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RESEARCH ARTICLE

The behavioral regulation of thirst, water collection and water storage in honey bee colonies

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ABSTRACT

This study investigated how a honey bee colony develops and quenches its collective thirst when it experiences hyperthermia of its broodnest. We found that a colony must strongly boost its water intake because evaporative cooling is critical to relieving broodnest hyperthermia, and that it must rapidly boost its water intake because a colony maintains only a small water reserve. We also clarified how a colony's water collectors know when to spring into action – by sensing either more frequent requests for fluid or greater personal thirst, or both. Finally, we found that the behavioral flexibility of a colony's water collectors enables them not only to satisfy their colony's current water needs but also to buffer their colony against future extreme water stresses by storing water in their crops and in their combs.

KEY WORDS: ***Apis mellifera*, Social physiology, Social thirst, Nest thermoregulation, Water homeostasis, Water collectors**

INTRODUCTION

Thirst is a sensation experienced by all terrestrial animals. It is the physiological signal that promotes water intake and so helps animals achieve osmotic homeostasis. The central mechanisms by which individuals produce and react to this vital signal are well studied in mammals (Bourque, 2008; McKinley and Johnson, 2004) and are increasingly being investigated in insects (Bernays, 1990; Nicolson, 1990; Cameron et al., 2010; Lin et al., 2014; Maxwell and Dubnau, 2014). In both mammals and insects, the processes that produce the sensation of thirst register small changes in the osmotic pressure (or volume) of an animal's blood, so individuals are constantly attuned to their water need and are stimulated to regulate their water intake accordingly (Bernays and Simpson, 1982; McKinley and Johnson, 2004). Though the mechanisms regulating thirst and water intake are reasonably well understood for individual organisms, little is known about these mechanisms for highly integrated groups that function as superorganisms, such as honey bee colonies.

A colony of honey bees needs water for several functions: to maintain body fluid homeostasis in the adult bees, to produce glandular secretions and dilute honey for feeding the brood, to cool the nest on hot days, and (in dry climates) to humidify the nest to prevent desiccation of the brood (Park, 1949; Nicolson, 2009; Human et al., 2006). Nectar is mostly water (35–85%, see fig. 2.12 in Seeley, 1995), so a colony's water needs are often met by its collection of nectar. Sometimes, however, a colony must collect pure water. These are times when the colony's nectar collection is meager, because of cold weather or a dearth of nectar-bearing flowers, or its water consumption is high, because of a high demand for brood food or a strong need to perform evaporative cooling. In either situation, the workers that are a colony's water collection specialists spring into action (Park, 1949; Lindauer, 1954, 1955; Robinson et al., 1984).

It is likely that whenever a honey bee colony has a high water consumption it must quickly boost its water collection. This is because colonies do not maintain large water stores in their nests. Rayment (1923) and Eksteen and Johannsmeler (1991) reported finding some water (amounts not specified) in the combs of colonies during droughts in Australia and South Africa. Lindauer (1954, 1955) suggested that a colony with fresh nectar stored in its combs might use this watery fluid as a buffer against rising colony thirst. A third means of water storage is the worker bee 'crop' ('honey stomach'). Park (1923, 1949) reported finding clusters of 'reservoir' bees – workers with swollen crops containing dilute nectar – on the periphery of a colony's broodnest after intense water collection.

A honey bee colony can achieve water homeostasis without maintaining a large water reserve because water, unlike nectar and pollen, is generally available (Seeley, 1995, p. 214). However, the reliance on external water sources requires rapid activation and deactivation of water collectors, as conditions change. Little is known about what activates a colony's water collectors. Perhaps water collectors become active when they are intensely begged for fluid but they have none to share because their crops are empty. Another possibility is that water collectors are activated by sensations of thirst after they have emptied their crops in response to their nestmates' requests for fluid. What is clear is that high nest temperatures by themselves do not activate water collectors, because these bees are often stimulated to fetch water even without a heat stress, such as when their colony is intensively rearing brood but low ambient temperatures prevent the bees from collecting water (Lindauer, 1954, 1955; Kiechle, 1961; Seeley, 1995, pp. 66-67).

More is known about what deactivates water collectors. When a water collector returns to her hive with a load of water, she offers her load to middle-aged bees that have positioned themselves near the nest entrance in order to unload bees returning with water. If the colony's need for water is high, then a water collector quickly (within 30 s) finds a bee to unload her. But if her colony's need for water is low, then she has difficulty finding a bee willing to accept her load. When it takes a water collector more than 5 min to find a nestmate willing to take her load of water, during which time the collector experiences dozens of unloading rejections, she stops her work and rests inside the hive (Kühnholz and Seeley, 1997).

Here, we address several mysteries about the mechanisms regulating water intake and water storage in honey bee colonies coping with the problem of broodnest hyperthermia. We begin by providing the first rigorous demonstration of the critical importance of water per se in regulating a colony's broodnest temperature. We then address the following two questions: (1) when a colony's need

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for water increases as a result of broodnest hyperthermia, what changes (behavioral or physiological) do the water collectors experience and how do these changes relate to their activation?; (2) when a colony experiences severe broodnest hyperthermia and greatly raises its water intake for evaporative cooling, does it also store water, in the bees or in the combs, or in both?

MATERIALS AND METHODS

Observation hive set-up in greenhouse

All experiments were performed with colonies living in a two-frame, glass-walled observation hive (46.0×50.5×4.5 cm) that we installed in a 6×8 m greenhouse at the Liddell Field Station at Cornell University in Ithaca, NY, USA (42°27.6′N, 76°26.7′W). We used this greenhouse to control the bees' access to water. The observation hive's entrance was configured so that the bees were forced to enter and leave from just one side of the combs (see Seeley, 1995, fig. 4.2); we refer to this as the 'front side'. Over the course of the study, we stocked the hive with two colonies; each consisted of a queen bee, approximately 3000 worker bees, and one frame of brood of all stages (eggs, larvae and pupae). Colony A was installed in the greenhouse on 17 June 2015 and was studied for 16 days. Colony B was installed in the greenhouse on 13 July 2015 and was studied for 14 days. In both colonies, the cells in the top frame of the comb contained primarily capped honey, but also some brood and pollen, whereas the cells in the bottom frame contained mostly brood, but also some pollen. We supplied the colonies with pollen (fresh pollen pellets that we dried, ground and spread in a shallow dish) *ad libitum*. The bees collected this pollen steadily, so the colonies continued rearing brood despite being confined in the greenhouse. The greenhouse contained no plants and the colonies were not fed sugar water, so the bees' only food supplies were the honey stores that they had in the hive and the pollen that we supplied. Neither colony had any opportunity to collect nectar.

