Comb phenotype of *Apis mellifera ligustica* is altered after exposure to chronic-localized heat stress

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Abstract:

Honey bees use a large array of thermoregulatory mechanisms to keep the temperature of their hives, particularly their brood, tightly controlled. Coordinated responses to acute heat stress, such as heat shielding, have been well studied. In the natural environment, however, colonies are more likely to be subjected to long-term circadian heat stressors than to a single acute event. In this study, we investigated how honey bees protect their hives from chronic-localized heat stress. Over 11 weeks, we heated sections of our hives to temperatures that endangered developing brood (42 ± 1°C) for 8 hours each day. We found that after four weeks heated areas contained significantly less brood ($P<0.001$) and significantly more non-brood ($P<0.0001$) than equally sized unheated areas. This supports the hypothesis that honey bees use a novel thermoregulatory mechanism, comb rearrangement, in response to chronic-localized heat stress. Our results provide insight into how honey bee thermoregulatory mechanisms operate in a natural context, as well as the speed and flexibility with which honey bees are able to rearrange their comb stores in response to changing thermal gradients in the hive.

Keywords: honey bees, heat stress, comb phenotype, thermoregulation

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The authors of this manuscript declare there are no conflicts of interest

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IPW was the primary contributor to manuscript preparation, and data analysis, and was a contributor to experimental design and data collection. ILK, AET, and RLK were contributors to data collection and data analysis. PTS contributed to experimental design and manuscript preparation.

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Introduction:

Social Insects display some of the most varied and complex behaviors of all animals. One of their most noteworthy abilities is the regulation of hive temperature, and the mechanisms they use to do so are as varied as social insects themselves (Jones & Oldroyd, 2006; Perez & Aron, 2020). The red wood ant, *Formica rufa* uses metabolic heat to warm their brood (Rosengren et al., 1987). *Macrotermes bellicosus* termites build nest mounds with a shape that varies depending on environmental temperature and humidity in order to keep internal conditions within an acceptable range (Korb & Linsenmair, 1998). Army ants like *Eciton burchellii* expand their bivouacs to create ventilation channels in response to high heat (Franks, 1989). In some vespid lineages, even larvae help warm the hive (Gibo et al., 1974).

The presence of brood, which must be kept within a species specific temperature range to thrive, is an important driver of hive temperature maintenance in social insects (Kronenberg & Heller, 1982). Honey bee brood, for example, only develop properly between 32-35°C (Simpson, 1961). Ants and termites can manage the temperature of their brood by transporting them along thermal gradients within their colonies throughout the day (Roces & Núñez, 1989; Penick & Tschinkel, 2008). Bees, however, construct highly ordered nests in which all stores, including honey, nectar, pollen, and brood are placed in hexagonal cells (von Frisch, 1974). This organization prevents them from moving their brood after it has been oviposited, meaning all brood temperature maintenance must occur wherever the brood are laid. Likely due in part to their inability to move their brood and their large colony size, eusocial bee species like honey bees employ the largest array of thermoregulatory mechanisms of all insects (Jones & Oldroyd, 2006; Jarimi et al., 2020; Maebe et al., 2021).

Heat stressors can range in duration from acute (minutes) to chronic (days), and in space from localized (a fraction of the hive) to dispersed (the entire hive). To combat acute-dispersed heat stress, honey bees fan their wings to create cyclic hive-wide air currents (Kronenberg & Heller, 1982; Peters et al., 2019) and often simultaneously regurgitate water around the hive to induce evaporative cooling (Seeley, 1985). To combat acute-localized heat stress, honey bees engage in heat shielding, where workers orient themselves with their tarsi on heated hive walls to absorb the heat into their bodies, and then move away to dissipate the heat to the periphery of the hive (Starks & Gilley, 1999; Bonoan et al., 2014). Heat shielding, fanning, and evaporative cooling are all effective at quickly lowering hive temperature.

Honey bees mitigate chronic-dispersed heat stress primarily by using passive mechanisms to reduce ambient heat in the hive (Jones & Oldroyd, 2006). They preferentially build their hives in insulated spaces that are relatively protected from temperature fluctuations, such as tree trunks or wall cavities (Seeley & Morse, 1976; Heinrich, 1979); and further insulate their brood by arranging their comb stores in a highly characteristic pattern where a dense central brood cluster is surrounded by honey and nectar, with a thin strip of pollen separating the two (Camazine et al., 1990, Seeley & Morse, 1976). Effective use of passive thermoregulation can reduce the colonies need to actively thermoregulate, thereby reducing its energy expenditure.

