Seeley 1985). In a survey of nearly 20% of the 2.44 million US *managed* colonies, starvation was the second most prominent cause of overwinter whole-colony mortality (VanEngelsdorp et al. 2008), greater than disease and parasite-caused mortality. However, even when hives store as much or more than needed for survival, even more is needed for colonies to reproduce new colonies (Winston 1980).

Colonies “reproduce” through fission, when colonies divide and swarm, and both the swarm and the mother colony that remains in the original nest consume stored honey. Swarming groups take a proportion of the hive’s honey with them when they swarm. The average individual in a swarm carries honey reserves that equal 50% of their body weight (Combs 1972). Thus, in a colony containing about 40,000 workers, roughly half of them swarm, taking with them about one kg of honey (Winston 1980, Seeley and Visscher 1985, and Heinrich 1981), which fuels nest selection and establishment (Heinrich 1981).

Honey bees do not get all their energy from honey; they feed on nectar when out of the nest. However, the performance and survival of colonies are inextricably tied to honey. Thus, understanding behavior, ecology, and physiology related to honey production, storage, and consumption are crucial for understanding honey bees.

Brood rearing may be as costly as foraging, but relatively little is known about its impact on honey consumption. Honey bee larvae (the only stage that is fed) receive food produced primarily from the synthesis of pollen and thus have a limited direct honey requirement (Haydak 1970). However, very precise temperature regulation of developing honey bees requires consumption of honey by the nurse bees to fuel the high thoracic temperatures used to warm the brood (Heinrich 1972, 2004). We do not know how colonies manage their resources when maintaining brood temperature, nor do we fully understand the temperatures that juveniles are reared or the impacts those temperatures may have on colony effort and resource consumption. This fundamental gap in our understanding of colony economics hinders our ability to measure colony energy and resource budgets. Resolving this gap is the primary goal of my thesis.

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Chapter 2: Honey bee larvae like it hotter and more consistent: larvae and pupae develop at different temperatures, but both are warmer than the brood nest

Abstract

Apis mellifera (honey bees) use passive and active behaviors to maintain remarkably constant brood nest temperatures, from 33 to 35°C, across a wide range of ambient temperatures. In addition to these colony-scale behaviors, a small subset of nurse bees behaves as "heater bees." Heater bees contract thoracic flight muscles to generate heat, but their thoraxes reach much higher temperatures than other bees responsible for brood care, ranging between 40 and 47°C. Heater bees focus their attention on incubating individual cells. We developed modified hives that allowed us to record the temperatures of undisturbed young in the brood nest area established by the colony. We found that the temperature at which heater bees regulate cells is above the overall average temperature range of the brood nest. Honey bees raised larvae at 36.38±0.02°C, substantially higher and with a narrower range than what has been reported for the brood nest, 33-35°C. Honey bees raised pupae at 35.18±0.04°C, also higher than the reported temperatures for the brood nest. Instead of simply maintaining brood nest areas at 33-35°C, honey bee juveniles appear to develop at extraordinarily precise, but different, temperatures for larvae and pupae.

Key Words: *Apis mellifera*, brood temperature, larvae, pupae, temperature regulation

Introduction

Most hymenopteran species control the temperature of their brood to some degree. Among these species, *Apis mellifera* (honey bees) use collective passive and active behaviors to maintain remarkably constant brood nest temperatures, from 33 to 35°C, across a wide range of ambient temperatures (Southwick & Heldmaier, 1987; Seeley, 1985; Jones et al., 2005). They construct nests in closed cavities and place developing young in the center, surrounded by honeycombs where they are insulated from external environmental conditions. Colonies place developing juveniles inside brood nest cells that are heated by colony activity. Some of this activity appears to be intentional warming of the brood nest area as adults produce heat by activating thoracic flight muscles in a shivering-like process, which they then transfer to larvae and pupae (Kleinhenz *et al*., 2003). If the brood nest becomes too hot, adults regurgitate and evaporate water by beating their wings (Seeley, 1985). Such collective regulation of brood nest temperatures provides optimum conditions for the timing and rate at which juveniles develop (Hölldobler & Wilson, 2009).

In addition to these colony-scale behaviors, a small subset of nurse bees behaves as "heater bees." These bees also contract thoracic flight muscles to generate heat, but their thoraxes reach much higher temperatures, ranging between 40 and 47°C (Kleinhenz et al., 2003, S.E. Debnam, *unpublished data*). Heater bees target specific cells for attention and place their superheated thoraxes near developing larvae and pupae in cells to transfer heat to the juveniles (Bujok et al., 2002). Heater bees display two distinct behaviors that suggest they are heating specific larvae and pupae in need of temperature control. First, they warm the brood from above by resting their heated thoraxes on the caps of brood cells, and second, they warm the brood by entering adjacent cells while their thoraxes are heated (Kleinhenz et al., 2003). The clear division of labor that results in heater bees and their cell-centric behavior suggests that the temperature of developing juveniles might be maintained at different or more precise temperatures than the general brood nest.

Honey bee juveniles mature through three stages: egg, larva, and pupa. Each stage requires a unique time frame and care regime. For example, larvae require constant feeding throughout a four to five day developmental period. In contrast, pupae do not eat and require between eight and thirteen days to complete metamorphosis, depending on the caste and gender of the individual. Might heater bees function to maintain the temperatures of cells specific to developmental stages and different from that of entire brood areas? Whether heater bees' unique behavior results in different temperature regimes for different juvenile stages is not known.

The temperature at which brood stages are raised can strongly influence development and adult behavior. For example, Lin & Wilson (1998) found that workers who reared below 33°C failed to develop ovaries. Groh et al. (2004) raised honey bee pupae at temperatures from 29 to 37°C and found that synaptic complexes in adult brains differed in response to different temperatures. In particular, the performance of the dance language that communicates forage location was reduced in both number and intensity for workers reared at 32°C but not between 35 and 36°C (Tautz et al., 2003; Becher et al., 2009). A tightly controlled brood nest temperature appears essential for the development and function of adult honey bees.

To determine the potential effects of heater bees on brood stage-specific temperatures, we 1) measured the temperatures of cells containing larvae and pupae with an infrared camera and thermocouples and 2) conducted experiments with pupae at different temperatures and measured their survival.

Methods

Larvae and pupae temperatures

We developed a modified ten-frame hive design to facilitate detailed measurements of brood temperatures. These hives were 58 cm x 28 cm x 68 cm, with an internal volume of approximately 40 L. These dimensions allowed for using ten standard Langstroth hive frames in each hive. A 15 cm diameter opening was cut and lined with aluminum sheeting on one side of the hives. Matching holes were cut into two frames, which allowed the internal opening of the "window" to view the surface of the third frame (Figure 1). Colonies functioned normally in these modified hives and reared their brood on the third frame. This hive design allowed us unrestricted access to the surface of a single brood frame. It also permitted the recording of brood temperatures without removing the frame, which changes thermal dynamics. The internal opening was plugged when the window was not in use, and the remaining space was filled with insulation.

We established six separate honey bee colonies in April of 2018 using 1.5 kg of worker bees and a mated queen sourced from a single honey bee package producer (Heitkam's Honey Bees, Orland, California). These colonies were mature by 15 June 2018, and measurements were taken from 10 July 2018 through 24 August 2018. Juveniles mature in 21 days, which allowed us to quantify two brood-rearing cycles from each brood frame. All colonies were monitored for diseases and pests throughout the experimental period. Of our six initial colonies, one contracted *Pestis americana larvae apium* (American foulbrood), and two did not raise juveniles in the viewing area, reducing our replication to three colonies.

Viewing areas were recorded with a Camkix® macro camera fitted with a ring light and a FLIR E60 thermal imaging camera. We used Camkix® macro camera images to identify the developmental stage (larva or pupa) of each cell's occupant, and the FLIR E60 provided the temperature of each cell. We collected temperatures from 450 larvae and 838 pupae among the three colonies.

After the recordings, we calibrated the thermal camera using a blackbody (CES100) with an emissivity of 0.97 ± 0.02. The camera temperatures were 0.87°C higher than identified with the blackbody calibration; thus, all temperatures were adjusted by this amount from the data collected from the window hives.

For pupae, the thermal camera could only record the wax cap's temperature as the pupa was beneath it. Therefore, we used a thermocouple (Barnant model # 600-1020) to measure the pupa's temperature under the cap and the wax cap of the same cell. To do this, we placed the wire junction of the thermocouple on the wax cap, then pressed the wire through the cap and contacted the pupa underneath. We recorded the temperature of the wax cap and the pupa of 92 cells in six colonies. We sampled cap and pupae temperatures for 38 cells from the initial three colonies set up for this experiment in August 2018. In July 2021, we collected temperatures from three new colonies established using the same protocol as in 2018 and sampled 54 more caps and pupae pairs in these colonies.

The thermometers used for this research were calibrated using boiling water. At the altitude of Missoula, Montana (978 m), water boils at 97°C. The FLIR and thermocouple recorded boiling water at 97.3 ± 0.2 and 96.6 ± 0.1°C, respectively. Individual cell temperatures were recorded using the camera's internal software preset to the emissivity of the honey bee (e = 0.97, Stabentheiner and Schmaranzer 1988).

The temperatures of whole brood nest areas were collected using the Nectar Technologies (Montreal, Canada) in-hive sensor. The sensor probe was placed in the brood nest and continually recorded temperature and humidity at 30-minute intervals. We used in-hive sensors in the three colonies we established in 2021 and collected data from July through August. We also placed an in-hive sensor into an empty hive to collect control data.

Temperature and brood survival

We conducted an experiment in which we raised pupae at the temperature measured for larvae and the temperature measured for pupae and compared survival. We used the temperatures collected from the FLIR E60 before the black body calculation was performed; thus, pupae survival was tested at 36°C and larvae at 37.2°C. These temperatures are 0.87°C warmer than our temperatures adjusted to the black body calibration.

We removed frames of capped pupae from the colony and placed them in temperature-controlled incubators. Pupae were used because they are not fed by nurse bees and can develop independently from the colony in the appropriate conditions. Larvae require feeding and would die if separated from nurse bees. However, if nurse bees remained in the experimental environment with larvae, they would counteract the temperature set by the incubators. We thus did not use larvae in this experiment.

Eight pupal brood frames (5 or 6 days after hatching) were selected from eight different colonies. A patch of capped cells was selected, counted, and placed in a five-frame hive and then in an incubator. We counted 875 pupae reared at pupal temperature and 1026 pupae raised at larval temperature. Pupal frames remained in incubators for 12 days, and then the number of emerged pupae was determined by counting empty cells from within the patch that was counted before the experiment. The empty cells represented the number of successfully emerged adult workers. These counts were used to assess survival percentage.

We used paired-sample t-tests and two-way ANOVA to determine differences between larval and pupal temperatures, cell capping temperatures, and pupal temperatures. To test pupal emergence at different temperatures, we used a paired samples t-test.

Results

Based on the infrared camera, the temperature at which honey bee colonies raised larvae, 36.38 ± 0.02°C, was substantially higher and within a narrower range than the 33-35°C reported for the brood nest area (Jones et al., 2005; Figure 2). The temperature at which honey bee colonies raised pupae was 35.18 ± 0.04°C, again higher than reported temperatures for the brood nest. There was no difference between the temperature of the capping and the pupa underneath (ANOVA, df = 5, 86; P=0.27). Notably, the temperatures we measured for larvae and pupae were different from each other (t-test, df = 1286, P<0.001, Figure 2).

We found that the mean brood nest temperature in other, but similar, experimental window hives measured with the Nectar Technologies in-hive sensor were 34.5 ± 0.01°C, with temperatures ranging from 32.9 to 36.2°C. For this comparison, the temperatures at which larvae and pupae were kept were higher than those for the brood nest areas. For a general comparison of how honey bee colonies maintain brood temperatures, in a hive of the exact dimensions without bees, the average temperature was 25.5 ± 0.28°C and ranged from 12.8 to 38.8°C (Figure 2).

When reared at the mean pupal temperatures that we measured (36.0°C), 76.4% of pupae emerged from cells as adults (Figure 3). In contrast, when pupae were raised at larval temperatures (37.2°C), only 34.7% of pupae emerged (Student's t-test = 3.997, df = 1, 12; P=0.002).