Water source and labeling of water collectors

We supplied the bees with a water source (see Seeley, 1995, figs 4.5 and 4.6). It was positioned 1 m from the hive and could be visited by 20+ bees simultaneously. We eliminated all other sources of water in the greenhouse. We trained water collectors to the water source according to standard techniques (von Frisch, 1967). Each bee that collected water was labeled for individual identification with a paint mark on her thorax or abdomen, or both.

Recording temperatures and heating the hive

Every 15 min throughout each experiment, we measured the temperature in two locations: inside the colony's broodnest and just outside the hive. To do so, we mounted thermocouples (copper–constantan, type T, Omega Engineering, Stamford, CT, USA) in the center of the front side of the lower comb (broodnest) and just outside the hive (ambient) and we took temperature readings using an Omega Engineering digital thermometer (model DP465). We heated the broodnest by positioning a 100 W incandescent bulb in a Luxo lamp 10–20 cm from the glass covering the front side of the lower comb. We adjusted the distance between the lamp and glass throughout the heating process to maintain a broodnest temperature of 40±2°C, except in experiment 1, in which the lamp stayed a fixed distance, ca. 10 cm, from the glass.

Experiment 1: importance of water in regulating broodnest temperature

To assess the importance of water in responding to broodnest hyperthermia, we deprived each colony of its water source while we heated the broodnest for 7.5 h with the heating lamp in a fixed position. For the first 2 h, we provided a water source; for the next 2.5 h, we removed the water source; and for the final 3 h, we restored the water source. This test was conducted with colony A on 3 July and with colony B on 20 July. To quantify a colony's responses to hyperthermia, every 15 min we measured the number of bees fanning their wings for nest ventilation and the number of active water collectors. Also, every 30 min we measured the size of the colony's 'beard' (the cluster of bees that have evacuated the hive and assembled just outside its entrance), the time to drink 0.2 ml of water (our measure of colony thirst) and the mass of water collected (except during the 2.5 h period of water deprivation).

Number of fanners

We made three counts every 15 min of the number of bees fanning their wings (for nest cooling) within a 20 cm wide×4 cm high area just inside the hive entrance.

Beard size

We counted the number of bees in the cluster hanging outside the hive entrance. Counts greater than 100 bees are estimates, not exact counts.

Time to drink 0.2 ml of water

We squirted 0.2 ml of water dyed blue (for visibility) onto the hive floor 5 cm inside the hive entrance and measured how long it took the bees to drink up this little puddle. Dye was from a McCormick Food Color and Egg Dye set.

Number of water collectors

We made a roll call of the bees (all labeled for individual identification with paint marks) that visited the water source during each 15 min segment of an experiment.

Water collected

We measured the mass of water collected by placing the water source on a scale (Ohaus triple-beam balance, 0.1 g precision) and recording its mass every 30 min.

Experiment 2: water collection response to hyperthermia

To measure a colony's water collection response during broodnest hyperthermia, we collected data (on broodnest and ambient temperature, number of fanners, beard size, time to drink 0.2 ml of water, number of water collectors and water collected) for 1 h. We then heated the broodnest to 40±2°C for 2 h and continued to collect data. Finally, we turned off the lamp and continued to collect data for another 1–2 h. Water was always available at the water source. This experiment was performed with both colonies, on 1 July for colony A and 15 July for colony B.

Experiment 3: behavior of water collectors in the hive

To see how the within-nest behavior of water collectors changes in relation to changes in their colony's need for water, we tracked individual water collectors inside the hive as their colony's need for water started low, then became high, and finally subsided. For each trial, we made repeated observations on 5–6 focal water collectors over a period of 4–5 h; each focal bee had been seen collecting water the previous day and had been labeled for individual identification. For the first hour, the colony's broodnest was not heated and the water source was not filled. For the next 2 h, the colony's broodnest was heated to 40±2°C, but the water source remained unfilled. For the final 2 h, the heating lamp was turned off and water was provided. Throughout the experiment, we made 30 s observations of

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focal water collectors, tracking them one at a time. We cycled through the focal bees, making 1–2 30 s observations of each bee every 10 min except when we were unable to find some individuals (when they were outside the hive or on the back of the comb). During each 30 s observation period, we recorded the in-hive activities of the bee using the following nine categories: standing, walking, grooming self, grooming other, waggle dancing, begging for food/water, being begged for food/water, giving or receiving food/water, and inserting head into cell. We defined standing as the act of a bee remaining motionless for at least 5 s, and being begged for food/water as getting touched on the mouthparts by another bee's mouthparts for 2 s or less. We counted only contacts lasting 2 s or less because those lasting 3 s or longer are likely to involve transfer of food or water (Farina and Wainselboim, 2001) and we wanted to record instances in which a bee received a request for fluid but she did not respond by regurgitating fluid, probably because she had none. We distinguished begging and being begged for food/water by noting which bee (the focal water collector or the bee interacting with her) extended her proboscis; the begging bee extends her tongue. We then calculated for each of the nine categories of behavior the proportion of all the 30 s observations made during a 30 min period in which a particular behavior was seen at least once, i.e. in what fraction of the 30 s observation periods over a 30 min period that behavior occurred. Thus, for each category of behavior, the proportion could vary between 0.00 and 1.00. This experiment was performed twice, on 2 July with colony A and on 16 July with colony B.

Analysis of crop contents

We analyzed the contents of workers' crops over a range of conditions to determine whether bees store water in their crops in response to broodnest hyperthermia. Bees were sampled in the evening, starting at 20:00 h, on days when we had performed an experiment. We opened the observation hive by removing the glass on the front side, located bees with distended abdomens and removed them one at time from the hive. Using flat-tipped forceps, we squeezed each bee's abdomen until she regurgitated a droplet of liquid onto the sample surface of a refractometer (Atago model no. 050106) and then we measured the percentage sugar of the liquid.

We analyzed the bees' crop contents after the following treatments: (1) no heating and water available *ad libitum* (control), (2) 2.0 h of heating and water available *ad libitum*, and (3) 7.5 h of heating, which included 2.5 h of water deprivation (as in experiment 1). Note: after the third treatment, we sampled the bees not only in the evening of the treatment day but also on the following morning. We did so to see whether crop contents would change overnight. Most bees sampled were unmarked (i.e. were not water collectors), but on some days with treatments 2 and 3, we also sampled water collectors to compare their crop contents with those of bees that were not water collectors.