Disturbances like bark being stripped from a tree housing a colony or a crack forming in a Langstroth hive can weaken a hive's capacity for passive thermoregulation (Griffiths et al., 2018). In serious cases this can cause hive areas that were once well suited for brood rearing to become inhospitable due to chronic-localized heat stress. Initially, it seems colonies subjected to such a disturbance have two costly options: increase the rate of active thermoregulation or abscond. Absconding is an energetically expensive and dangerous behavior where a colony leaves food and brood behind to completely rebuild its hive in a new location (Hepburn et al., 1999; Winston et al., 1979). While increasing use of active thermoregulatory behaviors such as fanning and heat shielding is energetically expensive (Peters et al., 2017), exposes bees to high temperatures that can decrease their lifespan (Li et al., 2019), and may leave colonies less able to respond to other disturbances (Ostwald et al., 2016). Given the high costs of both options, a colony's ability to mitigate dangerous changes in the internal hive environment is likely critical to its survival.

In this study we tested whether honey bee colonies use another behavioral mechanism, modifying the arrangement of their comb stores, to reduce the impact of chronic-localized heat stress in their colonies. By replacing temperature sensitive brood in areas subjected to chronic-localized heat stress with thermally resistant and potentially insulating honey nectar and pollen, a colony could reduce the overall effect the stressor has on the colony. To assess whether honey bees modify the arrangement of their comb in response to chronic-localized heat stress, we placed a heat stressor in the center of one side of one frame in eight two-frame observation hives and measured the comb contents of these areas plus an equally sized unheated area over 11 weeks. We predicted that the comb pattern would change over time, with thermally resistant stores like honey and nectar becoming significantly more prevalent in heated areas. A changing comb pattern in response to heat stress would indicate that honey bee colonies use alternate thermoregulatory mechanisms to minimize the impact of chronic-localized heat stress in the hive.

Materials and Methods:

Subjects

Eight *Apis mellifera ligustica* colonies housed in vertical two-frame observation hives were installed in an enclosure (4.5 m x 5.5 m) adjacent to Tufts Campus in Medford, MA (42°24'20" N 71°06'51" W) on June 7th, 2019. Observation hives were 53 × 54 × 5 cm with 6 mm thick plexiglass windows encasing each side of both frames (Bonoan et al., 2017, 2018). Two observation hives were arranged in each corner of the enclosure with tubing that allowed honey bees to freely move between their hive and the external environment. Hives were given a week to acclimate to their new location during which they were provided one liter of 1:1 water:sucrose solution. During this time, the colonies began to accumulate honey and nectar in the top frame, while the bottom frame was mostly filled with brood (Seeley & Morse, 1976). The enclosure was kept at approximately 20°C throughout the study using an in-wall A/C unit.

Chronic-localized heat stress treatment

On June 14th, two 15 × 20 cm heating pads (Zoo Med ReptiTherm®) were mounted on the plexiglass covering the same side of each hive (Starks & Gilley, 1999; Starks et al., 2005; Siegel et al., 2005). To limit the size of the test areas, a 5 mm thick insulation sheet with a 65 mm diameter hole cut in the middle was placed on the surface of each heating pad (Fig 1). The insulation sheet blocked heat from radiating into the hive from the heating pad beyond the central hole, which had a diameter large enough to cover approximately 200 comb cells. Heating pads were mounted on the center of the top and bottom frame of each hive with command strips, allowing for easy removal and reattachment (Fig 1). Experimental heating pads were powered on for eight hours a day for the duration of the study, and control heating pads were left unplugged. Treatment location was randomly assigned so that half the hives had experimental pads on the top frame and half had them on the bottom frame. Temperature controllers (InkBird ITC-306T) maintained the temperature of experimental heating pads at 42 ± 1°C from 6:00 AM to 2:00 PM for the duration of the study, simulating natural conditions with hot days and cool night. All electricity to the heating pads was cut off when they were powered down each afternoon.