Discussion

Honey bee colonies appeared to keep larvae 1.9°C and pupae 0.7°C warmer than general brood nest temperatures. Larval and pupal temperatures were each strikingly constant. These results suggest a new perspective on how honey bees might thermoregulate their brood. Measurements of whole-brood nest temperatures suggest that honey bees raise their brood within a range of 33-35°C for all developmental stages of juveniles (Jones et al., 2005). Our results suggest, by contrast, that honey bees regulate temperatures of specific juvenile stages much more precisely and keep them warmer than other nest areas. However, the relative contribution of heater bee activity vs. larval respiration to these differences is not certain, see Chapter 3.

Our experimental results emphasize the importance of different incubation temperatures for larvae and pupae. When pupae were incubated near the lower temperatures measured for pupae, 36°C, 76.4% of pupae emerged as adults. When incubated at 37.2°C, closer to the temperatures measured from the larvae, only 34.7% of pupae emerged as adults. Compared to the results of Groh et al. (2004), our mortality rates for the 36°C treatment appear to be relatively high, perhaps because we eliminated all colony care, which could have affected the responses of pupae to temperature. Nevertheless, these rates indicate that the small but consistent differences in temperatures at which colonies maintain larvae may be important for juvenile development. Likewise, Groh et al. (2004) conducted experiments with honey bee pupae at temperatures from 29 to 37°C. They found that synaptic complexes in adult brains increased in number and density when bees were raised at 34.5°C and that both neural development and adult emergence declined at 37°C, as did ours.

Larvae lack these synaptic complexes, and this may allow them to tolerate a higher temperature, which may promote more rapid development. Perhaps the sensitivity of pupal synaptic complexes is connected to the nurse bees keeping them slightly cooler. Many hymenopterans have a faster developmental rate in warmer temperatures (Porter, 1988, Spivak et al., 1992, Mohamed et al., 2006, Medrzycki et al., 2010), and faster development would increase the number of adults available to care for the nest, add to nest structures, and to forage during the most productive season. However, if the temperatures at which pupae develop the fastest also negatively affect neural development, perhaps the larval or egg phase are the only age classes that can be heated high enough to maximize developmental rates.

The highly consistent and high temperatures likely reflect the activities of all nurse bees in the brood nest but with supplemental heating from heater bees. The average thoracic temperatures of non-heater bees in the brood area are between 35.8 and 37.5°C (Basile *et al*., 2008, S. Debnam, *unpublished data).* However, heater bees are both warmer and appear to incubate individual cells to meet developing juveniles' highly specific and constant temperature requirements (see Bujok et al. 2002). Those temperature requirements seem to be determined by whether the occupants are larva or pupa. These precise temperature differences can occur even when larval and pupal-aged juveniles occupy adjacent cells (Figure 4). The high thermal capacity of beeswax probably maintains these sharp temperature differences (0.51 KJ/kg, melting point = 64°C, Amin *et al*., 2017).

Other hymenopterans show similar benefits from increased rearing temperatures. The solitary parasitoid wasp *Nasonia vitripennis* develops faster with increasing temperatures. Increasing temperatures from 15 to 30°C reduced development time from 44 days to 11 days (Grassburger & Frank, 2003). In the social wasp *Polistes fuscatus*, which construct exposed nests, temperature had a stronger effect on colony size than even nutrient availability (Nadeau & Stamp, 2003). And in the closely related *Apis dorsata*, pupal emergence and developmental rates were highest at 34°C. Lower temperatures delayed pupae development and adult emergence (Mardan & Kevan, 2002). In experiments utilizing *Apis mellifera*, Petz *et al*. (2004) found that respiratory rates of developing larvae increased substantially with increasing temperature up to 38°C. Clearly, temperature plays an essential role in the developmental rates of this insect order.

Our findings indicate that instead of maintaining the entire brood nest area at 34.5°C, honey bee juveniles develop at extraordinarily precise, but different, temperatures for larvae and pupae within an *overall* brood nest area temperature of 34.5°C. The differences in temperature of these different developmental stages suggest that there is some benefit, either to the young or to the colony as a whole, to this behavior.

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Figure Legends

Figure 1: A) Modified hive design illustrating the "window" through which the brood frame was imaged. The "window" was plugged when observations were not being made. B) View into the colony when the plug was removed, showing that nurse bees are raising brood in the colony's center. The "window" permits *in situ* brood observations without frame removal or colony disturbance. Colonies function normally in this hive design and fully utilize the two frames the "window" passes through.

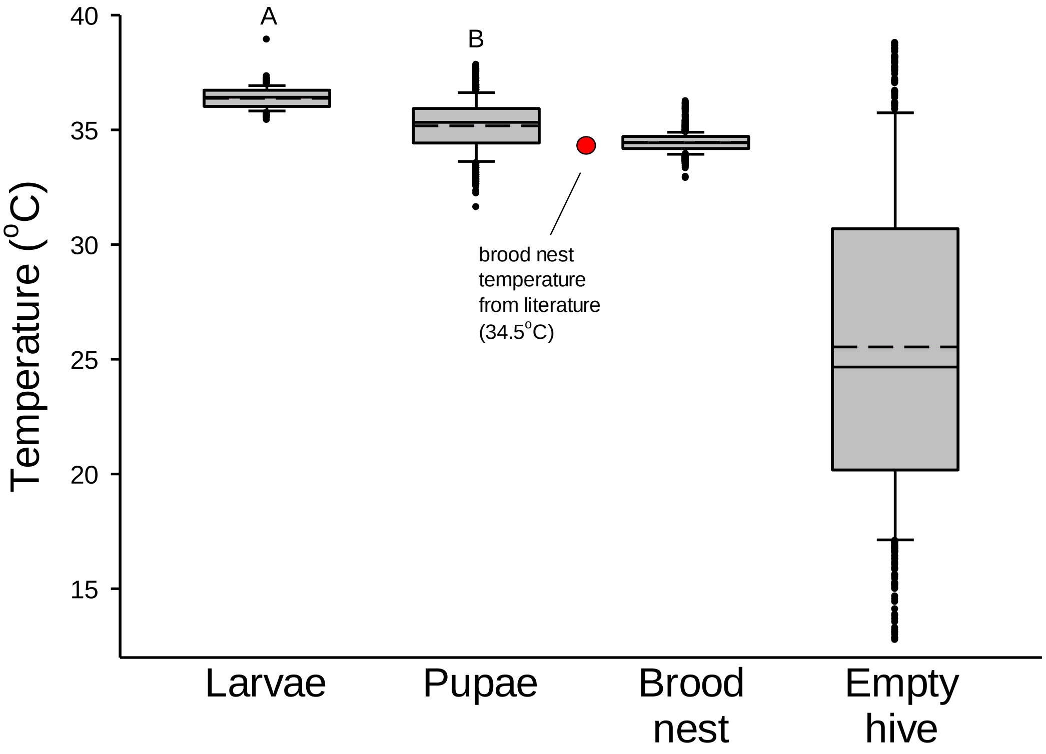
Figure 2: Temperatures of the larval and pupal stages, brood nest measurements from the window hives and unoccupied hives. Dashed lines represent the means, boxes represent data within the 1st and 3rd quartile, and whiskers represent minimum and maximum values. The red dot represents the mean brood nest temperature reported in the literature. Letters represent significant differences between larvae and pupae, but other statistical comparisons were not made because measurements were made at different times and with different hives.

Figure 3: Emergence percentage of pupae reared in incubators set to larval temperatures (37.3°C) and pupal temperatures (36°C). Dashed lines represent the means, the boxes represent the data that fall within the 1st and 3rd quartile, and the whiskers represent the minimum and maximum values. Dots outside the whiskers are outliers. The means are different between the adult emergence percentages (P=0.002). The pupae in our experiments had higher emergence rates when reared at 36°C than when reared at larval temperatures (37.3°C). Letters represent significant differences between pupal survival percentages.

Figure 4: This image illustrates the temperature differences between our colonies' cells with larvae and cells with pupae. Temperatures of individual cell types, or juvenile stages, are maintained despite proximity to other stages.

Figures

Figure 1



A
B
40
35
30
25
20
15
Temperature (°C)
brood nest temperature from literature (34.5°C)
Larvae
Pupae
Brood nest
Empty hive

Figure 2

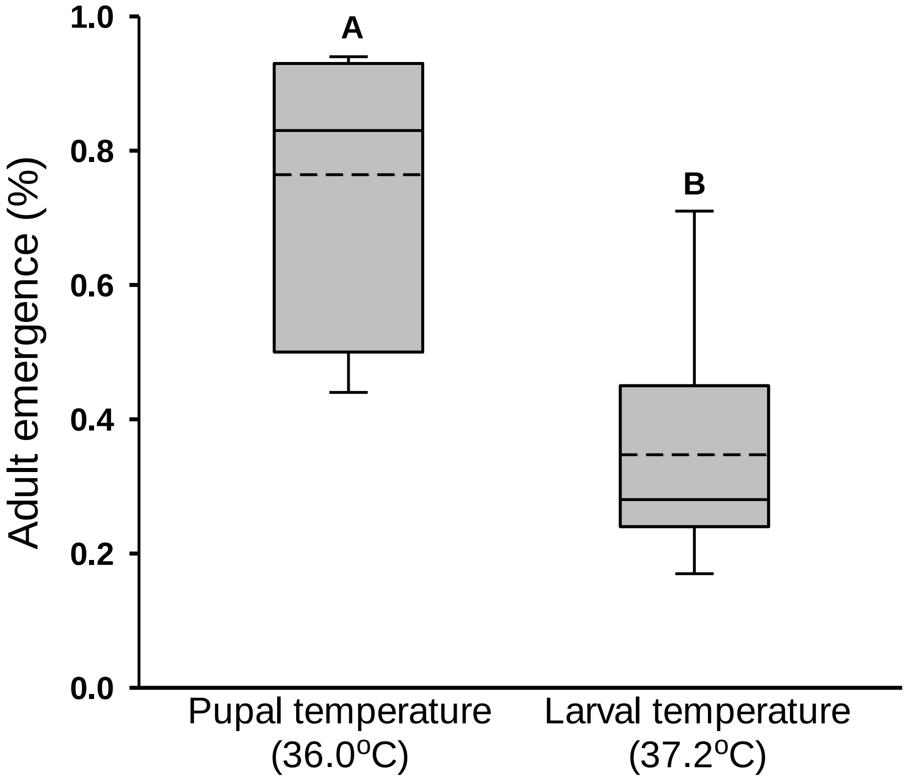

1.0
0.8
0.6
0.4
0.2
0.0
Adult emergence (%)
A
B
Pupal temperature (36.0°C)
Larval temperature (37.2°C)