Analysis of comb cell contents

In colony B, on the evenings of the days when the colony experienced the three treatments described above for the analysis of crop contents, we also analyzed the contents of cells in the lower comb that contained liquid. This comb contained most of the colony's broodnest and originally it had contained some of the colony's honey stores. But by the time we collected data on comb contents, the honey stores were gone in the lower comb because we had not given the colony access to sugar solution for more than 10 days. (There remained, however, large honey stores in the upper comb.) Therefore, when we sampled the cells containing liquid on the lower frame, we were confident that we were not simply sampling the colony's honey stores. We sampled up to 20 cells (fewer if we could not find 20 that contained liquid). Using a glass pipette, we extracted a droplet of the liquid in each cell and measured its sugar content with the refractometer.

Statistical analyses

To analyze the results reported in Figs 1–3, and Fig. S1, we used 2-tailed Welch's *t*-tests (no assumption of equal variances) to test for differences in the mean values of the variables monitored – broodnest temperature, number of fanners, number of bees in the beard, number of water collectors, colony thirst and water collected – between the three phases (before, during, after) of each experiment. In performing these tests, we used only the data collected during the last hour of each phase, i.e. when enough time had passed for each treatment to have an effect. Full reports of the results of these statistical tests are reported in Table S1. We used Mann–Whitney *U*-tests to check for differences between hive bees and water collectors in the sugar concentrations of their crop contents in a variety of conditions, as described in the Results (Fig. 4). We also used Mann–Whitney *U*-tests to check for effects of broodnest hyperthermia and water deprivation on the sugar concentrations of the liquids found in cells within the broodnest (Fig. 5). Summary values are reported as the mean±s.d.

RESULTS

Experiment 1: the importance of water in regulating broodnest temperature

Colonies A and B showed similar responses over the 7.5 h period of broodnest hyperthermia that we imposed on them, during the middle of which we emptied the water source (Fig. 1). When we began heating the broodnest at the start of each trial, its temperature rose steadily for the first 30 min, then it stopped rising. Colony A lowered its broodnest temperature by 4.0°C over the next 1.5 h. Colony B did not lower its broodnest temperature following the initial rise, but it did stabilize the temperature around 40°C. Meanwhile, both colonies showed increases in the number of fanning bees, the number of evacuees (in the beard) and colony thirst. And in both colonies, water collectors became active, especially in the second hour of heating.

When we emptied the water source, so the water collectors could no longer function, the broodnest temperature immediately began to rise in both colonies, at 2.6°C h^{-1} in colony A and 1.3°C h^{-1} in colony B, and eventually reached higher levels than before water deprivation (43.3 and 43.7°C, respectively; $P<0.001$ both colonies). The number of fanning bees remained high but dipped a bit in colony B ($P>0.30$ for colony A, $P<0.028$ for colony B), the number of beard bees increased ($P<0.001$ both colonies) and the colony thirst rose ($P<0.09$ and 0.15). The water collectors continued visiting the (empty) water source, which they probed feverishly but unsuccessfully.

When we refilled the water source, the water collectors quickly began bringing home water. The average rate of collection in colony A increased from 6.0 g 30 min^{-1} before water deprivation to 21.5 g 30 min^{-1} after deprivation ($P<0.02$). Likewise, colony B increased its water collection from 3.2 g 30 min^{-1} before deprivation to 22.8 g 30 min^{-1} after deprivation ($P<0.003$). Once the bees resumed collecting water, the broodnest temperature in both colonies started to fall. In colony A, the mean broodnest temperature dropped from 42.7°C to 36.6°C ($P<0.001$) in the 3 h after the water was restored (even though the hive was still being heated). Similarly, in colony B, the mean broodnest temperature dropped from 43.1°C

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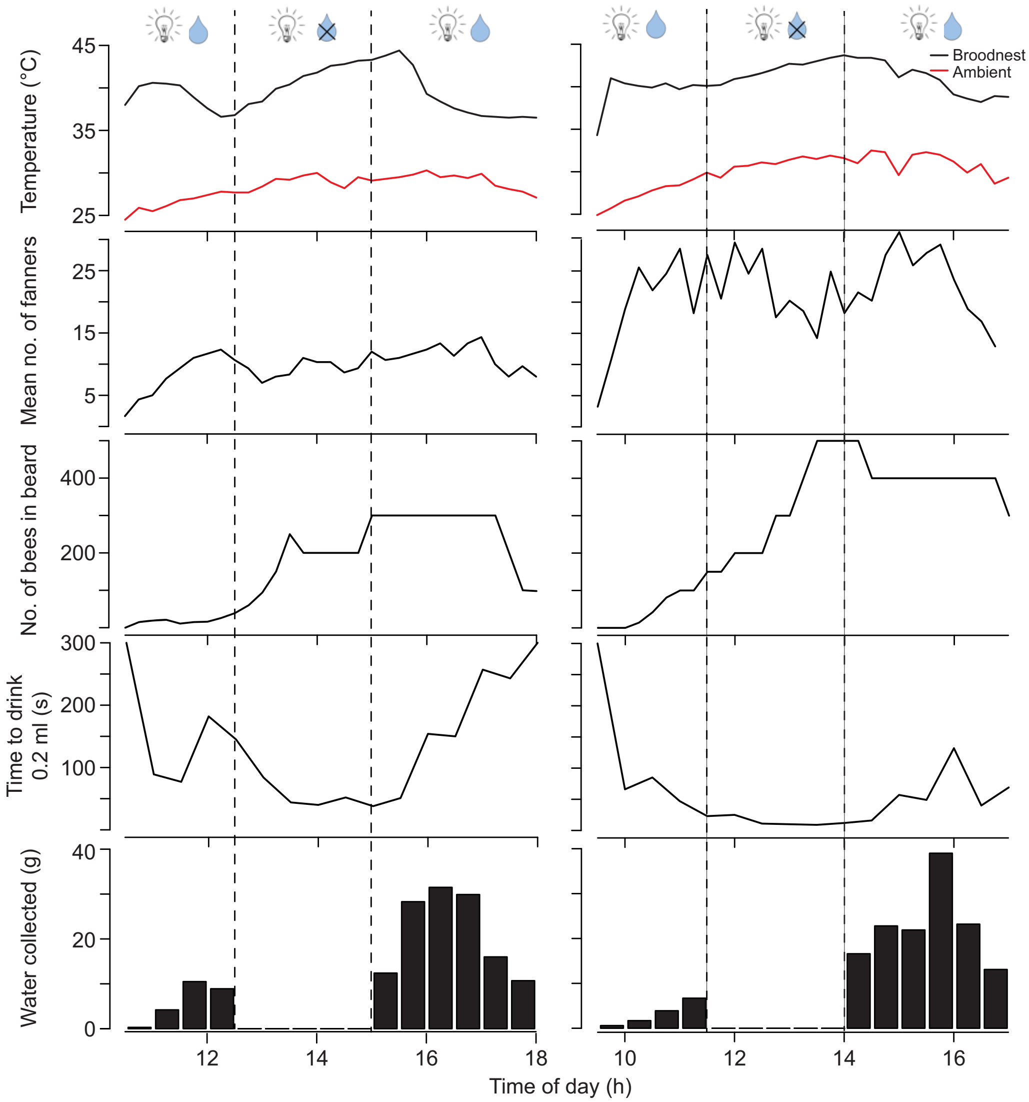