Comb cell content quantification

A circle denoting the exposure area of each control and experimental heating pad was drawn on the plexiglass above each experimental area using a black dry erase marker. The content of the filled cells within each area (approximately 200 cells each) was recorded by drawing dots on the plexiglass, with the color of the dot representing the content of the cell beneath (Carroll et al., 2017). Uncapped brood, capped brood, and pollen were all marked individually, while honey and nectar were recorded with the same color of marker since they both serve primarily as carbohydrate stores. Cell content counts have been previously used as metrics of colony health (Odoux et al., 2014; Sandrock et al., 2014). Splitting brood into uncapped and capped helps easily approximate their age: during the egg and larval stages, the brood cell is completely exposed to allow for feeding; after the eighth day, the larva begins to pupate and worker bees put a waxy seal, or "cap", over its cell (Winston, 1987). Cell content counts were taken in each control (N=8) and experimental (N=8) test area once per week for 11 weeks starting on June 14th, 2019. Photographs were taken of each traced record and cell contents quantified at a later date.

Statistical analysis

The impact of heat stress on the arrangement of cell contents in observation hives was analyzed in R (version 3.5.2, R Core Team, 2017) with a series of generalized linear mixed models (GLMMs) that employed zero-inflated negative binomial distributions to account for zero-inflation and overdispersion (Yang et al., 2017). Because carbohydrate reserves (honey and nectar) accounted for the bulk (>95%) of non-brood cell contents, honey, nectar, and pollen were lumped into a single "non-brood" category.

Treatment (heated or control), week, frame (top or bottom), and the interaction of treatment and week were included in each preliminary model as main fixed effects; week was also included as a zero-inflation fixed effect, and hive was also included as the only random effect. Total brood quantity (capped and uncapped), non-brood quantity, capped brood quantity, and uncapped brood quantity were all independently modelled as response variables to understand how brood abundance was affected overall as well as at different stages of development. We competed the full models against all possible nested models using Akaike's information criterion (AIC) and selected that which best explained the observed results for each response variable. Fixed effects in the winning model were tested for statistical significance using the function Anova from the R package car (Fox & Weisberg, 2019).

Results:

As the season progressed overall brood abundance declined, and non-brood abundance increased. This reflects usual honey bee behavior: brood rearing generally decreases as winter approaches and colonies begin to stockpile food for winter (Seeley, 1985; Winston, 1987; Odoux et al., 2014).

Total brood abundance was significantly affected by treatment ($P<0.001$, $\chi^2=11.87$), week ($P<0.01$, $\chi^2=7.57$), and their interaction ($P<0.001$, $\chi^2=12.38$). After three weeks, heated hive sections contained significantly less brood than unheated sections, and the magnitude of this difference increased over time (Fig 2). Total brood abundance was not determined by frame position ($P=0.26$, $\chi^2=1.25$).

Non-brood abundance was significantly affected by treatment ($P<0.0001$, $\chi^2=18.35$), week ($P<0.0001$, $\chi^2=50.16$), and frame ($P<0.001$, $\chi^2=12.05$). After three weeks, heated hive sections contained significantly more non-brood than unheated sections, and total non-brood abundance increased over time in both treatments (Fig 2). Significantly more non-brood was present on the top frames than the bottom frames, as is expected in healthy colonies (Seeley & Morse, 1976)

Capped brood abundance was significantly affected by treatment ($P<0.05$, $\chi^2=4.18$), and the interaction between treatment and week ($P<0.05$, $\chi^2=4.86$). After four weeks there was significantly less capped brood in heated than unheated areas, and this difference generally increased over time (Fig 2). Uncapped brood was significantly affected by week ($P<0.05$, $\chi^2=6.22$) and frame ($P<0.05$, $\chi^2=4.60$), with significantly more uncapped brood present on bottom frames. Uncapped brood was, however, not affected by treatment ($P<0.17$, $\chi^2=1.86$), suggesting that the queen continued to oviposit in both heated and unheated hive sections throughout the study.

Discussion:

In this study we provide the first experimental evidence that honey bees engage in a novel behavior, the alteration of the arrangement of their comb contents, in response to chronic-localized heat stress. We observed a significant decrease in brood quantity and a significant increase in non-brood quantity in heated comb areas over the course of our study (Fig 2). The effect of heat on comb arrangement became more pronounced as the season progressed, so that by week six non-brood became more prevalent than brood in heated frames and temperature overrode frame position in determining brood arrangement, i.e. brood were preferentially sequestered in control areas over bottom frames.