Figure 3

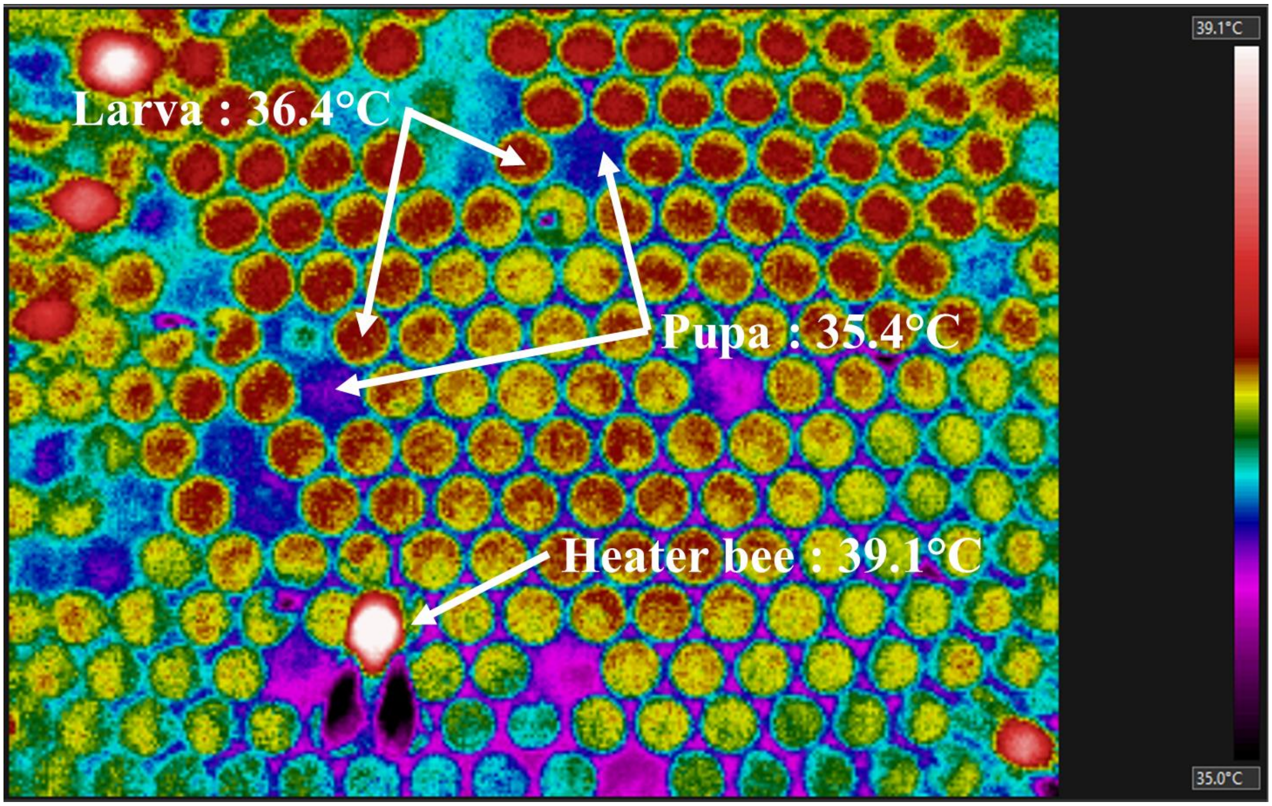


Figure 4

Chapter 3: Honey bee eggs, larvae, pupating juveniles, and pupae develop at slightly different temperatures

Abstract

Honey bees cope with temperature-dependent development by maintaining relatively high and constant temperatures in the brood nest, between 33-35°C. However, in Chapter 1, I found that honey bees maintain specific juvenile stages at even higher and more precise temperatures than previously known. In that experiment, honey bees raised larvae at 36.4 ± 0.02°C and pupae at 35.2 ± 0.03°C, a difference of 1.2°C. These remarkably hot, constant, and yet stage-specific temperatures may be controlled by heater bees which attend all stages of development over the 21 days of juvenile maturation. Yet honey bee development is far more complex than just the larval and pupal stages and can be further categorized into egg, larvae, pupating juveniles, and pupae. Might honey bees raise other stages of their young with stage-specific precision? We measured the temperatures of 150 brood cells daily through their 21-day maturation process in three specially designed observation hives. We found that eggs were maintained at 36.1 ± 0.03°C, larvae at 36.2 ± 0.02°C, pupating juveniles at 35.9 ± 0.03°C, and pupae at 35.8 ± 0.03°C. All stages were significantly different from all other stages, but importantly larvae were only 0.4°C different from pupae. We then conducted another experiment with brood frames without mature bees and in incubators at 34.5°C. Without nurse bees, the temperatures of eggs, larvae, and pupae were 34.4 ± 0.04°C, 34.7 ± 0.05°C, and 34.3 ± 0.04°C, with larvae different from all other stages, and a 0.3°C difference between larvae and pupae. When compared to the 1.2°C in Chapter 1, this 0.3°C difference suggests that heater bees may be a major driver of the differences between pupae and larvae. However, the 0.4°C difference between larvae and pupae in the second experiment reported in this chapter, vs. the 0.3°C difference, suggests that the larvae themselves may be the major contributor to the temperature difference between the life stages. Either way, our results suggest honey bee development may involve far more precise temperature during the development of juveniles than previously known.

Key Words: *Apis mellifera*, brood temperature, development, eggs, larvae, pupae, temperature regulation thermoregulation

Introduction

Honey bees (*Apis mellifera*) are one of Earth's most widely distributed species (Michener, 2000). This is due to a large degree to human introductions. Still, the ability of honey bees to persist and naturalize across almost all biomes (Hung et al., 2018) might be more due to their exceptional social behavior and organization that allows them to control their environments. A primary outcome of the social behavior of honey bees is the ability to control their nest cavity's internal environment to remarkably precise temperatures. The expansion of honey bees into temperate and cold environments likely depends on the thermoregulation of the nest.

Over much of their global distribution, honey bee colonies maintain consistent nest temperatures well above ambient air temperatures for much of the year. In temperate climates, where average winter temperatures can fluctuate between -17 and 7°C (Baker, 1994), nest temperatures are regulated to between 10 and 18°C (Owens, 1971; Southwick, 1987), and this variation is not correlated with ambient temperature (Owens 1971). Elevated nest temperatures during cold months demonstrate homeostatic feedback that maintains relatively constant temperatures over a year, whereas most solitary insects are not homeostatic and are dormant in cold months.

Within the nest, temperatures in brood areas are even more tightly regulated than in the hive as a whole. Juveniles develop without defects when raised at temperatures between 33 and 36°C (Heinrich, 2013; Seeley, 2014). Honey bee colonies use passive and active behaviors to maintain remarkably constant brood nest temperatures, from 33 to 35°C, across a wide range of ambient temperatures (Southwick & Heldmaier, 1987; Seeley, 1985; Jones et al., 2005). To accomplish this, bees use their flight muscles to generate the heat necessary to regulate nest cavity temperatures. Pupae raised in temperature regimes outside the 33-35°C norm die at higher rates, develop defective morphologies, and behave differently as adults (Himmer, 1927; Koeniger, 1978; Becher, 2010).

In honey bee colonies, a specific age class of bees care for the young. These "nurse" bees protect, feed, and maintain appropriate temperatures for the optimal development of juveniles (Seeley, 1981). A subgroup of nurse bees that are particularly active at maintaining brood temperature are called "heater bees." Heater bees can be readily distinguished from other nurse bees via thermal imaging because they superheat their thoraces to 42-46°C (S.E. Debnam, *unpublished data*; Kleinhenz, 2003). Through thermal imaging technology, the heating profiles of heater bees have been characterized in experimental hives with small populations (Bujok, 2002; Kleinhenz, 2003). These small-colony measurements are invaluable because they provide detailed descriptions of heater bee behaviors, but they may not show how brood nest areas may be cared for in whole, fully functional colonies when they are maintaining brood populations, working on nest structures, and foraging for resources.

Worker honey bee juveniles progress through three main stages as they develop over 21 days – egg, larva, and pupa. Development through these stages involves many complex morphological changes (Figure 1). First, eggs are laid into wax cells in the brood nest, and after three days, they become larvae. Cells with larval stage juveniles are not capped; only after larvae progress through five instars over five days are the cells capped with wax. For 24 to 37 hours after capping, the larvae spin within the cell as they wrap themselves in a silk cocoon (Myser, 1954). Then these prepupae become motionless as they progress through their final ecdysis resulting in a rudimentary adult body arrangement comprised of a head, mouthparts, antennae, thorax, legs, wings, and abdomen. These structures continue to develop in the pupae for another eight days, at which point a fully formed adult worker emerges (Rembold & Kremer, 1980). Considering this complex development of juvenile honey bees, might colonies show even more control over stage-specific temperatures and exceptional precision as has been found for larvae and pupae?

Our current understanding of brood nest temperature requirements and heater bee temperature profiles is substantial, but we lack a thorough understanding of accurate, small-scale *in situ* temperatures at which the different honey bee brood stages are maintained throughout the full course of their development. To our knowledge, variation in the temperature of individual brood stages throughout the 21 days of development has not been quantified. Here we provide the first in-depth temperature profile throughout the developmental progression of honey bee juveniles *in situ*.

Methods

We established three honey bee colonies in April 2021 from commercially produced honey bee packages. Each package was sourced from the same producer (Olivarez Honey Bees, Inc., Orland, California) and contained one mated queen and 1.5 kg of worker honey bees. These colonies matured by June 15, 2021, and measurements were taken from July 3 through August 12, 2021, when brood production in the Missoula area was at its peak. It takes 21 days for a honey bee worker to grow from egg to mature adult, which allowed us to quantify two brood-rearing events from each frame.

We developed a modified ten-frame hive design to facilitate detailed measurements of brood temperatures. These hives were 58 cm x 28 cm x 68 cm with an internal volume of approximately 40 L. A 15 cm diameter opening was cut and lined with 10 cm of aluminum sheeting on the bottom of one side of the hives. Matching holes were cut into two frames, allowing the internal opening of the "window" to view the surface of the third frame (Figure 2). Colonies functioned normally in these modified hives and reared their brood on the third frame. This hive design allowed us unrestricted access to the surface of a single brood frame and permitted the recording of brood temperatures without removing the frame, which changes thermal regulation. The internal opening was plugged when the window was not in use, and the remaining space was filled with insulation. To our knowledge, this is the first design that granted *in situ* observations of the thermal profile of the brood-rearing of honey bees.

We collected images of brood frames from the three colonies with a Camkix® macro camera and a FLIR E60 (MODEL # E64501) thermal imaging camera. The thermal camera was calibrated using a blackbody (CES100) with an emissivity of 0.97 ± 0.02. The camera temperatures were 0.87°C higher than identified with the blackbody calibration; thus, all temperatures were adjusted by this amount. Absolute temperatures were calculated via the FLIR thermal camera internal software with an emissivity value set to 0.97 (Stabentheiner, 1987). The Camkix® macro camera imaging process identified the brood developmental stage in each cell, and the thermal camera recorded the temperature of each cell. We collected temperature images of the 50 cells in each of the three colonies each day from the time eggs were laid in the cells until the entire cohort's emergence. Queens were allowed to lay eggs freely, resulting in asynchronous juvenile starting points. To ensure that we collected data from the full development of juveniles, measurements occurred over 40 days.

For developing pupae, which are located in a cell under a wax cap, the thermal camera could only record the temperature emitted through the cell cap. Because we did not know how the cap might change the temperatures we recorded, we used a thermocouple (Barnant model # 600-1020) to compare pupae temperature under the cap and the wax cap on the same cell. We first placed the wire junction of the thermocouple on cell caps, recorded cap temperatures, then pressed the wire through the cap, contacted the developing pupae underneath, and recorded the pupal temperature in 92 cells in six colonies. We sampled cap and pupae temperatures for 38 cells from three colonies in August 2018, and again in July 2021, we sampled an additional 54 cap and pupa pairs.

The temperatures of whole brood nest areas were collected using the Nectar Technologies (Montreal, Canada) in-hive sensor. The sensor probe was placed in the brood nest and continually recorded temperature and humidity at 30-minute intervals. We used in-hive sensors in the three colonies we established and collected data from July through August. We also placed an in-hive sensor into an empty hive of the same design to collect control data.

To separate the potential effects of nurse bees on differences in juvenile temperatures from the effects of the different juvenile stages themselves, we conducted a second experiment in which we placed brood frames in incubators without nurse bees. One frame of brood containing eggs, larval, and pupal juveniles were removed from each of the three different colonies. Nurse bees were removed from the frames, and each frame was surveyed to determine the position of eggs, larvae, and pupae on the frames. Each frame was placed into an incubator kept at a temperature of 34.5°C, the average brood nest temperature of our honey bee colonies. A single drawn wax frame was also included in the experiment as a control. The time frame over which we could measure temperatures was limited because larvae must be fed and could begin starving after two hours, affecting their metabolism and survival. Thus, these four frames remained in the incubator for two hours, and thermal images were recorded using the FLIR E60 camera every 30 minutes. After each recording, the order of the frames was randomly changed so that no single frame remained in the same position as during the previous thirty minutes. After two hours, the brood frames were returned to their hives, and 24 hours later, the frames were inspected to determine the condition of developing juveniles. None of the frames showed evidence of mass abortions, indicating that the juveniles in the frames survived the experiment. The images were then processed, and the temperatures for ten juveniles from each age class from each frame were determined using the FLIR Tools software. On the control frame, the temperature of 30 random cells was determined using the same software.