Fig. 1. Two tests of the importance of water to honey bee colonies in resisting broodnest hyperthermia. Shown are measurements from two colonies (left: colony A, observed 3 July 2015; right, colony B, 20 July 2015) in which the broodnest was heated with a 100 W lamp throughout the experiment (indicated by the bulb). Water was provided *ad libitum* at a source outside the hive except during a 2.5 h period in the middle of the experiment.

to 38.7°C (P<0.001). Fanning effort remained high in both colonies (P>0.92 and P>0.69). Likewise, beard size stayed high in both colonies (P>0.69 and P>0.23), even to the final hour of the experiment. In both colonies, the thirst level declined strongly (but not quite significantly, P<0.09 and P<0.15); the average time to drink 0.2 ml of water rose from 43 s to 267 s in colony A, and from 10 s to 80 s in colony B.

Experiment 2: water collection response to hyperthermia

This experiment examined the multifaceted responses of the colonies to a 2 h period of broodnest hyperthermia under natural conditions, i.e. with water always available (Fig. 2). Before heat stress, both colonies had stable and normal broodnest temperatures: on average, 34.3°C for colony A and 33.0°C for colony B. Once the heat stress began, we observed in both colonies a rapid rise in broodnest temperature, the number of bees fanning near the entrance and the number of bees in the beard outside the hive. However, in both colonies, the number of water collectors, the level of colony thirst and the water collected per 30 min rose only gradually during the period of hyperthermia.

By the end of the broodnest heating, the average temperatures of colony A and colony B were higher than before heating (40.6 and 39.8°C, P<0.001 for both colonies). Likewise, in both colonies the number of fanners was higher (P<0.002 for both colonies), the number of bees in the beard was greater (P<0.004 and P<0.02), the level of thirst was higher (P<0.009 and P<0.06), the number of water collectors had increased (P<0.001 and P<0.0002), and the rate of water collection was greater (P<0.40 and P<0.03).

Once the heating lamp was turned off, the broodnest temperatures fell back to their original levels (34.4 and 33.3°C, P<0.001 for both colonies), as did the number of fanners (P<0.002 and P<0.001) and the number of bees in the beard (P<0.004 and P<0.02). In both colonies, the level of thirst declined strongly, but the change was significant only for colony A (P<0.009), probably because we observed colony B (P<0.57) for only 1 h after turning off the lamp. The two other indicators of water need – the number of water collectors and mass of water collected per 30 min – also decreased, but only the number of water collectors in colony A was significantly lower by the end of the observation period (number of water collectors, P<0.001 and P<0.79; mass of water collected, P<0.43 and P<0.50).

Experiment 3: behavior of water collectors in the hive

This experiment examined how the behavior of water collectors changes when colony thirst increases, to help us understand how

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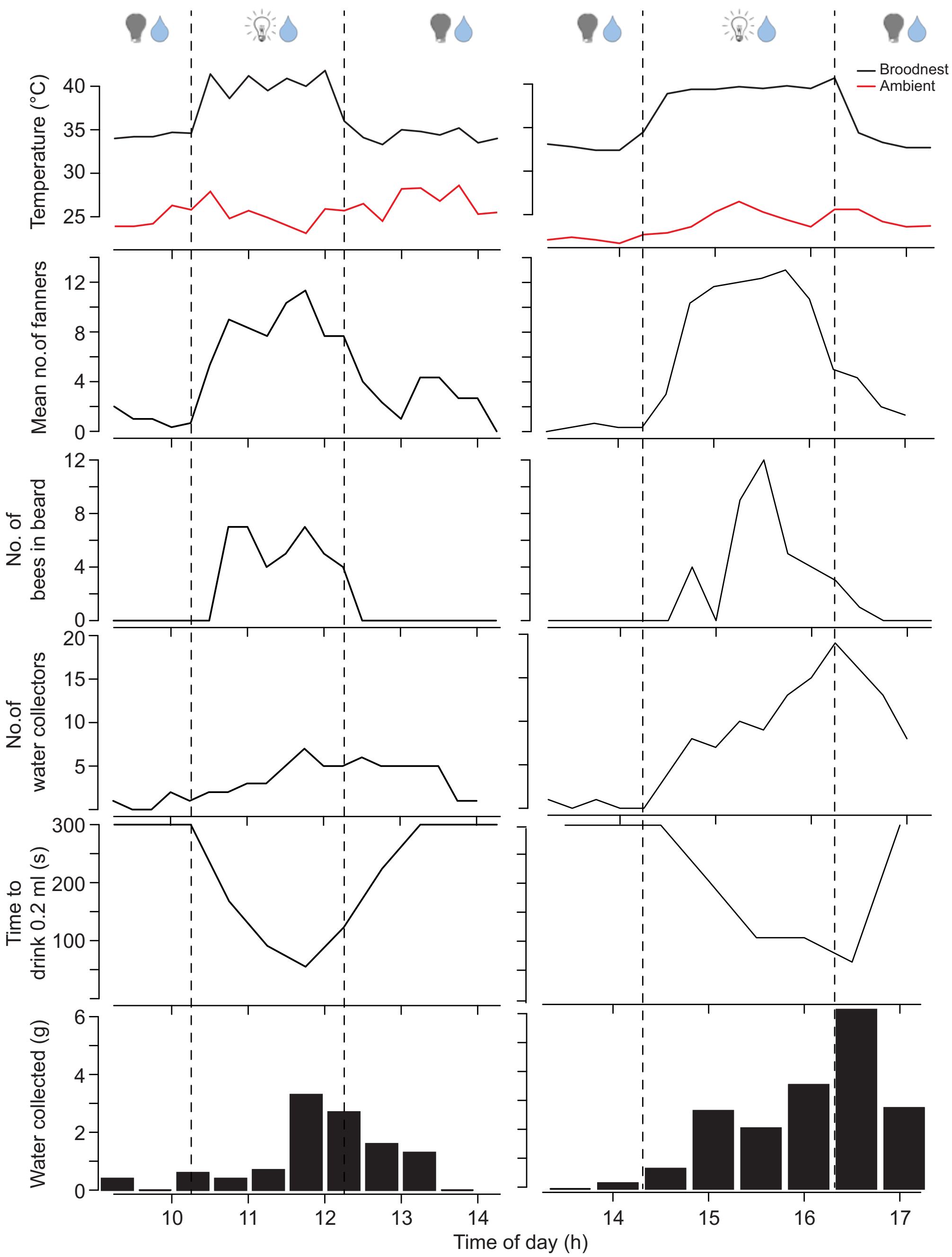