Prior to this study, there was little insight into how honey bees respond to localized heat stress in a natural setting. Previous investigations observed that honey bees engage in heat shielding when exposed to acute-localized heat stress (Starks & Gilley, 1999; Starks et al., 2005) by absorbing heat into their bodies and dissipating it to the hive periphery (Siegel et al., 2005; Bonoan et al., 2014). While acute-localized heat stress does occur, the situations that lead to it are unlikely to be quickly remedied. If a flaw forms in the structure housing a colony, such as a crack in an Langstroth hive or protective bark being stripped from a tree, the area underneath is likely to have its insulating properties permanently weakened. While bees can patch structural flaws with propolis (Ghisalberti, 1979; Anjum et al., 2019), doing so takes time and may not bring ambient temperatures back down to baseline depending on the extent of the damage. We showed that as localized heat stress transitions from acute to chronic, bees respond by decreasing brood abundance in heated areas. Honey bees are known to engage in more heat shielding and fanning near brood comb than they do in proximity to other stores like honey (Simpson, 1961; Starks & Gilley, 1999; Bujok et al., 2002; Stabentheiner et al., 2010). Therefore, reducing brood quantity in heated areas likely decreases the need for workers to preform risky (Li et al., 2019) and energetically expensive (Peters et al., 2017) active thermoregulatory behaviors.

While our experimental setup did not allow for the direct observation of behavior while the heating pads were powered on, this study provides insight into possible mechanisms driving in the changes in comb arrangement we observed. Honey bee colonies usually maintain a stereotypic comb arrangement because individual bees follow simple behavioral rules (Camazine, 1991; Johnson, 2009; Montovan et al., 2013). The three behavioral rules most relevant to this study are 1) the queen lays eggs preferentially in the center of the comb structure near other brood, 2) workers first try to deposit food stores near the center of the comb and move upwards if they are unable to find space, and 3) workers preferentially consume pollen and nectar that is stored near brood (Camazine, 1991; Johnson, 2009; Montovan et al., 2013). These behavioral rules essentially pit the queen and workers against each other for space in the center of the comb. The characteristic comb pattern of food above brood persists in non-stressed hives because centrally stored food is consumed quickly, usually within a few days (Montovan et al., 2013), while brood remain in a cell for three weeks once laid. This means central cells filled with brood persist for much longer than central cells filled with non-brood, allowing the central brood cluster to remain stable while excess food is transported upwards.

It has been hypothesized that the rules stated above are influenced by thermal gradients within the hive (Camazine et al., 1990; Montovan et al., 2013). Consistent with this hypothesis, we found that profound changes in comb arrangement occurred in heated hive areas over the course of this study. We believe that the changes were driven by workers reducing the depletion of nectar stores in heated areas, thereby increasingly blocking the queen from ovipositing within them. We can be confident that the observed changes in comb arrangement were not a result of a complete cessation of ovipositing in heated areas because of the continued, albeit reduced, presence brood in those areas. Surprisingly, we found no difference in uncapped brood quantity between treatments but a significant reduction in capped brood in heated areas (Fig 2). It is tempting to speculate that ovipositing behavior was not affected by heat stress, and that the changes we observed were due to increased brood mortality in heated hive sections. Brood are negatively impacted by heat stress (Fukuda & Sakagami, 1968) and are particularly temperature sensitive during the transition from larvae to pupae (Wang et al., 2016), but we were unable to directly observe rates of ovipositing and brood mortality. Based on our results, it is reasonable to conclude that the changes we observed occurred despite continued competition for space between the queen and workers in heated hive areas.

Significant differences in the amount of brood stored in heated and control areas emerged after four weeks of heat stress, while significant differences in the amount of non-brood became apparent after just three weeks (Fig 2). This delay tracks nearly perfectly with the development of worker brood, which take three weeks from oviposition to eclosure as adults (von Frisch, 1914; Winston, 1987), and implies that as cells in heated areas become vacant after brood eclose, worker bees are significantly more likely to store nectar within those cells. Since the observed changes persist for the duration of the study, this also implies that honey bees are less likely to consume and deplete nectar stored in heated areas, therefore allowing those stores to persist in heated areas for extended periods of time.