Data were analyzed using a linear mixed-effects model with temperature as the response variable; brood developmental stage (egg, larvae, and pupae) as a fixed factor, and the individual colonies as a random factor. Further effects of developmental stage and colony were analyzed using QQ plots and Shapiro-Wilk tests. This allowed us to determine the level of variance between the three levels of brood development and the different colonies over time. For the nurse bee removal experiments, because the data failed the Shapiro-Wilk test for normality, we used a Kruskal-Wallis one-way ANOVA on ranks with age class and colony as fixed factors to compare temperatures among the three juvenile stages.

Results

The four developmental stages of honey bee juveniles each had distinct temperature profiles (Figure 3). Eggs, larvae, pupation, and pupae stages were maintained at 36.1 ± 0.03, 36.2 ± 0.02, 36.0 ± 0.03, and 35.8 ± 0.03°C, respectively ($P < 0.001$ among all stages). The average of these juvenile stages was 36.0 ± 0.02°C. These temperatures were higher than the mean brood nest temperature measured with the Nectar Technologies in-hive sensor, which ranged from 32.9 to 36.2°C. The average temperature in a hive without bees was 25.5 ± 0.28°C, ranging from 12.8 to 38.8°C. Moreover, the mean temperatures on the different days of the experiment ranged from 23 to 34°C with an average of 30.9°C, suggesting that colonies maintain precise homeostasis for different juvenile stages regardless of ambient conditions.

When nurse bees were removed and frames kept at a constant temperature of 34.5°C, the temperatures of eggs, larvae, and pupae were 34.4 ± 0.04°C, 34.7 ± 0.05°C, and 34.3 ± 0.04°C respectively (Figure 4). The mean temperature of the larvae was higher than that of the other two stages ($P < 0.001$), whereas pupae and eggs did not differ ($P = 0.07$). The average temperature of the control frame was 34.4 ± 0.03°C, not different from eggs or pupae ($P = 0.96$, $P = 0.57$), but cooler than larvae ($P < 0.001$). At T_1, larvae temperature was 34.5 ± 0.05°C, which was equal to pupae temperatures ($P=0.13$). However, T_2 and T_3 larvae were warmer than pupae by 0.5°C ($P<0.001$). And by T_4, larvae temperatures had increased to 0.7°C above pupae temperatures ($P<0.001$).

Statistical details for juvenile stage and colony on temperature are as follows. Eight data points were extreme outliers and were removed. Normality was assessed with a QQ plot and Shapiro-Wilk test. The effect of colony on temperature at the different juvenile stages was statistically significant ($p<0.001$), and yet minimal variation is explained by the generalized eta-squared = 0.015 (see Lakens 2013). The effect of stage (day) on the temperature was very statistically significant ($p<6.53e-31$) with a large effect (eta-squared=0.114, F=10.77). When the stages are grouped into broader categories (egg, larvae, pupation, and pupae), stage explains more variation in the temperature (eta-squared=.240, F=54.54). Additionally, a pairwise comparison indicates that the four stages are significantly different from each other, with the most robust differences occurring between larvae and pupation ($p=6.69e-18$) and the least (but still significant) differences occurring between egg and larvae ($p=6.2e-2$), and pupation and pupae ($p=3.89e-5$).

Discussion

Our key finding was that all four stages of juvenile honey bees were at different temperatures throughout their development. All were substantially warmer than the highest temperature we measured for the brood nest in general. This suggests that honey bees might regulate their developing juveniles at higher and far more stage-specific temperatures than previously known. This is emphasized by the differences between our first and larger set of measurements (Chapter 1), showing that larvae were 1.2°C warmer than pupae. The mature bee removal experiment showed an average of 0.3°C difference between larvae and pupae. However, our second set of measurements (Figure 3) showing a 0.4°C difference between larvae and pupae with mature bees present and an average of 0.3°C with adult bees removed (also statistically significant) raises the possibility that the larvae themselves drive at least some of the temperature difference. In support of this, when mature bees were present in colonies, eggs were significantly warmer (0.2°C) than pupae. However, when mature bees were absent, the eggs and pupae temperatures did not differ significantly (0.1°C). This suggests that heater bees might account for the very small difference between eggs and pupae.

The incubator temperature was set at 34.5°C, and the temperatures measured for control frames (no juvenile or mature bees) was 34.4 ± 0.03°C. Thus, it appears that nurse and heater bees are responsible for at least part of the differences we report in Figure 1. Still, we cannot reject the possibility that the larvae generated part of the differences. The egg and pupal stages did not show any ability to generate heat as they equilibrated to the control frame's temperature within 30 minutes (T_1). They maintained temperatures similar to that of the control frame throughout the experiment, suggesting that they may rely solely on the heat provided by the nurse and heater bees.

There are several issues to consider about larvae-generated heat. First, our measurements in the nurse bee removal experiment ended after two hours because of concern over juvenile mortality and its effects on temperatures. Our measurements do not indicate decreasing temperatures over time from any of the juvenile stages; however, larvae temperatures equilibrated to the control frame at the T_1 recording and were equal to the pupae at that time. The larvae showed an increase in temperature as the experiment progressed, resulting in a 0.7°C difference between the larvae and the pupae by the end of the trial, again suggesting that larvae may have generated heat through increased activity and metabolic output.

A concern with this is that insect larvae are, in general, thought to generate exceptionally little heat (Cooley et al. 2016). However, there is a very large difference in body sizes between the beetle larvae examined by Cooley et al. (~10 g) and the honey bee larvae in our experiments (~ 75 mg). It may be that insect larvae would need to be larger than 10 g to self-heat, questioning whether the much smaller honey bee larvae in our experiment could produce the temperatures we found with nurse bees removed. Honey bee larvae may have much higher mass-specific metabolic rates, enough to heat themselves slightly. If honey bee larvae have a high mass-specific metabolic rate, and temperature of a larva is constant, then the rate of heat input from metabolism would have to be greater than the rate of heat loss via all other processes combined (evaporation, convection, conduction, infrared emission), resulting in the slight elevation in temperature that we found. However, both metabolic rates and degrees of heat loss in honey bee juveniles are yet to be determined.

Temperature drives the development rates of the vast majority of insect species, thus controlling the timing and abundance of insect populations (Ratte, 1984). Unlike most other species of Hymenoptera, honey bee juveniles do not depend on favorable moments in the abiotic environment for complete development, but instead, across most biomes on Earth, raise their young in 21 days due to environmental engineering by the adults. Maintaining brood nest temperatures at a very precise 33-35°C has been thought to be a crucial mechanism for this consistency. These general brood nest temperatures are undoubtedly necessary, but our results suggest that honey bees might achieve their temporal developmental precision through far greater stage-specific temperatures.

Recognizing the potential role of larvae-generated heat, honey bee adults accomplish their contribution to precise temperature regulation through the combined efforts of both nurse bees, who provide elevated brood nest area temperature within the nest cavity (Stabentheiner et al., 2010), and heater bees, who concentrate their efforts on heating individual juvenile cells through specialized behavior (Bujok et al., 2002; Kleinhenz et al., 2003). Heater bees select specific cells to either heat the capping, thereby warming the developing juvenile underneath, or they enter vacant cells within the brood nest, allowing the transfer of heat to surrounding cells and their juvenile occupants. These unique behaviors require that heater bees have some mechanism for determining the temperature of the juveniles they are heating.

Our results were obtained from three colonies between July and August when daily temperatures in the Missoula, MT area were, on average, 25.5°C, with range of 12.8-38.8°C. Thus, colonies must cool and warm brood. However, we sampled daily at 1400 hours resulting in a very narrow range between 23 and 34°C with an average of 30.9 ± 0.6°C. At these temperatures, the colonies in our experiments should have always been heating the brood. Importantly, we do not know how colonies heat different brood stages when faced with thermal challenges outside of this range.

We know of few other studies that link colony temperatures to development rates for Hymenoptera, but Riaño et al. (2014) found that temperatures inside *Bombus atratus* (bumblebee) colonies in Columbia were consistently higher than ambient, but ranged from 6.8 to 26.2°C. They also reported that the developmental time from oviposition to adult worker emergence was 29 ± 5 days, 14 ± 4 days for larvae, and 9 ± 4 days for pupae. For honey bees, the variance in the timing of total development, and for these stages, would be far less than one day.

An implication of the patterns we observe is that honey bees must be able to detect minuscule differences among brood cells in temperature and perceive the specific developmental stage in the cell and its requirement. Little is known about how honey bees sense temperatures, but electron microscopy indicates that their antennae have coelocapitular sensilla with thermosensitive neurons (Yokohari et al., 1982; Yokohari, 1983). Kohno et al. (2010) reported a transient receptor potential A (HsTRPA) channel (AmHsTRPA) on these sensilla, apparently honey bee or Hymenoptera-specific. Interestingly, heat activates this channel with a threshold temperature of 34°C. Ablation of antenna and injection of AmHsTRPA inhibitors reduces the ability of honey bees to respond to temperature. These mechanisms do not explain how honey bees might detect and control the temperatures of brood cells with the precision our data suggest, but they provide a direction for study. The channel reported by Kohno et al. (2010) also functioned as a chemo-sensor, and honey bees have profoundly sensitive abilities to detect a wide range of chemicals. If temperature sensitivity parallels this known chemical sensitivity, this may explain how honey bees maintain very precise temperature differences among developmental stages.

Another possibility is that nurse, and heater bees respond to temperature-related chemical cues from juveniles instead of to temperatures themselves. Many studies have illustrated the effect of brood-produced pheromones on adult bee behaviors (Alaux et al., 2009; Schultner et al., 2017; Maisonnasse et al., 2010; Pankiw & Page, 2001). For example, artificially altering brood pheromonal levels increased all aspects of brood production behaviors in both workers and queens, resulting in larger colony sizes (Sagili & Pankiw, 2016). Additionally, He et al. (2016) found that starving larva altered their pheromone output in a way that stimulated increased feeding from nurse bees. Perhaps juveniles can signal via pheromones that they are too warm or too cool, stimulating the appropriate responses from heater bees. If so, larvae must also be able detect miniscule differences in their body temperatures and then communicate this to adults. Regardless of the mechanism, honey bee juveniles appear to develop at remarkably stage-specific precise temperatures, much more precise than previously known.

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Figure Legends

Figure 1. Life stages of juvenile honey bees. **Abbreviations:** L1 – L5 = 1st through 5th larval instar; LS1 – LS2 = Larvae spinning cocoon sealed under capping; PP1 – PP2 = prepupa, final larval ecdysis (LS1 – PP2 = pupation); P = pupa (w = white eyes, p = pink eyes, d = dark eyes, dl = dark eyes, light cuticle pigment, dm = dark eyes, medium cuticle pigment, dd = dark eyes, dark cuticle pigment). Modified from Rembold and Kremer (1980).

Figure 2. A) Modified hive design illustrating the "window" through which the brood frame is imaged. The "window" is plugged when observations are not being made. B) View into the colony when the plug is removed, revealing nurse bees raising the brood in the colony's center. The "window" permits *in situ* brood observations without frame removal or colony disturbance. Colonies function normally in this hive design and fully utilize the two frames the "window" passes through.

Figure 3. Temperatures of developing honey bee juveniles. Eggs (grey circles) are maintained at 36.1 ± 0.03°C across the three-day period, larvae (black squares) are maintained at 36.2 ± 0.02°C across the five-day periods, pupating juveniles (white diamonds) are maintained at 36 ± 0.03°C across the three-day period, and pupae (gray triangles) are maintained at 35.8 ± 0.03°C across the eight-day period. These four means are different ($P < 0.001$). The dashed line shows the average temperature we recorded for the whole brood nest.

Figure 4. Temperature of developing honey bee juveniles with nurse bees removed from the frames. When placed in an incubator for two hours with a temperature of 34.5°C, egg temperatures were 34.4 ± 0.04°C, larvae temperatures were 34.7 ± 0.05°C, and pupae temperatures were 34.3 ± 0.04°C. The larvae temperature was different from the eggs and pupae ($P < 0.001$), but the eggs and pupae were not different ($P = 0.07$). Each box is drawn from the

first through the third quartile. The line represents the median temperature. The whiskers represent the minimum and maximum temperatures recorded for each juvenile stage. The dots are outliers, and the letters denote significant differences.