Fig. 2. The multifaceted response of a honey bee colony to broodnest hyperthermia. When a colony's broodnest overheats, the workers increase their fanning to ventilate the nest, they partially evacuate the nest (forming a 'beard' outside its entrance) and they raise their water intake for evaporative cooling. Shown are measurements from two colonies (left: colony A, observed 1 July 2015; right, colony B, 15 July 2015) in which the broodnest was heated with a 100 W lamp for 2 h in the middle of the experiment. Water was provided *ad libitum* at a source outside the hive throughout the experiment.

these bees sense that their colony's water need has increased. On 30 June, colony A was given a control treatment: the broodnest was not heated and water was continuously available. The colony's broodnest temperature stayed normal (35.1°C) and its thirst remained low (Fig. 3, left). The colony always took ≥300 s to drink the 0.2 ml of water squirted in the entrance. Also, none of the 12 bees that had collected water the day before (when the hive was heated for a different experiment) visited the water source, so it was unsurprising that the water source lost only 1.4 g during the 4 h experiment. The most commonly observed behavior for the water collectors was standing; it was seen in most (average proportion of 0.77±0.10) of the 30 s observation periods made during each 30 min segment of this control treatment. Walking was less common (0.20±0.10). The behavior of a water collector being begged for fluid was rarely observed (0.004±0.01).

On July 2, when colony A was given a water deprivation treatment, the water collectors (bees that had collected water the day before when the hive was heated) behaved initially as they had during the control treatment (Fig. 3, right). During the first hour, when the broodnest was not heated and water was not provided, their most common behavior was standing (in 0.73±0.22 of the 30 s observation periods), walking was the next most common activity (0.32±0.15) and being begged for fluid was exceedingly rare (0.02±0.05). Also, only 2 of the 12 bees that had worked as water collectors the previous day made more than one trip to the (empty) water source.

The behavior of these 12 water collectors changed conspicuously when the colony was heated while still deprived of water. The broodnest temperature rose to an average of 40.5°C ($P<0.001$) and colony thirst became strong, indicated by the plunge in time to drink