When modified to account for behavioral changes caused by thermal gradients within the hive, the rules honey bees use to arrange their stores can explain the observed changes in comb arrangement. If, as we observed, nectar consumption is reduced in areas subjected to chronic-localized heat stress, then the amount of time nectar remains in those areas, and therefore the overall quantity of nectar in those areas, will increase. Since the queen preferentially oviposits near existing brood, as the amount of nectar in heated areas increases, the likelihood of the queen ovipositing within those areas decreases. Then, since workers preferentially consume nectar stored near brood, as the amount of brood in heated areas decreases, the likelihood of nectar in those areas being consumed also decreases. Combined, these factors could create a feedback loop that increases the amount of time nectar stores persist in heated areas and decreases the probability of brood being oviposited in heated areas. This feedback loop would allow honey bee colonies to adaptively respond to localized stressors that cause hive areas to become inhospitable for brood by replacing sensitive brood with resistant food stores in response to dangerous conditions.

As the global climate crisis continues, it is increasingly important that beekeepers are equipped with effective techniques for managing heat stress in their hives. Future studies could determine the temperature thresholds needed to induce a change in comb phenotype, and degree to which localized heat stress affects hive wide comb phenotype. It would also be useful to determine the degree to which full sized honey bee colonies are capable of similar comb rearrangement and how overall colony productivity, i.e. foraging intensity and brood production, are affected by heat stress. We found that as localized heat stress transitions from acute to chronic, honey bees engage in passive thermoregulation via comb rearrangement to lessen the need for active thermoregulatory behaviors like heat shielding. This indicates that honey bees can modify their behavior based on localized circumstances, allowing them to deviate from the normal rules of comb arrangement. We also found that it is the workers, not the queen, that primarily drive changes in comb phenotype.

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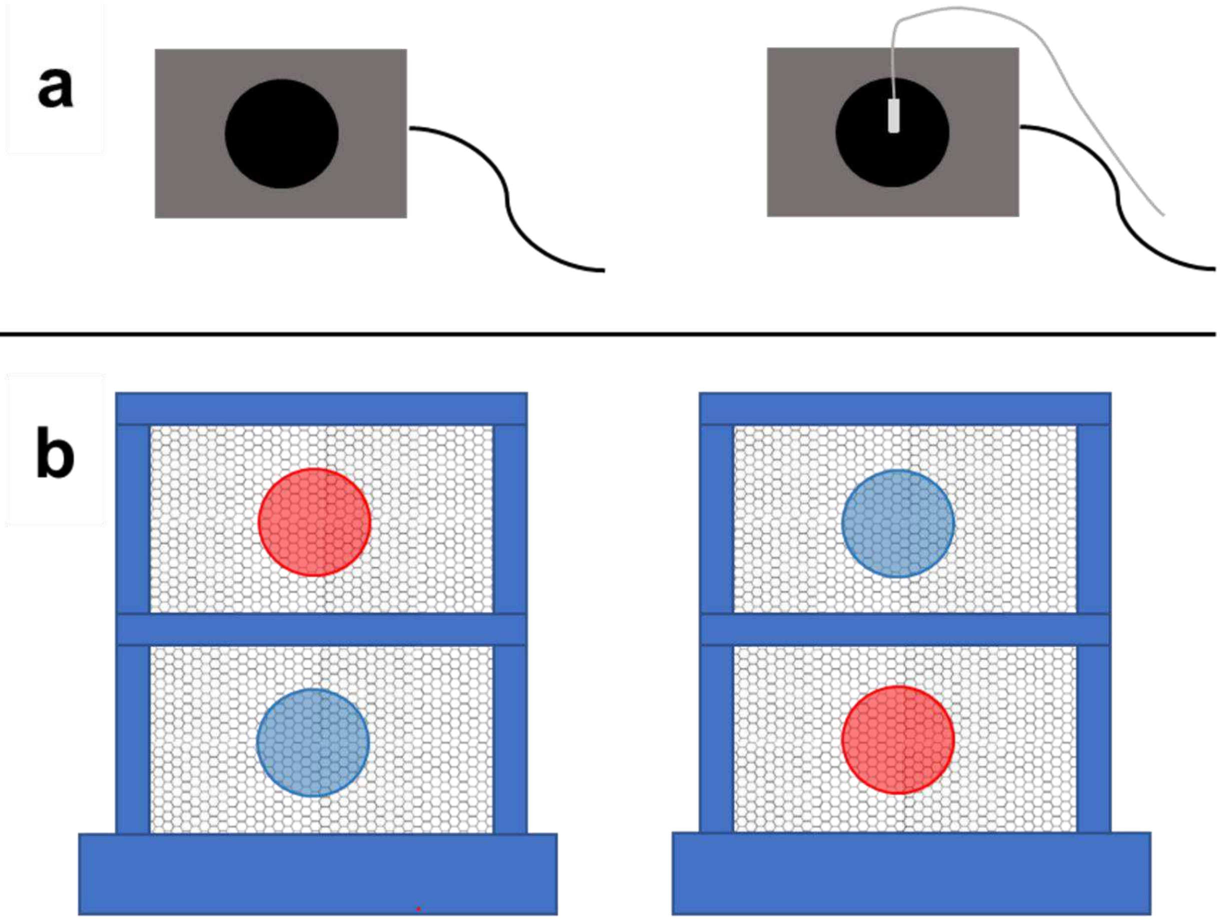