Figures

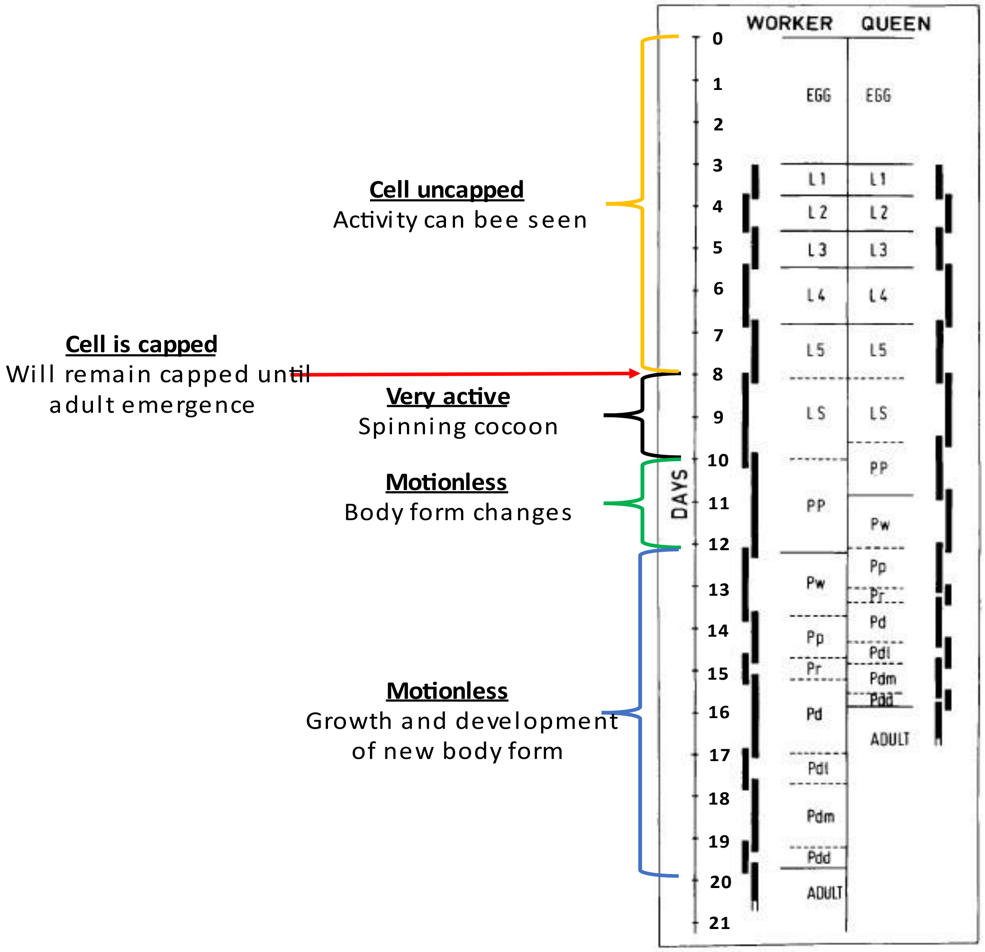

Figure 1

Figure 2

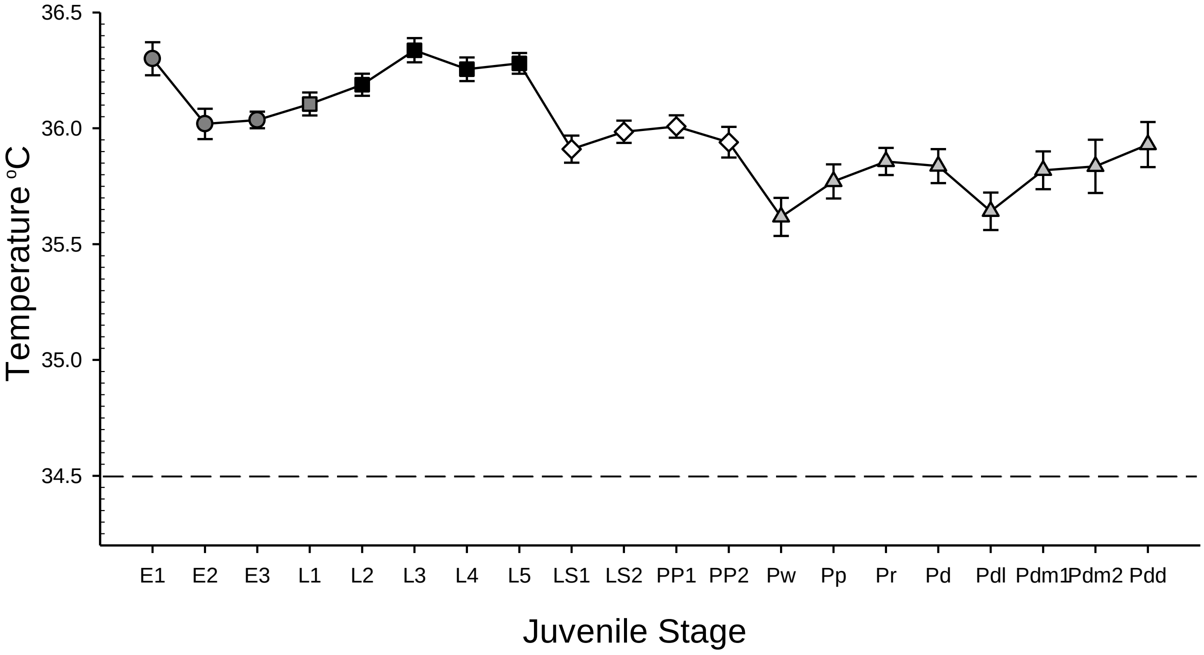
36.5
36.0
35.5
35.0
34.5
Temperature °C
E1
E2
E3
L1
L2
L3
L4
L5
LS1
LS2
PP1
PP2
Pw
Pp
Pr
Pd
Pdl
Pdm1
Pdm2
Pdd
Juvenile Stage

Figure 3

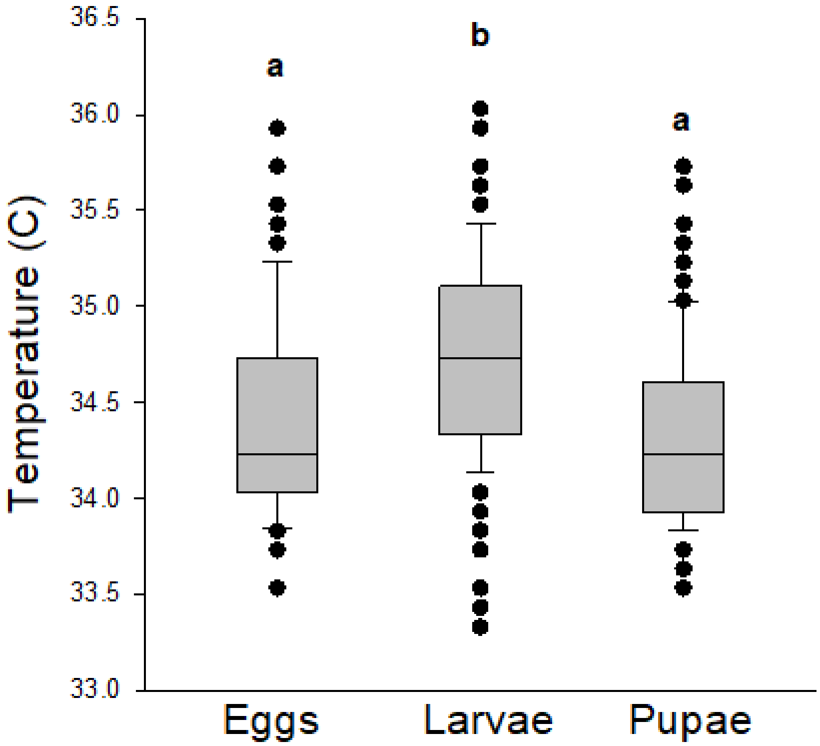
36.5
36.0
35.5
35.0
34.5
34.0
33.5
33.0
Temperature (C)
a
b
a
Eggs
Larvae
Pupae

Figure 4

Chapter 4: Energetic costs of raising brood in honey bee colonies are high, but heater bees are cheap

Abstract

Little is known about the energetic costs to insects of raising young. Honey bees collectively raise young, or brood, through a series of complex behaviors that appear to accelerate and synchronize the timing of brood maturation. These include maintaining the brood nest at warmer and consistent temperatures (33-36°C) and the exceptional activity of heater bees. Heater bees are among the larger group of nurse bees that care for brood by rapidly contracting thoracic muscles to generate high body temperatures, from 42 to 47 °C. Heater bees move among brood cells and display this behavior to regulate the temperatures of individual larvae and pupae. To explore the energetic costs of raising brood in general, and the cost of heater bees specifically, we constructed three sets of experimental hives. One set was designed to estimate the numerical allocation of individuals to the heater bee task. The second set was designed to contain only brood, which eliminated foraging and allowed us to quantify stored honey use when rearing juveniles at 10 and 30°C. The final set was used to measure the respiration rates and energy expenditure of individual bees displaying resting, walking, heating, and agitated behavior. By integrating honey used by brood-only experimental colonies with whole-colony measurements of honey storage in the literature, we estimated that raising brood costs colonies half of their annual energy budgets stored as honey, or approximately 43.7 ± 0.9 $kg \cdot yr^{-1}$. We estimated that roughly 2% of colony individuals perform the task of heater bee. Respiration rates of heater bees (19 mW) were more than those of resting bees (8 mW), but similar to those of walking bees (20 mW) and about half of those that were agitated (46 mW). The energetic cost of heating was more than an order of magnitude lower than reported values for the energetic cost of flying. By integrating data from our three experimental hives, we estimate that the annual cost of raising brood is quite high, however, we estimate that heater bee behavior and physiology, though extreme, may require only about 7% of the annual honey stored by a colony.

Key Words: *Apis mellifera,* brood care, division of labor**,** honey use, insect physiology, metabolism, temperature

Introduction

Temperature has a profound effect on the metabolism, developmental rates, fecundity, and survival of insects (Huey & Kingsolver 1989, Angilletta & Angilletta 2009). Most insects regulate their temperatures via individual behavior and physiology, but social species can collectively regulate the temperature of individuals and colonies via highly complex behaviors (Hölldobler & Wilson 2009). Such collective regulation can provide good conditions for the maturation of brood and in particular the rate at which young develop (Hölldobler & Wilson 2009). By controlling the timing and rate of brood development, social species can allocate resources in adaptive ways as conditions change seasonally and thereby reproduce (by fission) at the appropriate time. For example, *Polistes* wasps preferentially nest in microhabitats with warmer temperatures. This preference leads to the earlier production of workers, which enhances both colony initiation and reproductive output later in the year (Jeanne & Morgan 1992). Many social ant species construct mounds that act as solar collectors. They translocate developing brood into warmer parts of the mound areas to provide conditions that support rapid development. This increases the growth of individual colonies and contributes to reproductive swarming (Roces & Nunez 1995, Bollazzi & Roces 2002, Kadochova & Frouz 2014).

In the context of collective behavior, many social species display a striking division of labor in which different individuals, at least during periods of development, are allocated to tasks such as resource acquisition and storage, reproduction, care of developing offspring, or control of the nest environment (Hölldobler & Wilson 2009), such as described above for temperature. Division of labor in all societies is expected to produce more efficient resource use and greater productivity, a fundamental economic tenant (Romer 1990). This is also proposed for social insects (Breshers & Fewell 2001). Most social insects do not technically divide labor among specialized individuals but instead participate in polyethism, in which an individual's lifespan is divided into temporal periods of different tasks (Gordon 2016). Flexibility among tasks in insect societies goes beyond age polyethism, as the behavioral tasks of individuals change depending on external conditions and the requirements of the colony (Gordon 2016). Regardless, understanding the benefits and costs of temporally discrete divisions of labor requires careful evaluation of the specialized labor categories.

Task specialization and colony control of temperatures often converge in the care of developing brood. Bees and wasps invest considerable time and energy into brood warming (Heinrich 1977, Makino & Yamane 1980, Fahrenholz et al. 1989). Adults produce metabolic heat at high rates, through the activation of thoracic flight muscles and honey bees then deliver that heat to larvae and pupae by positioning themselves on brood cells or by entering empty cells in the brood area. This heat is also preserved through adult clustering behavior. The metabolic heat is then transferred to the brood. Honey bees (*Apis mellifera*) not only maintain optimal whole-colony temperatures during seasons with suboptimal ambient temperatures, but they also maintain precise temperatures for the different brood stages through the labor-dividing behavior of heater bees (Bujok et al. 2002; Chapters 2 & 3). Heater bees contract thoracic flight muscles to generate temperatures of 42-47°C and place their superheated thoraxes near developing larvae and pupae in cells to transfers heat to the juveniles.