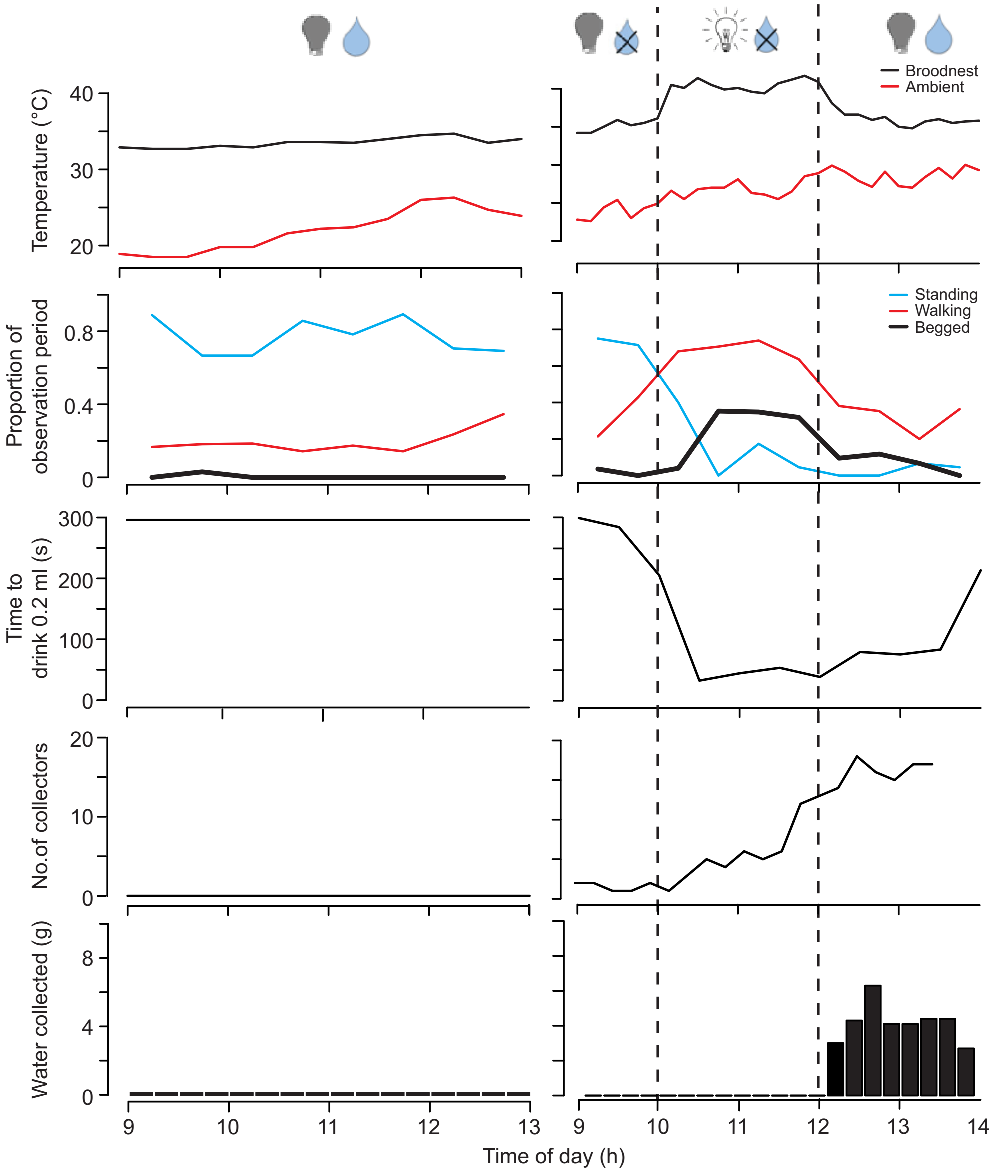

Fig. 3. Changes in the behavior of the water collectors in a honey bee colony when it experienced broodnest hyperthermia but was deprived of water. Shown are measurements from colony A on 2 days: left, control treatment on 30 June 2015, when the broodnest was not heated and water was provided *ad libitum*; right, experimental treatment on 2 July 2015, when the broodnest was heated and water was withheld for 2 h, and then broodnest heating ended and water was provided *ad libitum* outside the hive.

the 0.2 ml injections of water (264 s before heating, 46 s after heating; $P<0.02$). Also, the water collectors became more active: standing was less commonly observed than before (0.16±0.20, $P<0.001$) and walking became the most commonly observed activity (0.60±0.20, $P<0.001$). The most prominent change, however, was the surge in water collectors being begged for fluid by other bees. The fraction of 30 s observations in which this behavior was seen jumped from 1 out of 56 (average proportion of 0.02±0.05) before hyperthermia to 15 out of 45 (average proportion of 0.26±0.15) during hyperthermia ($P<0.001$). Shortly after the water collectors began to get begged intensively (ca. 10:45 h), the number of active water collectors increased markedly ($P<0.025$). All 12 of the previous day's water collectors appeared at the water source, which they feverishly probed for water (in vain).

At 12:00 h, the water source was refilled and the water collectors immediately began bringing home loads of water. Some also began performing waggle dances, so the number of water collectors soon grew to nearly 20 ($P<0.001$). Over the final 2 h of the experiment – when the heating lamp was off and water was available – the broodnest temperature dropped to normal and the colony's thirst diminished. The number of water collectors stayed high, but the fraction of the 30 s observation periods in which they were begged for fluid dropped to 0.07±0.12 ($P<0.001$) and the fraction of times they were observed walking declined to 0.36±0.27 ($P<0.002$). In general, the activity level of the water collectors dropped as their colony's thirst subsided.

When we repeated this experiment with colony B, we saw the same pattern of changes in the behavior of water collectors between times of water deprivation and times of water available as we saw with colony A (see Fig. S1).

Analysis of crop contents

By examining the crop contents of bees, we found that at the end of a day in which we induced broodnest hyperthermia and provided unlimited access to water, the crop contents of water collectors were more dilute than those of hive bees (Fig. 4A; Mann–Whitney $U=98.5$, $N=12$ water collectors, $N=10$ hive bees, $P<0.015$). We also found that hive bees were more likely to have dilute crop contents at the end of a day with broodnest

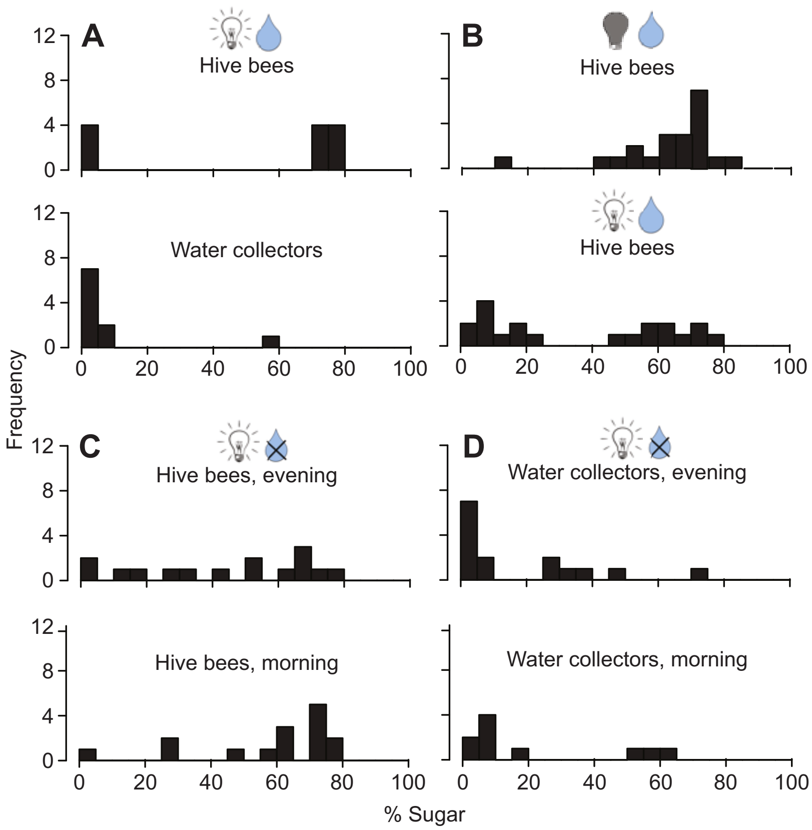

Fig . 4. Analysis of the crop contents of hive bees and water collectors in various contexts. The sugar concentrations of the bees' crop contents vary depending on how much each bee had fed on the honey stores inside the hive and how much water she had obtained, directly or indirectly, from the water source outside the hive. (A) Comparison of hive bees and water collectors at the end of a day with broodnest hyperthermia and water available *ad libitum*. (B) Comparison of hive bees at the end of a day with and without broodnest hyperthermia; water was available *ad libitum* on both days. (C) Comparison of hive bees in the evening after treatment and the next morning, following a day with broodnest hyperthermia plus a 2 h period of water deprivation. (D) Same comparison as in C, but for water collectors.

hyperthermia than at the end of a day without this stress, when water was provided *ad libitum* on both days (Fig. 4B; Mann–Whitney U=325.5, N=21 control, N=20 heat treatment, P<0.003). And we found that in a colony that received the treatment of broodnest hyperthermia+water deprivation, neither the hive bees nor the water collectors had crop contents that were more dilute in the evening of the treatment day than in the morning of the following day (Fig. 4C: hive bees, Mann–Whitney U=76.5, N=15 for evening and morning, P<0.15; Fig. 4D: water collectors, Mann–Whitney U=57.5, N=15 evening, N=10 morning, P<0.35). Overall, however, the water collectors were more likely to have dilute crop contents than the hive bees, both in the evening and in the morning after treatment (Fig. 4C,D; evening, Mann–Whitney U=52, N =15 water collectors and hive bees, P<0.015; morning, Mann–Whitney U=126.5, N=10 water collectors, N=15 hive bees, P<0.005).