Fig. 1

Graphical representation of experimental setup (not to scale). a) Two heating pads were attached to each hive. To reduce the size of the heated area, each heating pad was covered in 5mm thick insulating pad, except for a 65 mm diameter hole. The heating pad that was powered on for the duration of the study had an attached temperature probe (right). The heating pad that was powered down had no probe (left). b) Two frame observation hives were used for this study. Half of the colonies had heated sections on the top frame, and half had heated sections on the bottom frame.

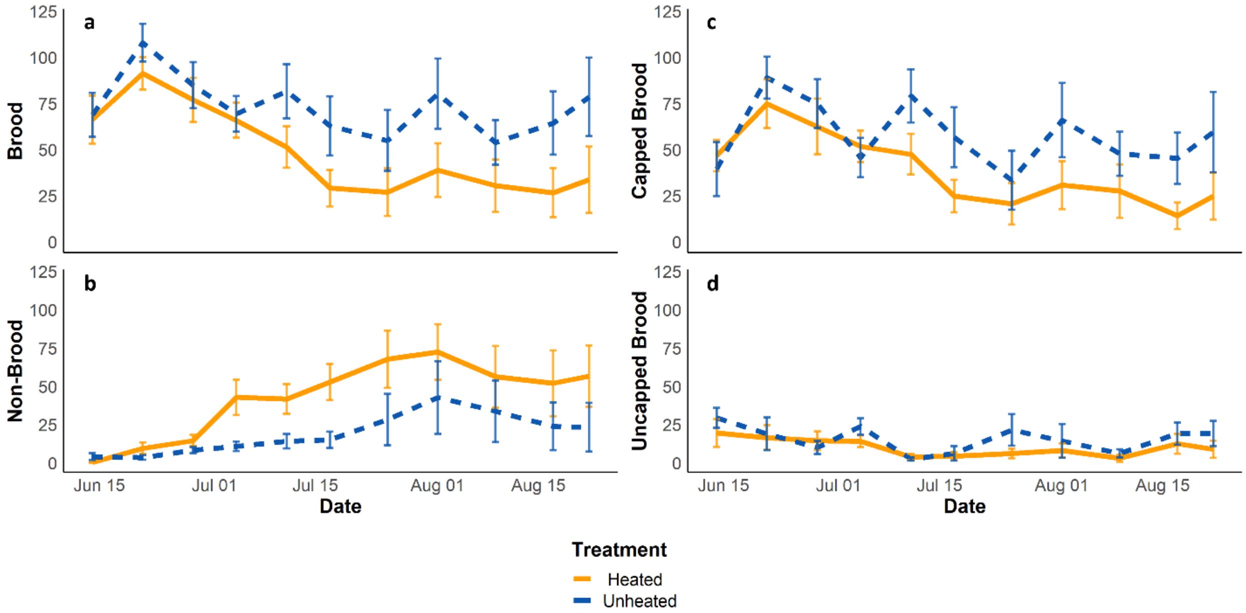

Fig. 2

Average abundance of cells containing a) overall brood, b) non-brood, c) capped brood, and d) uncapped brood between treatments over 11 weeks. Treatment significantly affected quantity of brood, non-brood, and capped brood, and did not significantly affect quantity of uncapped brood. N=8 observation hives, bars show standard error. Figure created in R using ggplot (Wickham, 2016).