In addition to such specialized behavior, honey bees make an exceptional effort to maintain the temperature of whole colonies and brood nests within narrow ranges, regardless of ambient temperatures (Southwick & Heldmaier 1987). For example, the average temperature of the entire brood nest is between 33 and 36°C across the global distribution of the species, and this is thought to provide ideal conditions for brood development (summarized by Seeley 1985).

Also, heater bees move throughout the brood nest area and maintain highly consistent temperatures for developing individuals within cells (Bujok et al. 2002). In hives constructed to allow precise measurements of individual developing brood, we found that pupae were maintained at 36.0 ± 0.03°C and larvae at 37.1 ± 0.02°C, over a range of ambient temperatures (S. Debnam, *unpublished data*). This remarkable precision, both warmer and less variable than the average temperature of the brood area as a whole (Chapter 2), is due to the activity of heater bees. Currently, it is not known what percentage of the colony is necessary to maintain precise brood temperatures, nor do we know the metabolic effort exerted by heater bees during brood warming. Also, the energy required to raise brood in general is not known.

Strong thermoregulation occurs despite substantial resource limitation. Despite, or maybe because of, the effort invested into maintaining optimal temperature ranges, between 77 and 88% of wild colonies perish from starvation during their first winter as they deplete their stores of honey (Seeley 1985). In a survey of nearly 20% of the 2.4 million managed colonies in the U.S., overwinter starvation was the second most prominent cause of colony mortality (VanEngelsdorp et al. 2008), greater than that from diseases and parasites. These results indicate that understanding the precise energetic costs of heater bees should provide insight into the overall energetics of honey bee colonies. Furthermore, understanding overall colony energetics is equally as crucial as honey governs, to a large extent, both overwinter survival and spring reproductive swarming.

The average managed colony in the United States produces about 100 kg of honey annually, requiring a colony to conduct about 4 million flights at an average distance of 3 km at a cost of 4.6 joules/km (Southwick and Pimentel 1981). Brood rearing may be as costly as foraging, but little is known about the impact of brood rearing on total colony honey resources, and thus its general effect on colony energetics. Neither do we know if the highly specialized thermoregulatory behaviors of heater bees are an important part of the total cost of brood rearing. These fundamental gaps in our understanding of colony economics hinders our ability to evaluate colony energy and resource budgets. Here, we (1) determine colony-level honey consumption when colonies are experimentally manipulated to only contain brood, and 2) quantify metabolic costs of heater bee activity.

Methods

We used a three-pronged approach to evaluate the honey cost and energetic expenditure required to raise larvae and pupae. First, we measured the rate of honey consumption in experimental brood-only colonies. A colonies' annual honey requirement may be as high as 111 kg (Seeley and Visscher 1985 and Seeley 2009); we aimed to establish how much of this annual budget is expended on raising brood. Second, we measured the proportion of individual bees that colonies allocate to heater bee activity. Third, we measured the rate of energy expenditure of heater bees during heating and compared this expenditure to that of other activities. This combination of approaches allowed us to explore how bee microeconomics behaviors, expenditures of individuals) scale up into colony macroeconomics (colony energetics).

Colony Honey Consumption

To quantify the energy required to raise brood, we removed foragers, queens, and drones from eighteen colonies, leaving a single brood frame in each with only its nurse bee population to provide care. Visually, these nurse bees continued to perform typical brood care behaviors during the experiments. All colonies with larval brood attempted to rear queens in specialized cells, which is the response to the absence of queens. Colonies were housed in modified nucleus hives that prevented the colony members from foraging - they could not add resources to the experimental hives. The eighteen colonies were divided into subgroups. Seven of these experimental colonies contained larvae and were placed in incubators with external ambient temperatures maintained at 30°C and four other larval colonies had external temperatures maintained at 10°C. Three pupal colonies were placed in incubators with external temperatures maintained at 30°C and four other pupal colonies where at maintained at external temperatures of 10°C. Sample sizes were not equal due to power failure at the facilities which eliminated five experimental colonies.

Ten and 30°C were selected because those represent approximately the spring and summer mean air temperatures of the contiguous United States (NOAA; 2018) and allowed for measurements of honey consumption at low and high ambient temperatures. Colony level honey consumption during the larval stage was measured until 48 hours after cells were capped at which time larvae had completed cocoons and experienced their final larval ecdysis into the pupal stage (Rembold et al. 1980). We maintained colony-level honey consumption experiments during the pupal stage until capped pupae had emerged as adults, which took between twelve and fifteen days.

Colonies in modified hives exchanged oxygen and carbon dioxide through a 3-cm diameter aperture covered with a hardware screen with a 0.32 cm mesh. All hive components, including the frames of honey, and the brood frames, were weighed before being added to the colony. To determine the precise ratio of honey consumed to the number of brood raised, we counted the number of brood successfully raised to the next age class. Frames with pupae were surveyed using a Plexiglas panel cut to the dimensions of a Langstroth hive frame. A grid divided into 2.54 x 2.54 cm squares was placed over the panel. Setting the gridded panel over brood frames allowed a survey of the area of each frame that contained brood. An average of 5.5 brood cells was contained in a square. Counting the number of all capped cells at the beginning of the experiment and subtracting the number of remaining capped brood cells after allowed us to determine successful pupal emergence. For the experiments designed to quantify colony level honey consumption during the larval stage, we surveyed the frames to generate the number of capped cells representing the transition to the pupal stage after eight days.

We also weighed all hive components after brood were successfully reared to the next age class. By subtracting initial from post-trial weights, we determined the amount of honey consumed during the experimental period. Dividing honey consumption values by the number of individual juveniles reared, generated a per-individual cost of raising brood, for each of the two age classes. Honey, as measured in this experiment, included that used as food by nurse bees and the subsequent feeding of juveniles by nurse bees, which is mostly pollen. Experimental colonies did not add wax combs to the frames or hives.

Heater Bee Allotment

Six colonies were housed in observation hives at the Fort Missoula research facility in Missoula, Montana, U.S.A, in an insulated and temperature-controlled mobile laboratory (temperature 25±2.4°C). This temperature required continuous brood warming by the colony. Bees were allowed to forage freely throughout the experiment in herbaceous communities outside the mobile lab by exiting through a plastic tube. Each colony was composed of a single brood frame (~ 220 cm^2 of visible brood area) and a honey frame (~3-4 kg). Colony populations were equalized as much as possible with similar numbers of attendant nurse bees, foragers, drones, and a queen. The glass from the observation panel was replaced with transparent plastic film to enable thermographic recording (Supplementary Figure 1). This film did not block thermal radiation. We measured objects ranging from 30-35°C with the camera, and temperature readings were identical with and without the film.

A FLIR E60 (model number E64501) thermal imaging camera was placed 45 cm from the brood frame, and thermal video recordings were collected for one hour once per day starting at 1400 hours. Colony 1 contained a mix of larvae and pupae, colony 2 had only pupae, and colony 6 contained only larvae. For colonies 3, 4, and 5, a video was recorded for six days to capture the transition from larval to a mixture of larvae and pupae, and finally only pupae.

Individual bee thoracic temperatures were recorded using the camera's internal software preset to the emissivity of the honey bee's thorax ($e = 0.97$, Stabentheiner & Schmaranzer 1988). Temperatures were encoded to a color scale on the video recordings such that the heater bee's thorax appeared white on the scale (mean heater bee thoracic temperature was 42.2 ± 1.3°C). The heater bees were distinguished on the videos via these superheated thoraxes. Each one-hour video was viewed, and the number of heater bees was counted at each minute. This generated 60 individual counts from each recording. These data were used with the literature (McLellan 1978, Seeley 1985) providing the total numbers of bees in whole colonies to estimate the proportion of the colony’s population allocated to the heater bee task.

The total brood area and the brood stage were evaluated using the gridded Plexiglas panel before each recording. The number of heater bees and total brood area were used to determine the number of heater bees per cm^2.

Heater Bee Energy Expenditure

Heater bees did not exhibit heating activities when removed from the brood frame. To measure their metabolic rates, therefore, we modified a 10-frame Langstroth hive body to view colony activity and to measure metabolism. A window was cut into one side of the hive and covered with transparent plastic to prevent the bees from escaping and to allow us to view them during metabolic measurements. We also cut a 10-cm hole in the side of the hive through which we placed a gas exchange chamber (described below) over active heater bees while they were on brood frames. This hole was sealed with black plastic when experiments were not being performed and covered with a flexible black sheet that allowed experimenter access but prevented bee escape during experiments. This hive was filled with a single frame of honey (~1-2 kg) and a single frame of brood with attendant nurse bees. Colonies were allowed to forage via a plastic tube to the outside throughout the experiments. Finally, we designed small metabolic chambers to measure gas exchange of heater bees functioning within our modified Langstroth hive (described above). We placed the metabolic chambers over individual nurse bees as they actively warmed the brood in place on brood frames during August and September 2020.

The metabolic chamber was constructed from a 25-mm diameter Plexiglas tube, open on one end equipped with a 12 mm rubber gasket (Figure 1). The chamber had a total volume of 27 ml, and was fitted with two lines, one for gas inflow and one for recovery. CO_2-free air was flooded into the chamber at 0.98 liters per minute. Once chambers were sealed over bees, we waited for the chamber to equilibrate to ambient CO_2 levels before measuring metabolic rates. The response time of this system was, on average, 17.2 seconds. CO_2 in the outflow (was measured using an infrared gas analyzer (Licor LI7000) plumbed in dual-channel mode. The analyzer was calibrated regularly with zero and span gases (pure N_2 and 2000 ppm CO_2 in N_2, respectively). We controlled for background CO_2 signals by placing the chamber over cells containing empty wax but without nurse bees. Corrected CO_2 production rates were calculated as CO_2 levels from active heater bees minus CO_2 levels from empty cells and then converted into rates of energy use (milliwatts).

Heater bees display two distinct behaviors when heating brood. First, they warm the brood from above by resting their heated thoraxes on the caps of brood cells, and second, they warm the brood by entering adjacent cells while thoraxes are heated (Kleinhenz et al. 2003). When the metabolic chamber was placed over heater bees on top of the cells, they stopped heating behavior and immediately became agitated and tried to escape. However, heater bees did not react to chambers being placed over them when they were heating the brood within adjacent cells. Thus, we collected heating bee metabolic rates from heater bees servicing pupae from empty cells adjacent to the pupae. Heater bees were identified for these samples by their position in the cells and pulsating abdomens as described by Kleinhenz et al. (2003).

We measured the respiration rates of 40 heater bees and selected another 75 bees to measure resting rates to compare to heater bee respiration. Five individual bees were removed from each of 15 different colonies from August through September 2021. These bees were stored in 15 ml centrifuge tubes until respiration was measured. Our goal for these 75 bees was to measure resting metabolism, but these bees displayed three distinct behaviors: walking, resting, and agitated. Bees were left in the centrifuge tubes for 40 minutes, after which bees were placed into metabolic chambers on the benchtop along with a single drop of honey. Walking was classified as movement using legs but without wing movements. Agitated behavior was defined as combined walking, rapid wing movement, and flight within the chamber. Bees were identified as resting once they discovered the honey and remained motionless while feeding. All bees became agitated when trapped in chambers *in the hives,* therefore resting, walking and agitated behaviors had to be measured on a benchtop.

Data Analysis

Due to high variability and unequal sample sizes across treatments, we analyzed honey use and proportions of heater bees using Kruskal-Wallis tests. We performed further pairwise comparisons between the groups utilizing the Mann-Whitney U test. We used ANOVA to determine the effects of mobile lab temperature and brood development on the number of heater bees as well as for pairwise comparisons of the different activities.