Analysis of comb contents

When we gave colony B a control treatment (broodnest not heated and water available) during the day, we found in the evening only four cells containing liquid in the bottom comb. The liquid in all four cells had a sugar concentration greater than 70% (76±1%; Fig. 5A). But when we heated the broodnest of colony B for 3 h and provided water during the day, we found in the evening 10 cells that contained liquid in the bottom comb, with sugar concentrations ranging from 0 to 74% (46±25%; Fig. 5B). Finally, in three trials in which we heated the broodnest of colony B for 3 h and emptied the water source, we found over the three evenings a total of 52 cells with low sugar concentrations (below 20%) and just three cells with high sugar concentrations (above 20%: 5±10%; Fig. 5C). Thus, we found that on the evenings of days when the bees experienced broodnest hyperthermia and were deprived of water during the period of hyperthermia, the bees had stocked cells in their broodnest with sugar solutions that were much more dilute than those that we found on the evening of days when they experienced broodnest hyperthermia but were given water for evaporative cooling (Fig. 5B,C; Mann–Whitney U=49, N=10 with water, N=55 without water, P<0.001).

DISCUSSION

To better understand how a honey bee colony generates and responds to a collective need for water, we investigated what happens inside a colony when its broodnest gets overheated.

The importance of water in preventing broodnest hyperthermia

Honey bee colonies possess multiple interlocking mechanisms for regulating the temperature of their nests, including ventilation, evacuation and evaporative cooling. Combined, these processes can greatly lower a colony's broodnest temperature, as Lindauer (1954, 1955) found when he placed an occupied hive in a lava field in direct sunlight. Even with the temperature outside the hive reaching 60°C, Lindauer's colony maintained a temperature inside the hive of no more than 36°C. In our study, we demonstrated the critical significance of water collection for nest thermoregulation when we removed a colony's water source while we heated the broodnest, and then watched broodnest temperature steadily rise to a dangerously high level (up to 44°C). Only when water was available were the colonies able to keep broodnest temperature from rising to such high levels. Water has long been recognized as an important component of thermoregulation in honey bee colonies (Chadwick, 1922; Lindauer, 1954, 1955), but this study is the first to demonstrate its critical importance separate from the effects of nest ventilation and nest evacuation.

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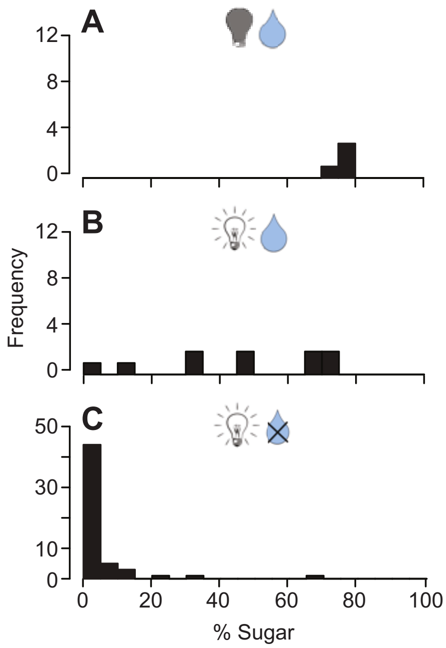

Fig. 5. Sugar concentration of liquids found in cells within the broodnest on evenings following various treatments during the day. The sugar concentrations of the cells vary depending on how much the bees who deposited the liquid in each cell had fed on the honey stores inside the hive and how much water they had obtained, directly or indirectly, from the water source outside the hive. (A) No broodnest hyperthermia and water available *ad libitum*. (B) Broodnest hyperthermia for 3 h and water available *ad libitum*. (C) Broodnest hyperthermia for 3 h and water withheld during hyperthermia (combined data for three separate treatment days).

Water collection response to hyperthermia

We described a colony's integrated response to broodnest hyperthermia when water is always available, as normally occurs in nature. As expected, the increase in broodnest temperature was accompanied by increases in cooling behaviors. In both colonies, when the broodnest was heated to 40°C – approximately 4°C above the limit of acceptable brood temperature – the bees responded almost immediately by increasing their fanning efforts and by partially evacuating the hive, forming a beard outside the entrance. The level of thirst, number of water collectors and rate of water collection also all rose, but they did so gradually over the 2 h period of broodnest hyperthermia (Fig. 2). The fact that the colonies developed thirst only gradually in response to the hyperthermia implies that they possessed reserves of fluids for nest cooling, buffering them against sudden fluctuations in temperature and water demand.

The marked delay between the start of heating and the initiation of water collection, which was also found by Kühnholz and Seeley (1997), implies that water collectors are not responding directly to the increase in nest temperature when they become active. This is not surprising, as the water collectors normally stand near the hive entrance, away from the broodnest, which was the focus of the heat stress in our experiments. So, what external (behavioral) cues or internal (physiological) signals are water collectors sensing as indicators of their colony's need for more water?

How water collectors sense the need to begin collecting water

Lindauer (1954, 1955) suggested, and Kühnholz and Seeley (1997) demonstrated, that what a water collector experiences when she returns to the hive and seeks to unload her water provides her with reliable information on the colony's water need. The higher this need, the fewer the unloading rejections that a water collector experiences and the faster she delivers her water load. These cues of unloading ease (number of rejections and speed of unloading) are reliable indicators of colony thirst to bees that are already engaged in collecting water. However, because only bees already engaged in collecting water can sense these cues, something else must stimulate water collectors to begin collecting water. What might this be?

We addressed this mystery by observing bees that had previously functioned as water collectors and looking for changes in how they were treated by their nestmates when we raised the colony's need for water by heating the broodnest. As the colony's need for water increased, the bees that had previously worked as water collectors were increasingly begged for fluid by their nestmates (Fig. 3; Fig. S1). This result suggests that being begged more often stimulates the inactive water collectors to resume their water collection work. Schulz et al. (2002) withheld food (honey and pollen) from colonies and observed that the frequency of begging interactions rose with increasing severity of starvation, and that it fell as the need for food was met. This pattern of being begged more frequently for food as a function of starvation level is strikingly similar to the one we observed as a function of dehydration level.

It is also possible, however, that water collectors are reactivated to water collection not as a response to being begged for fluid per se but as a response to sensations of personal thirst that they feel after they have regurgitated to hivemates the dilute sugar solutions they had in their crops (Fig. 4A). The physiological basis of thirst in individual honey bees, and thus of the control of water ingestion by individuals, remains unknown. Water ingestion/collection might be initiated by reduced abdominal volume in the water collectors, as in blow flies (*Phormia*) (Dethier and Evans, 1961) and locusts (*Locusta*) (Bernays, 1977), or by an elevated hemolymph osmotic pressure in the water collectors, as in the blow fly *Lucilia cuprina* (Barton Browne, 1968). An important next step in understanding the sociophysiological mechanisms that underlie water collection in honey bee colonies is investigating what exactly stimulates water collectors to fly out of the hive and fill their crops with water.

Storage of water in workers' crops and combs

Honey bee colonies store resources to cope with an unpredictable environment, most notably pollen and honey to avoid starvation. To protect the broodnest from overheating when the weather suddenly turns hot, a colony must be able to begin evaporative cooling even before water collection begins; hence, it must provision itself with water in some capacity beyond its immediate need. In our second experiment (Fig. 2), we found evidence that a honey bee colony does indeed maintain a store of water for evaporative cooling: the level of colony thirst and the rate of water collection increased only gradually in both of our study colonies after the onset of broodnest hyperthermia. This reserve of cooling fluids in our study colonies must have been rather small, however, because in our first experiment (Fig. 1) we saw that both colonies started to lose control of the broodnest temperature as soon as they lost access to an external supply of water.

Where does a colony maintain a store of water for evaporative cooling? One possibility, as suggested by Lindauer (1954, 1955), is that a colony's store of unripened nectar can function as a buffer against overheating. A second possibility is that there exist 'reservoir' bees, that is, bees with water-filled crops. Still a third possibility is that bees deposit water in some of the cells of the combs. Park (1923) reported finding reservoir bees in a colony in Iowa in early spring, when bees were able to obtain water for rearing brood only on occasional warm days. On these days, Park observed water collectors flying out *en masse* from a colony living in an observation hive. These bees gathered water from grass blades and puddles, and upon return to the hive they transferred their loads to other bees that served as water reservoirs, so called because these bees had greatly distended abdomens. These reservoir honey bees resembled what occurs in the honeypot ant (*Myrmecocystus mexicanus*), the social insect that is famous for its internal fluid storage. A subset of the workers in colonies of this species, known as 'repletes', function as living nectar storage vessels with extremely distended abdomens (Hölldobler and Wilson, 1990). Snelling (1976) described *M. mexicanus* workers in Arizona whose engorged crops contained water rather than nectar, and called them 'aquapletes'. Conway (1977) found that 2% of the repletes in a colony of *M. mexicanus* collected in Colorado stored extremely dilute nectar and suggested that these workers function as water storage vessels in the same way that the majority of repletes serve as nectar storage vessels. In the situation of variable water demand coupled with variable water supply, the reservoir honey bees may function in the same way that the aquaplete honeypot ants function in arid environments.

Our analysis of the crop contents of worker bees over a range of conditions suggests that water-filled bees are most prevalent after a period of heat stress combined with water deprivation, much as Park's reservoir bees appeared after several days of intense brood rearing combined with chilly weather that restricted water collection. Our water collectors generally had more dilute crop contents than hive bees sampled at the same time. Also, in the trials in which we heated the broodnest but provided water *ad libitum*, the water collectors were more likely to contain dilute crop contents than the hive bees (Fig. 4A). It makes sense that a water collector that may not have found a bee willing to unload her once the colony's thirst had declined would retain a full load of water into the evening, available for unloading as needed. Curiously, however, there was no significant difference in the concentration of crop contents between water collectors and hive bees at the end of days when colonies experienced both heat stress and water deprivation (Fig. 4C,D, top); many bees in both groups had crops containing dilute sugar solution! It appears that in such extreme situations a sizable fraction of a colony's workforce, including many of the hive bees, will engage in the task of water storage. On the morning following the day on which heat stress plus water deprivation was applied, there were still bees with distended abdomens containing dilute sugar solutions, mainly water collectors but also some hive bees (Fig. 4C,D, bottom). Presumably, these bees can survive overnight despite having little metabolic fuel in their crops (see Visscher et al., 1996) because they are resting quietly in the hive.

We also found evidence of the second possible method of water storage in response to extreme broodnest hyperthermia. After experiencing a serious heat stress, the bees in colony B stored water in the comb cells located in the unloading area inside the hive entrance. Water storage in the combs was especially strong in the evening following a day of heat stress combined with water deprivation (Fig. 5C), when most of the cells on the bottom comb that we found filled with liquid proved to contain water or a dilute sugar solution. Perhaps some eager and hurried water collectors quickly dumped their loads in cells between trips instead of waiting to be unloaded. No stored water was found the following morning, indicating that either the bees had used it overnight or it had simply evaporated. Bees living in cool temperate regions are generally thought not to store water in their combs, and certainly they do not do so as extensively as they store honey and pollen. Perhaps this is because any water stored in open comb cells is prone to loss through rapid evaporation, especially inside the warm nest of a honey bee colony, so the best way for a colony to store water is inside the crops of bees. It is now clear, though, that temporary water storage in combs is part of the social physiology of honey bee colonies (see also Rayment, 1923; Eksteen and Johannsmeler, 1991). Whether it serves mainly to help these superorganisms cope with the need to strongly cool their nests, both night and day, in hot climates, or to recover from an extreme thirst (water deficit) earlier in the day is an intriguing topic for future study.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.M.O. analyzed the data and prepared the figures. All authors (M.M.O, M.L.S. and T.D.S.) designed the study, collected the data and wrote the manuscript.

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Data availability
The data recorded in this study are found at the following link, as part of the eCommons Digital Repository at Cornell University: http://hdl.handle.net/1813/43783.

Supplementary information
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