Results

Colony Honey Consumption

The amount of honey consumed by experimental colonies did not differ between brood stages or ambient temperature treatments (P=0.65; Figure 2). Considered together, colonies consumed an average of 0.4±0.3 g of honey to raise an individual from the larval to the pupal stage, and another 0.5±0.4 g from the pupal stage to emergent adult. Colonies with pupae at 10°C tended to consume more honey than colonies with pupae at 30°C, as might be predicted, but there was a great deal of variation suggesting that substantially larger sample sizes would be needed for a more accurate understanding of costs. The colonies in our experiment consumed roughly 0.9±0.1 g of honey to raise individual brood from the larval to the emerged adult stage, thus, we estimate that the cost of producing approximately 1000 bees is 1 kg of honey.

Heater bee Allotment

Colonies that were used to determine heater bee allocation were maintained (inside the mobile lab) at an average temperature of 23.2 ± 1.5°C. The average ambient temperature (outside the lab) was 27.7°C with a maximum of 33.4°C and a minimum of 19.4°C. Across all colonies and brood stages, there was an average of 28.2 ± 0.2 active heater bees and an average

222.8 ± 1.6 cm^2 of observable brood area per colony, resulting in a density of 0.12 heater bees per cm^2 of brood area.

The average number of active heater bees increased from 25.6 ± 0.4 heater bees on larvae to 27 ± 0.4 heater bees on mixed brood, and 30.1±0.4 heater bees on pupae ($P<0.001$). In experimental colonies, brood areas averaged 241.9 ± 2.9, 205.1 ± 2.1, and 216.2 ± 2.5 for larvae, mixed brood, and pupae stages, respectively. Larval brood area differed from mixed brood and pupal brood area in pairwise comparisons (all $P<0.001$). There was no difference between mixed brood and pupal brood area ($P=0.384$). This resulted in heater bee to brood area ratios of 0.099, 0.127, and 0.132 bees per cm^2 for larvae, mixed brood, and pupae, respectively (all stages differed; $P<0.001$, Figure 5). These numbers were affected by colony and mobile lab temperature ($P< 0.001$). As the temperature in the lab increased 1°C, the number of heater bees per cm^2 decreased by 0.34±0.15 ($R=-0.60$, $P=0.025$).

Heater Bee Respiration Rates

Honey bees displayed different metabolic outputs depending on their activity. When resting, walking, heating or displaying agitation, honey bees generated 7.7 ± 2.2, 23.5 ± 5.2 18.6 ± 5.5, and 42.5 ± 10.3 mW respectively (Figure 3). These were different for all pairwise comparisons ($P<0.001$) except for walking vs. heating which was not different ($P=0.18$).

Discussion

Our results indicate that raising brood is costly, consuming perhaps half of a colony's annual honey budget. Using colony numbers from Kostarelou-Damianidou et al. (1995; Supplementary Table 1) and our per brood honey consumption values, we estimate that the annual cost of raising juveniles may be as high as 43.7 ± 0.9 kg honey (Supplementary Table 1).

The total honey consumption of a colony may be between 85 and 111 kg·yr^{-1} (Seeley 1985, Southwick and Pimentel 1980), thus rearing brood might cost a colony half of this total. Numbers from Kostarelou-Damianidou et al. (1995) are within the ranges reported by others (Nolan 1925, Jay 1974, Avitabile 1977, McLellan 1978, Page 1981).

The highly specialized behavior of heater bees, however, did not contribute much to these high costs, based either on the respiration rates of individuals or on estimates of stored honey consumption by heater bees. Colony brood area and adult populations fluctuate throughout the year but are largest in the summer. Brood area can range between 1200 to 4500 cm², and adult populations range from 15,000 and 35,000 individuals. Using the average estimated brood area and adult population sizes (see Nolan 1925, Jay 1974, Avitabile 1977, McLellan 1978, Page 1981, Seeley 1985, Kostarelou-Damiandou et al. 1995), we estimate that in most colonies 0.5 – 3.5% of adults function as heater bees. Early spring allocation would be the highest (roughly 3.5%) as the smallest number of adults supports the largest annual brood areas. Midsummer colonies, which have the largest populations, have roughly the same brood area (~4500 cm²) as in the spring, but may only allocate 1.7% of their population as heater bees. The metabolic rates of heater bees were roughly two times higher than those of resting bees, no different than walking bees, and less than "agitated" bees. And based on other studies of the respiration rates of flying bees (Suarez 2000, Woods et al. 2005), heater bee metabolic rates are about 30x lower than those of flying bees. This large difference may be linked to the metabolic requirements of mechanical flight. The high metabolic investment necessary to maintain lift is not needed for non-mobile thoracic shivering and heating. In other words, of the total energy dissipated in metabolism, flying bees convert more of it into mechanical energy whereas heater bees turn it more directly into heat. These estimates suggest that heater bees accounted for energy expenditure roughly equivalent to 15% (~7 kg) of the stored honey consumed during brood rearing (43.7 kg·yr^{-1}) or about 7% of the total annual honey budget of a colony (85-111 kg·yr^{-1}), a relatively small cost for a significant behavioral and physiological adaptation.

The low cost of heater bees, relative to the overall cost of raising brood, suggests that the lower, and very precise, temperature at which pupae are raised (36.0 ± 0.02°C), relative to larvae (37.1 ± 0.03°C; Chapter 2) is not for energetic savings. These temperatures are higher than the 33-36°C internal temperature of brood nests measured by others, and the 33 to 36.3°C internal temperature of brood nests measured in our experimental hives. The precision of these higher temperature differences suggests a developmental advantage of keeping pupae and larvae warmer than the ambient brood temperature, and larvae particularly warm.

There are several important caveats for our estimates. First, the respiration rates of heater bees were measured, by necessity, using bees that were active within the brood area, whereas resting, walking and agitated bees were measured on a countertop. If the wax substrate on which heater bee respiration was measured resulted in less O_2 flow out of the cuvette than the countertop, then we would have underestimated the energetic costs of heater bees. However, we measured O_2 flow rates in empty chambers on wax and countertops prior to every measurement and subtracted the rates from the following measurement with a bee in the chamber. The rates on the wax did not different from the rates on the countertop (P=0.71). Second, our counts of heater bees were almost certainly undercounts because heater bees could operate within open cells and could be missed in the tallies. However, Bujok et al. (2002) found that most heater bees remained on the surface of the brood and Kleinhenz et al. (2002) even the individual bees that heated inside cells were on the surface of the combs much more than in the cells. Thus, our counts are likely to be reasonable.

Honey bee colonies consume 0.9 g of honey to raise an individual bee from larva to adult. This occurs over eighteen days resulting in a daily honey consumption rate of approximately .05 g of honey per reared bee. During periods when colonies support many juveniles, upwards of 15,000 during the spring, colonies consume roughly 14 kg of honey. Perhaps mitigating this cost, colonies maintain low number of juveniles throughout most of the year (Seeley and Visscher 1985). Colonies support fewer than 200 juveniles from mid-October through mid-January in temperate areas at a total cost of about 0.2 kg. Late January through mid-March, brood levels begin to increase to around 1000 cells at a total cost of about 0.9 kg, (Avitabile 1978), after which time juvenile counts continue to grow until they reach their peak in May and June, when colonies may support as many as 25,000 juveniles at a total cost estimated at 9.0 kg per month (Nolan 1925, Page 1981). Brood numbers drop off after July, with most colonies maintaining only about 600 juveniles by September at a total cost of about 0.6 kg of honey. In temperate regions, this brood rearing dynamic matches environmental resource availability.

A number of factors could increase or decrease our estimates of honey consumption. For example, Harbo (1993) estimated that 0.2 g of honey was required to rear one a bee to the pupal stage, and again data from Kostarelou-Damianidou et al. (1995) provides an estimate of 7.6 $\text{kg}\cdot\text{yr}^{-1}$ honey to raise brood. This is equivalent to 24,300 kcal, which seems low since commercial bee keepers provide roughly 1,800 kcal of supplemental energy to their hives *per day*, in the form of sugar syrup, to maintain the survival of their colonies during early spring brood rearing (S.E. Debnam, *personal observations*). Furthermore, Harbo (1993) measured honey consumption from the egg stage to an undefined point in the capped pupal stage, but not to adult emergence. We measured honey consumption from the larval stage to emergence but did not include the three-day egg stage. If maintaining egg stage temperatures consumes as much honey as other stages, our estimates of annual honey costs to raise brood would increase to 53 $kg \cdot yr^{-1}$.

We did not consider energy in the pollen brought to the colony by foragers, thus underestimating the total resources of a colony. Our experimental design, which separated nurse bees from the rest of the colony, eliminated heat that the rest of the colony members may have provided, which could lead to an overestimate of honey used. We also separated the brood frames from the rest of the hive, which may have eliminated insulation provided by a complete nest structure. Also, our experiments prevented bees from access to the outside, and efforts to escape may have increased activity levels and resource consumption.

Surprisingly, juveniles in the pupal phase were nursed by 0.13 heater bees per cm^2 in the brood area vs. 0.09 bees per cm^2 for larvae, significantly different. This occurred even though pupae were very consistently kept a degree cooler than larvae (S. Debnam, *unpublished data*). Perhaps colonies invest in more heater bees to prioritize pupae over larvae because they have already invested a great deal of energy into pupae and maintaining very precise temperatures is crucial. Schmickl & Crailsheim (2001) found that during periods of low resource availability, nurse bees cannibalized larvae and eggs instead of pupae, preserving the oldest brood stage in which cumulative investment has been high.

Ambient temperatures might affect the number of heater bees required to maintain larval and pupae temperatures. Our experimental colonies were exposed to a very narrow range of temperatures inside the mobile lab - 23.3 ± 2.4°C - even though outside temperatures fluctuated between 19.4 and 33.4°C. However, the modest ranges of temperatures colonies were exposed to in the lab affected the number of heater bees attending the brood. As the temperature in the trailer increased 1°C, the number of heater bees per cm^2 decreased by 0.3 ± 0.2 (R=-0.60, P=0.025). We did not measure whether colony allocation to heater bees changed over a wider range of ambient temperature, but it is likely that decreases in ambient temperature would correspond with an increased allotment to heater bees. Stored honey consumption, however, did not increase with lower ambient temperatures in our honey consumption experiment.

Our low estimate of costs of heater bees may be because of the temperature regulation performed by all bees in colonies. Internal temperatures in hives outside of the brood nest area can range from 17 to 37°C (Saville et al. 2000, Meikle et al. 2017, S.E. Debnam, *unpublished data*) but brood are kept at 33 to 36°C. In our experiments, the average thoracic temperatures of non-heating bees in the brood area were 37.4°C in comparison to 42.2°C for heater bees. Non-heating bees comprised about 97% of the workers on brood substrate, with roughly 3% allocated to heater bees. Thus, the collective impact of the heat generated by all nurse bees in the brood area may have overwhelmed the specific contribution of heater bees, which appear to focus their effects very tightly to the brood cells themselves (see Bujok et al. 2002). The high cost in stored honey of raising brood may be due to the overall investment in nurse bees, and less to heater bees.

Based on their behavior, social insects invest heavily in brood care. The division of labor, nest construction, active feeding, and heater bees are examples of behaviors that may optimize the raising of juveniles. However, the energetic costs of the behaviors are poorly understood. Our results provide an estimate of brood raising costs of about half of the annual stored honey of a colony. These costs appear to be primarily in nest construction, feeding of larvae, and the elevated temperatures of nurse bees in general. The relatively few specialized heater bees appear to be relatively low-cost, and rather than provide a substantial source of heat for the brood, they might provide the exact and highly consistent temperatures so that juveniles develop rapidly and emerge in unison.

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Figure Legends

Figure 1: The metabolic chamber in use on the benchtop. The chamber contains a resting bee consuming honey.

Figure 2: Honey consumption by experimental brood colonies at different temperatures and brood stages. Error bars show one SE and shared letters indicate no significant differences.

Figure 3: Metabolic rates of bees during different behavioral activities. Error bars show one SE and shared letters indicate no significant differences.

Figures

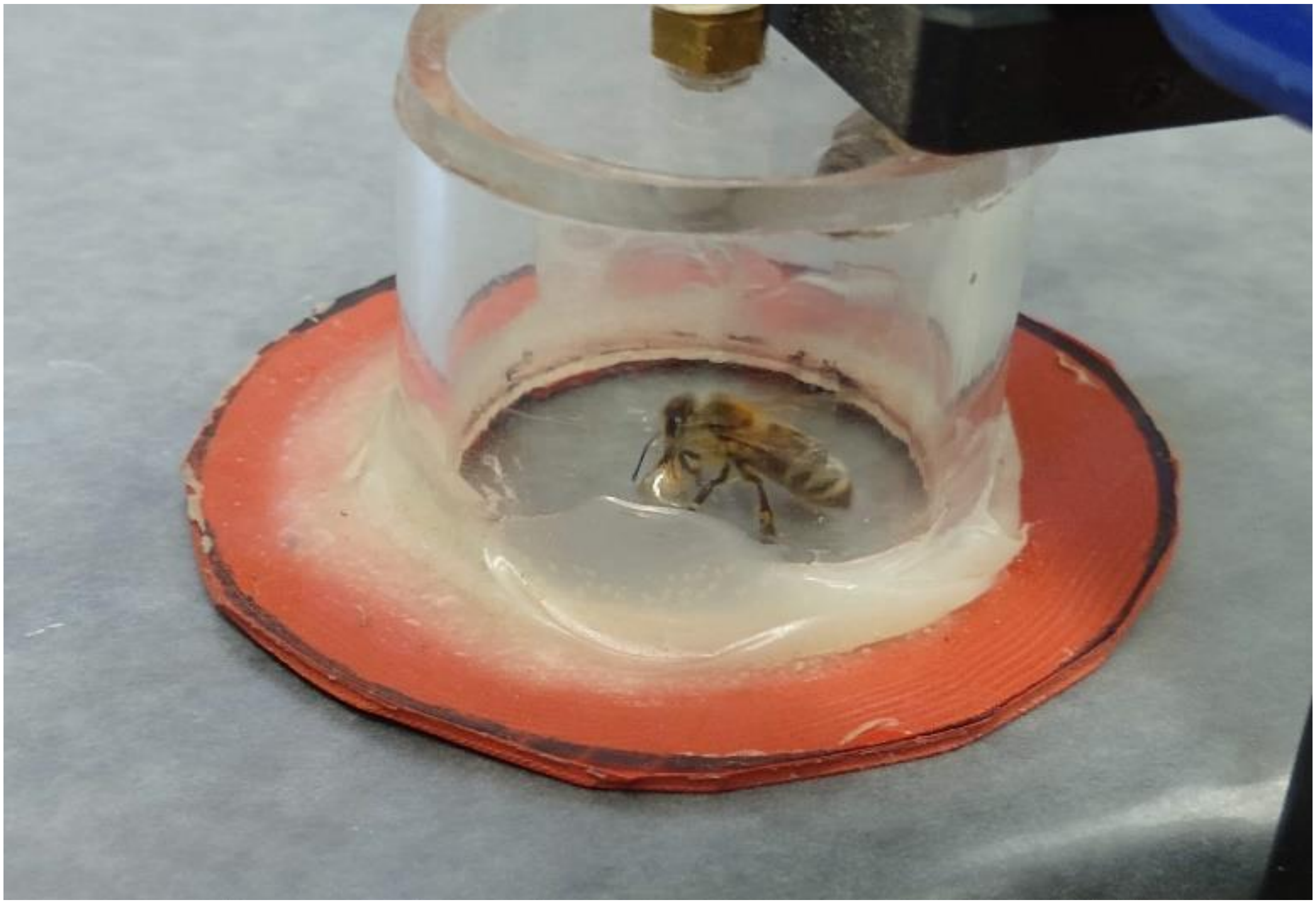

Figure 1

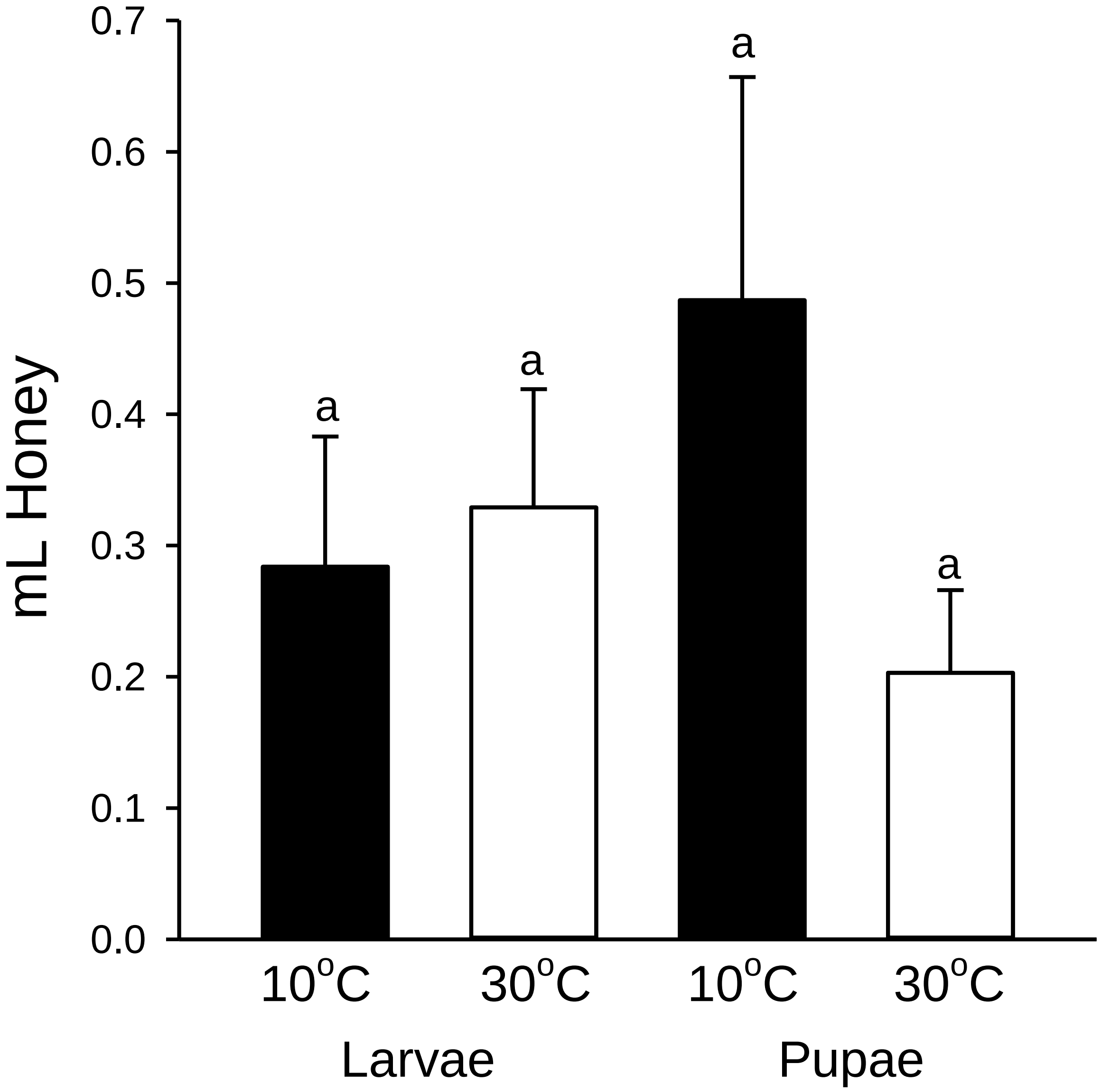
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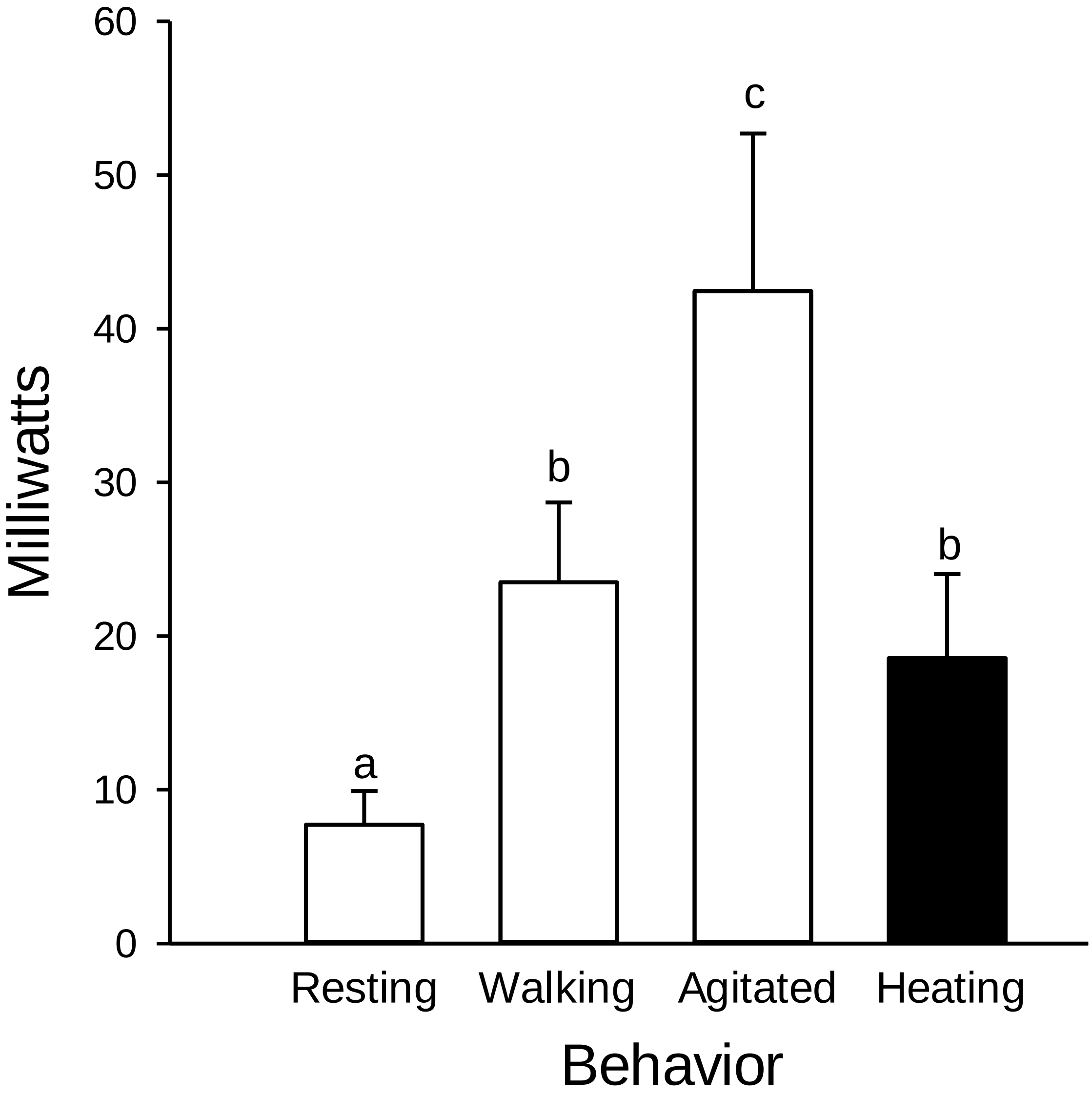
Figure 2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Walking
Agitated
Heating
Behavior

Figure 3

Supplementary Information

Table 1: Modified results from Kostarelou-Damianidou et al. (1995). Average Brood nest size was the average throughout their three year experiment. The number of brood were generated by multiplying the average brood nest size by 2.48, Kostarelou-Damianidou et al. reported number of brood cell per cm². Honey use was then calculated using the number of brood multiplied by 0.9, the honey used in our experiments to raise brood from the larval to the emerged adult stage.

Month	**Average brood nest size (cm²)**	**# of brood**	**Honey use (kg)**
Jun	1083	4637	4.3
Jul	1802	7712	7.2
Aug	1349	5775	5.4
Sep	707	3026	2.8
Oct	946	4047	3.8
Nov	193	825	0.8
Feb	184	789	0.7
Mar	784	3357	3.1
Apr	1431	6125	5.7
May	2461	10531	9.8
Total			**43.7**

Supplementary figure 1. Observation hive for measurements of the proportion of nurse bees allocated to heater bee tasks.

Supplementary Figure 2: Hive used for measurements of metabolic rates. Here the metabolic chamber is being used to sample an actively heating bee.